AN INVESTIGATION OF PLANT-DERIVED CARDIAC GLYCOSIDES AS A POSSIBLE BASIS FOR APOSEMATISM IN THE APHIDOPHAGOUS HOVERFLY *ISCHIODON AEGYPTIUS* (WIEDEMANN) (DIPTERA: SYRPHIDAE).

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INTRODUCTION

The chemical defences of insects against predators are either passive or aggressive. Passive defence is achieved through crypsis, and aggressive defence is maintained by a conspicuous or 'aposematic' (Poulton, 1890) appearance that advertises some noxious quality of the insect harmful to a predator. Aposematism is mutually beneficial to both the bearer and its predator, whereas crypsis only benefits the prey species. It is therefore not surprising that the fascinating array of chemical defences in insects is both diverse and widespread (Roth and Eisner, 1962).

Noxious or unpalatable qualities of an insect have either an intrinsic basis as original synthesis of toxic compounds by an insect or, an extrinsic basis from the incorporation of some toxic feature of the environment into the body of the prey insect (Eisner, 1970). Many examples of noxiousness with an intrinsic basis have been described in insects (Weatherston, 1967), particularly the glandular and non-glandular secretions of Coleoptera (Eisner, 1970; Tschinkel, 1975). However, significant advances have recently been made in the appreciation of the close association between insects and plants as an extrinsic source of unpalatability in insects (Brower and Brower, 1964; Brower *et al.*, 1967; Dethier, 1970; Ehrlich and Raven, 1964; 1967; Fraenkel, 1959; 1969; Rothschild, 1972; 1973; Southwood 1973; Whittacker and Feeny, 1971).

Since 1964, L.P. Brower has worked in conjunction with a number of biologists, chemists and pharmacologists on a fascinating investigation into the extrinsic basis of aposematism in the Monarch butterfly *Danaus plexippus*. This investigation has elegantly displayed the relationship between the toxin-rich larval food plants of the family Asclepiadaceae and the adult butterfly in the maintenance of chemical

defence against predation. Brower and his colleagues worked on the premise, suggested by Slater in 1877 and elaborated and argued over by Haase in 1893 and Poulton in 1914, that aposematic insects incorporate the toxic properties of poisonous plants fed on by their larvae as the basis for their advertised unpalatability. In 1914 Poulton accurately predicted that the "work of the chemist" would clarify the basis of aposematism in D. plexippus that feeds as larvae on toxic plants of the family Asclepiadaceae. Fifty years later the research on D. plexippus has proven that the butterflies ingest and sequester ten of the cardioactive steroids present in the larval food plant. These steroids or cardiac glycosides are toxic to avian predators and hence provide the extrinsic basis of protective aposematism in the Monarch butterfly, (Brower, 1969; Brower and Brower, 1964; Brower et al., 1967; 1968; 1972; Brower and Glazier, 1975; Brower and Moffitt, 1974; Duffey, 1970; Erickson, 1973; Parsons, 1965; Reichstein, 1967; Reichstein et al., 1968). Cardiac glycosides, as the basis for aposematism, have also been found in other phytophagous insect groups such as Orthoptera, Hemiptera, Homoptera, Coleoptera and other Lepidoptera, (Duffey and Scudder, 1972; Feir and Suen, 1971; Reichstein, 1967; Rothschild and Parsons, 1962; Rothschild et al., 1970a; 1970b; 1973a; 1973b; Scudder and Duffey, 1972; von Euw et al., 1967; 1971).

The concept that predatory insects will ingest and possibly incorporate the toxic properties of aposematic prey as an extrinsic basis for their own aposematism was first suggested by Eisner et al., (1962). Rothschild et al., (1973a) reported the ingestion and possible storage of cardiac glycosides by coccinellid and chrysopid predators of the aposematic aphid Aphis nerii feeding on a plant rich in cardiac glycosides. A. nerii was previously shown by Rothschild

et al. (1970a) to contain some of the cardiac glycosides present in the host plants. On the basis of the findings of Eisner and Miriam Rothschild and their co-workers, that some predatory insects do ingest and use toxins present in their prey as an extrinsic source of their advertised unpalatability, this thesis was designed to indicate a possible explanation for the apparently aposematic appearance of most aphidophagous hoverflies (see figures 1 and 23).

Aphidophagous syrphids are described as 'wasp-like' (Oldroyd, 1964; Colyer and Hammond, 1968; Waldbauer and Sheldon, 1971), implying protection from predation by a superficial resemblance to sting-protected Hymenoptera. Adult aphidophagous syrphids are nectar and pollen feeders and the larvae prey largely upon Homoptera. This dissertation suggests the thesis that aphidophagous syrphids sequester plant toxins, ingested via their larval prey, as a possible extrinsic basis for aggressive chemical defence in the black and yellow adult syrphids. To test this thesis the common, conspicuously black and yellow syrphid, Ischiodon aegyptius (Wiedemann) (see figures 1 and 23), was investigated for sequestration of cardiac glycosides ingested by its larvae that prey on the aposematic, host specific aphid, Aphis nerii Boyer de Fonscolombe. A. nerii feeds on plants of the cardiac glycoside-rich family Asclepiadaceae. Three asclepiad species were used to investigate the possibility of a variation in the cardiac glycoside content of asclepiad-reared insects as shown by Brower et al. (1967), for the Monarch butterfly.

The project is presented divided into three major sections. The first two sections are concerned with the asclepiad-aphid-syrphid association. The first section indicates the biological nature of the asclepiad-aphid-syrphid association, with field and laboratory observations. The second section describes the cardiac glycoside content of the association

with a chromatographic separation of cardiac glycosides in extracts of the three asclepiads, aphid and syrphid. As cardiac glycosides have a specific affinity and toxicity for vertebrate heart muscle (Fieser and Fieser, 1959) the last section is concerned with a pharmacological indication of the toxicity of asclepiad/A. nerii-reared I. aegyptius extracts on the exposed myogenic hearts of two potential vertebrate predators of the syrphid. The three sections are preceded by a short taxonomic account of the identity of the most common, local, conspicuous, aphidophagous syrphids.

Aposematism has not been previously suggested as a defence strategy in Diptera, although Reichstein et al. (1968) report that a tachinid parasite of the Monarch butterfly was found to show cardiac glycoside-like activity. No comment was made on the possible significance of the cardiac glycoside activity in the tachinid. Rothschild et al. (1972) also analysed the larvae of a syrphid and a cecidomyid, collected as predators of A. nerii on the cardiac glycoside-rich Oleander, for the presence of cardiac glycosides, but with negative results.

Diptera are such fascinating and adaptable insects, with an incredible variety of morphological, behavioural and physiological adaptations, that the thesis, of aposematism with an extrinsic basis, presented here is not too far-fetched a concept, despite the aphid link that removes the possibly protected insect one step further away from its source of protection, the plant.

2. TAXONOMY OF THE LOCAL APHIDOPHAGOUS SYRPHIDAE.

Several aphidophagous syrphid species were frequently collected in and around Grahamstown at the start of this project. Some of these species were found as larval predators of *Aphis nerii* on the asclepiad *Gomphocarpus physocarpus*. As this plant/aphid association was chosen as a possible source of plant-derived cardiac glycosides as the extrinsic basis for an aposematic, anti-predator strategy of a conspicuous, aphidophagous syrphid, it was considered necessary to identify the commonest, local aphidophagous syrphids.

The identity of most of the African syrphids is difficult to determine with any certainty. Ethiopian syrphids have principally been worked on taxonomically by Bezzi (1915), Curran (1927; 1938) and Efflatoun (1922, Egyptian syrphids). Aphidophagous species and genera have been described from the Ethiopian region by Bezzi (1920), Bigot (1884 - *Allograpta*), Hervé-Bazin (1914), Keiser (1971 - Madagascan syrphids), Loew (1857; 1858 - *Allograpta*, *Betasyrphus*, *Metasyrphus*, *Episyrphus*), Stuckenberg (1954a; 1954b - *Paragus*), Walker (1856 - *Betasyrphus*) and Wiedemann (1824; 1830 - *Betasyrphus*, *Ischiodon*, *Asarkina*). Vockeroth (1969) has published a very useful revision of the world genera of the Syrphini, the dominant tribe of aphidophagous syrphids.

The first source of confusion in a consideration of the Syrphidae is the division of the family into subfamilies. The family itself is a well defined group of cyclorrhaphous flies with the 'vena-spuria' or free vein of the wing as the most obvious diagnostic feature of the adult (Coe, 1953). Syrphid larvae are distinguished from those of the other cyclorrhaphous families by the fused posterior respiratory processes of

the second and third instar larvae (except Mesogramma polita) and by the segmental spines (Heiss, 1938). Coe (1953) divided the British Syrphidae into 11 subfamilies, based on the division by Hull (1949) of the world-wide Syrphinae into 14 subfamilies. Shiraki (1968) groups the Syrphidae of Japan into as many as 18 subfamilies. Syrphids are considered to form a superfamily by Glumac (1960), which is divisible into four families. On the basis of larval features Hartley (1961) groups the subfamilies of Hull and Coe into only 2 subfamilies, the Syrphinae and the Milesiinae. The principal criterion for the division of the Syrphidae into 2 subfamilies is based on the presence or absence of piercing mouth parts in the larvae.

The larvae of syrphids are of three types (Heiss, 1938); Saprophagous - the larvae live and feed in water or decaying organic matter or sap exuding from trees (Hartley, 1961). These larvae have also become phytophagous in one group (Woodville, 1970). Scavengers - the larvae feed on detritus in the nests of hymenoptera (Donisthorpe, 1927; Fraser, 1946). Aphidophagous - the larvae feed principally on colonial homoptera (Clausen, 1940; Coe, 1953; Heiss, 1938; Lundbeck, 1916; Schneider, 1969). The presence of piercing mouthparts distinguishes the larvae of the Syrphinae from those of the Milesiinae. Piercing mouthparts are associated with the aphidophagous habit of the larvae. The larvae of the Syrphinae are aphidophagous and those of the Milesiinae are of, or derivable from, the more primitive saprophagous larval type (Hartley, 1961; Heiss, 1938).

The adults of the two subfamilies are separated by Vockeroth (1969) on the presence or absence of hairs on the humerus (humeral callus of the prothorax (Oldroyd, 1970)). The Syrphinae have a bare, and the

Milesiinae a haired, humerus. The Syrphinae are further subdivided by Vockeroth (1969) into six tribes, Paragini, Chrysotoxini, Toxomerini, Melanostomini, Bacchini and Syrphini, based on the tribal division by Hull (1949). Although all the larvae of these tribes are considered by Hartley (1961) to be of the carnivorous type, Heiss (1938) records the larva of a species of Toxomerini as a phytophage. Heiss considers this phytophage to be a larval development from the aphidophagous habit. The larval habitat of the Chrysotoxini is unknown, but the larvae have been found in an ants' nest (Dixon, 1960) and in a compost heap or hidden in moist soil (Coe, 1953). Larval Chrysotoxini are apparently of the aphidophagous type, which is indicated by the presence of mouth hooks (Dixon, 1960), but they probably feed as saprophages.

The most frequent aphidophagous syrphids encountered locally around Grahamstown were species of the tribe Syrphini, which is a group of active flies, conspicuously patterned with black and yellow stripes, spots or lunules. Adults and larvae of the small black or black and orange species of Paragini were also frequently encountered. At certain times of the year adult Bacchini and the Melanostomini were abundant. The larvae of a species of Baccha were found as a predator of the psyllid Ctenarytaina eucalypti on Eucalyptus globulus. However, the Syrphini are almost certainly the dominant aphidophagous syrphid fauna in South Africa.

The species of Paragus which were found as larvae preying on Aphis nerii on Gomphocarpus physocarpus, with aphidophagous Syrphini larvae, were identified as adults from the keys and descriptions published by Stuckenberg (1954a; 1954b). Paragus minutus Hull, P. longiventris Loew and P. tibialis (Fallén) were identified as adults

which emerged from the puparia of larvae collected on A. nerii-infested G. physocarpus around Grahamstown. P. borbonicus Macquart and P. capricorni Stuckenberg were collected from A. nerii colonies on G. physocarpus at Lake Sibaya in Kwa Zulu.

The Syrphini contrast sharply with syrphids in the other Syrphinae tribes which consist of apparently cryptic adults, or adults that bear striking resemblances to wasps (see figures 23 and 24 and Waldbauer and Sheldon, 1971). By their behaviour the small inconspicuously black and orange Melanostoma and Paragus species and the black Baccha species are possibly cryptic. (The Toxomerini are new world flies, and no Chrysotoxini were found locally.) The Syrphini are larger flies that commonly have a wasp-like appearance with their black and yellow stripes. This taxonomic study is therefore restricted to the commonest local Syrphini as the most likely flies to show plant toxin-based aposematism. It should be stressed that these assessments of cryptic or conspicuous colouration and behaviour are largely intuitive.

The genera of Syrphini that occur in and around Grahamstown were identified, according to Vockeroth (1969), as Betasyrphus Matsumura, Metasyrphus Matsumura, Asarkina Macquart, Allograpta Osten Sacken and Ischiodon Sack. Of these genera Metasyrphus and Ischiodon are mono specific in South Africa. Adults of Metasyrphus cognatus (Loew) (see figure 23) were identified from the keys in Bezzi (1915) and Curran (1938) and compared with the brief original description of Loew (1857) which indicates a similarity with the palaearctic M. corollae (Fabr.). The keys in Bezzi (1915) and Efflatoun (1922) identify the very distinctive Ischiodon aegyptius (Wiedemann) (as Xanthogramma aegyptium) which also compares with the brief original description of Syrphus aegyptius from

Egypt by Wiedemann (1830). Vockeroth considers 'aegyptius' to be grouped with the Asian and Australian species 'scutellaris' in the genus Ischiodon and not as they have been previously grouped as Syrphus, Xanthogramma or Sphaerophoria. The males of I. aegyptius have a large spine on each hind trochanter which readily distinguishes the species. Both Vockeroth (1969) and Efflatoun (1922) give descriptions of I. aegyptius which agree with the specimens caught and reared in Grahamstown. Of the genus Allograpta, A. calopoides (Curran), A. calina (Curran) and A. rotundicornis (Loew) were caught in Grahamstown and identified from the keys in Bezzi (1915, as Xanthogramma rotundicorne) and Curran (1938, as Syrphus rotundicornis, S. calopoides and S. calinus). Adults of A. rotundicornis are extremely similar in appearance to I. aegyptius and occur equally commonly. The two syrphids are simple to distinguish as A. rotundicornis has a lateral yellow stripe on the mesonotum which extends from the humerus to the scutellum. The yellow stripe of I. aegyptius only extends as far as the dorsal suture. The wings of A. rotundicornis are considerably longer than the abdomen whereas the wings of I. aegyptius reach only as far as the tip of the abdomen.

Several species of the very distinctive genus Asarkina were also common locally. However, as no eggs, larvae or pupae of these very fast flying, large, black and yellow flies were found it seemed pointless to delve into the confusion which obscures the identity of these handsome hoverflies.

Larvae of I. aegyptius and several Betasyrphus species were most frequently encountered in aphid colonies. The identity of the Betasyrphus species was investigated with locally collected material and

specimens from the collection in the Albany Museum, Grahamstown, which were kindly loaned by Mr. C. Jacot-Guillarmod and Mr. F.W. Gess.

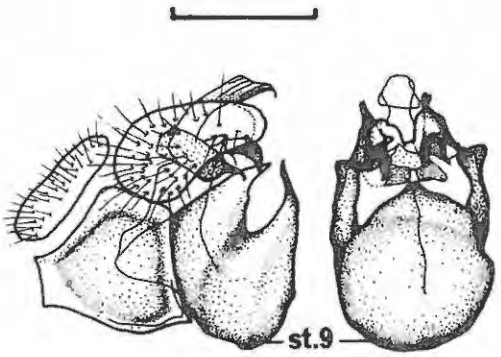
Bezzi (1915) distinguishes six species and two additional forms of Betasyrphus which are characterised by the possession of hairy eyes. The same group of species are recognised by Curran (1938) who included a further two species, one a new designation. Of these Betasyrphus species, B. capensis (Wiedemann, 1830) has characteristic abdominal markings and is simple to identify from Bezzi's and Curran's keys. The rest of the Betasyrphus species commonly found in Grahamstown have either grey abdominal markings, like B. claripennis (Loew) or orange abdominal markings like B. adligatus (Wiedemann). The larvae of syrphids allied to B. claripennis were not found as frequently in colonies of A. nerii on G. physocarpus as larvae of syrphids closely allied to B. adligatus. According to the keys of Bezzi and Curran and the original descriptions of Betasyrphus species the ethiopian species with grey abdominal markings are, B. claripennis (Loew, 1857), B. hirticeps (Loew, 1857), B. luci (Curran, 1938, the same as the B. hirticeps in Bezzi (1915)), B. intersectus (Wiedemann, 1824, which Bezzi (1915) considers synonymous with B. claripennis) and possibly the characteristic B. inflaticornis (Bezzi, 1915). The Grahamstown species in this group ie probably B. claripennis as it has a face with white pile, four grey bands on the abdomen (tergites 2-5), a shining thorax and pure hyaline wings (Bezzi, 1915).

The Betasyrphus species allied to B. adligatus, characterised by orange abdominal markings, were found as at least three distinctive groups in Grahamstown. Of the three B. adligatus-like groups one is separated with the keys of Bezzi and Curran as possibly B. adligatus. However, the species also agrees with the description of B. congolensis

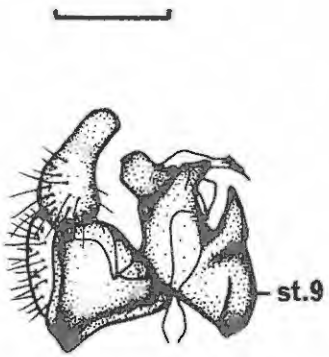
by Hervé-Bazin (1914). None of the Grahamstown syrphids key out as, or agree with the original descriptions of, B. tricolor (Walker, 1856, preoccupied and is now B. saundersi van der Goot, 1964) or B. melas (Bezzi, 1915). As original type material was not available for comparison the three groups of B. adligatus-like syrphids are arbitrarily termed Betasyrphus species A, B and C. Species B may be B. adligatus.

Specimens of the Betasyrphus species, I. aegyptius, A. rotundicornis and M. cognatus were sent to Dr. J.R. Vockeroth at the Biosystematics Research Institute in Ottawa, Canada and to Dr. B.R. Stuckenberg at the Natal Museum in Pietermaritzburg, South Africa for species determination and identity confirmation. Unfortunately both Dr. Vockeroth and Dr. Stuckenberg did not have an opportunity to verify my suggestions or indicate the identities of the syrphids, although they both asked to see the specimens that were sent. Dr. Stuckenberg did however suggest that differences in the orange fascia or bands on the tergites may separate B. saundersi from B. adligatus. From the differences in the orange fascia the three groups of B. adligatus-like syrphids are separated as species A, with orange bands that cover two-thirds of tergites 2 and 3 and a thin grey band on tergite 4. Betasyrphus species B has orange bands on tergites 2, 3 and 4, those on tergites 2 and 4 are half as wide as the orange band on tergite 3 which covers half the width of the tergite. Betasyrphus species C is distinguished by a thin grey band on tergite 2, tergite 3 is almost completely orange and tergite 4 is orange on the basal half.

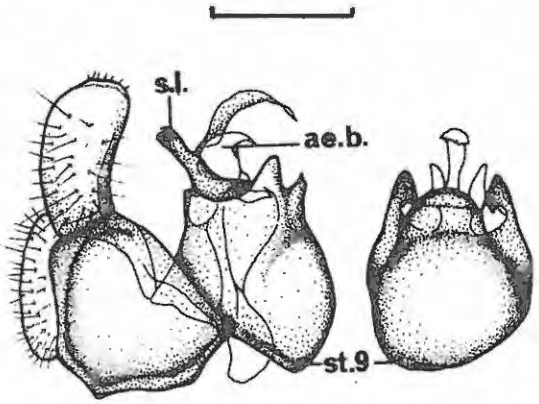
The separation of adults of Betasyrphus species A, B and C was reinforced by differences in photographs of face profiles and the angle of dorsal divergence of the eyes of the holoptic males (features



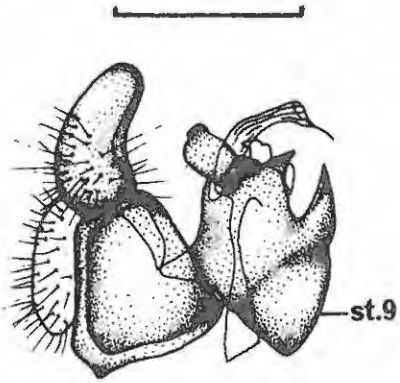
a). Betasyrphus sp. A.



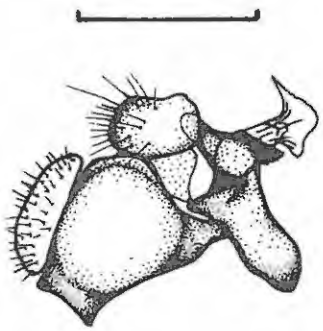
b). Betasyrphus sp. C.



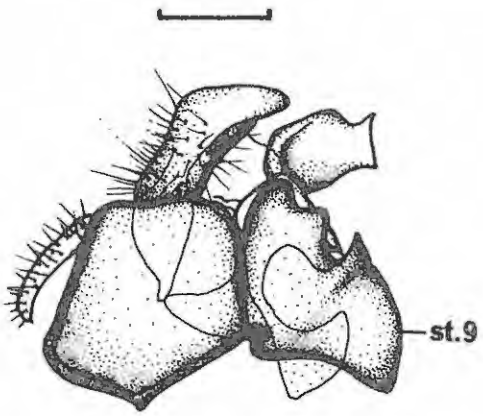
c). Betasyrphus sp. B.



d) Betasyrphus claripennis.



e). Allograpta rotundicornis



f). Metasyrphus cognatus.

Figure 2. Male terminalia of Betasyrphus species A, B, & C, B. claripennis, Allograpta rotundicornis and Metasyrphus cognatus. Lateral views of segment 9 and associated structures with ventral view of sternite 9 on right of a) & c). Interpreted according to Vockeroth (1969). Dorsal = left of lateral view; scale lines = 0,5mm; st.9 = sternite 9; s.l. = superior lobe; ae.b. = aedeagal base.

suggested by Dr. B.R. Stuckenberg, personal communication). Differences in the male terminalia also helped distinguish Betasyrphus species A, B and C. Male terminalia of B. claripennis, M. cognatus, I. aegyptius and A. rotundicornis were also examined for a comparison with the figures of the same or closely allied species in Vockeroth (1969).

Male genital capsules (segment 9 and associated structures, Vockeroth, 1969) were removed from fresh syrphid specimens with a needle, or from pinned specimens after the tip of the abdomen was softened in hot water. The isolated genitalia were cleared in 10% potassium hydroxide for 24 hours (Fluke, 1950). The cleared genitalia were examined in glycerine under a binocular microscope. To expose the superior lobe the tergite and sternite of segment 9 were pulled slightly apart. The prepared genital capsules were drawn with the aid of a camera lucida. The structure of the male terminalia was interpreted according to Vockeroth (1969). Male terminalia of Betasyrphus species A, B and C, B. claripennis, M. cognatus and A. rotundicornis are illustrated in figure 2 in the orientation of Fluke (1950) as a lateral representation of tergite and sternite 9 and associated structures. Two ventral views of sternite 9 are also illustrated in figure 2 to show the difference in depth of the notch in sternite 9 between Betasyrphus species A and B. The very large, exposed terminalia of I. aegyptius compared exactly with the illustration of the terminalia of I. aegyptius in Vockeroth (1969, figure 63), and so are not illustrated here. A. rotundicornis has male terminalia (figure 2e) which are very similar to the figure in Vockeroth (figure 85) of the terminalia of the American A. obliqua. Sternite 9 of M. cognatus terminalia (figure 2f) is more elongate than sternite 9 figured by Vockeroth (figure 30) of the closely allied European M. corollae, otherwise the terminalia are similar.

The genitalia of Betasyrphus species A and C (figure 2a and b) are similar and resemble the typical Betasyrphus condition with a deep notch in sternite 9 as figured by Vockeroth (figure 37) for B. serarius, the type species of the genus. B. claripennis (figure 2d) also has a deeply incised ninth sternite. Betasyrphus species B is clearly distinguished by the shallow notch in sternite 9 (figure 2c) and by the thin elongate superior lobe and enlarged, round, hooked aedeagal base.

Although Betasyrphus species A and C have similar male terminalia the two species may be separated by characteristic differences between features of the head. Species A has an extensively glossy black frons and antennal tubercle with a region of gold dusting clearly limited to the eyes. The gold dusting covers no more than one third of the frons and tubercle. The eyes in the male of species A are joined for a distance longer than the length of the ocellar triangle and the general body pile and scutellar hairs are dark yellow. Species C is distinguished as the frons and antennal tubercle is almost completely covered with gold dusting and the eyes are joined in the male for a distance shorter than the length of the ocellar triangle. The face of species C is also produced more than that of species A, and is black haired. The face of species A is yellow haired. These features were compared on a series of 20 specimens of species A and 4 of species C, reared from larvae collected from colonies of A. nerii on G. physocarpus.

In Kenya, Schmutterer (1974) encountered B. claripennis, B. hirticeps, B. adligatus and three unidentified Betasyrphus species allied to B. adligatus. The Betasyrphus species of Schmutterer's work were identified by Dr. Vockeroth in Canada. It would therefore have been interesting to know Vockeroth's opinion on the identities of the

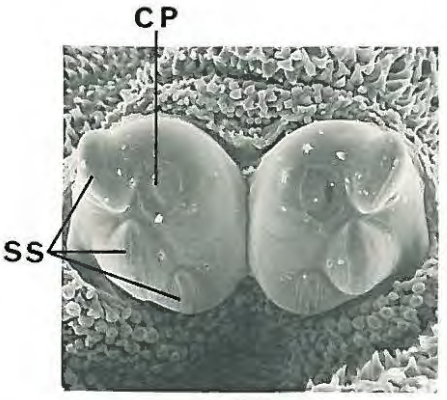
Grahamstown Betasyrphus species particularly in the light of comparison of Kenyan and South African syrphid material. Unfortunately Schmutterer and Vockeroth did not reply to questions on the criteria used for identification of the Kenyan Betasyrphus species, although Dr. Vockeroth did mention in a letter that he has a tentative key to the Kenyan Betasyrphus species.

Schmutterer investigated aphid-syrphid associations in Kenya and hence also notes larval characteristics in his 1974 paper. Characteristics of the developmental stages can be used to identify syrphid material collected from aphid colonies.

Identity of developmental stages - Schmutterer (1974) describes the larvae of his Betasyrphus species "B" and "C" as light to dark grey with whitish, dorsal cross lines. The larvae of species "C" are distinguished with a browner ground colouring than species "B". This description agrees with the appearance of the arbitrarily named Betasyrphus species A and C of this work, which were found commonly on A. nerii-infested G. physocarpus. Larvae of Betasyrphus species A were as common as the bright green larvae of I. aegyptius in A. nerii colonies. Betasyrphus species A probably corresponds with Schmutterer's Betasyrphus species "B". Species C of this work may also equal Schmutterer's Betasyrphus species "C".

Characteristic features of eggs, larvae and puparia have been used to identify and separate syrphid species. Scott (1939) and Chandler (1968) used the differences in chorionic sculpturing of aphidophagous syrphid eggs to separate species. The white eggs of aphidophagous syrphids are elongate oval in shape, with an inconspicuous micropyle, and

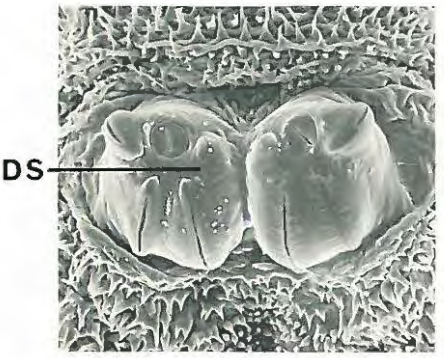
x100



x100

a) Ischiodon aegyptius.

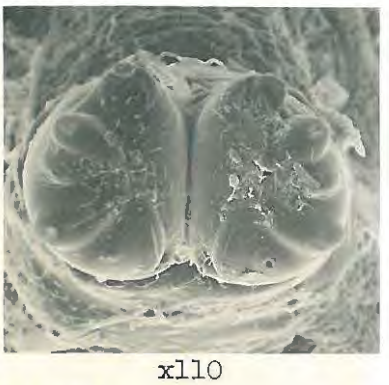
x130



x110

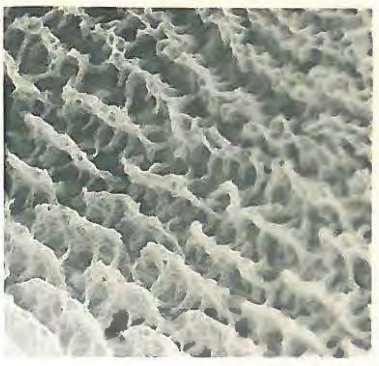
b) Metasyrphus cognatus.

x70



x110

c) Allograpta rotundicornis.



x500

d) I. aegyptius.

x500

e) M. cognatus.

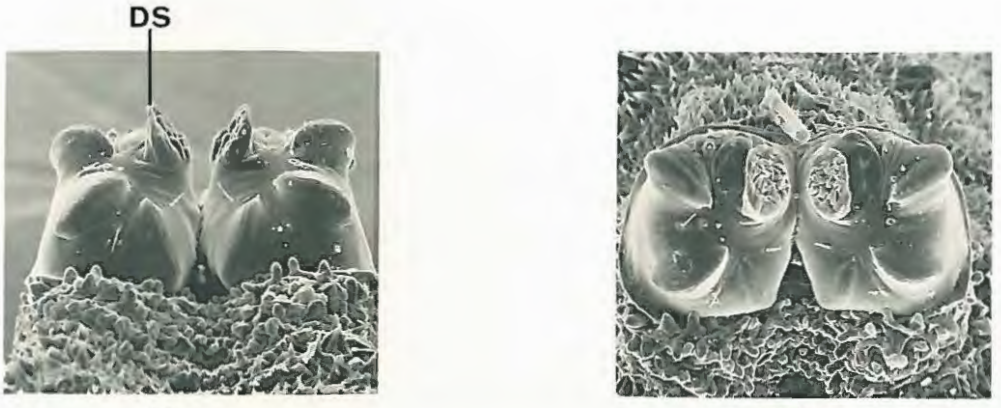
Figure 3. Scanning electron micrographs of the posterior respiratory process of the puparia of three aphidophagous syrphid species (a,b & c) and the dorsal chorionic sculpturing of the egg of two of the syrphid species (d & e). Ventral view of respiratory process on left & spiracular plate on right of a,b & c. (CP = circular plate; SS = spiracular slits; DS = dorsal spur).

are attached by the flattened ventral surface. The dorsal surface of the egg shows species-specific chorionic sculpturing and patterning.

Features of the fused posterior respiratory process of third instar larvae and the unchanged respiratory process of the puparia were used to identify larvae and pupae of aphidophagous syrphids by Dixon (1960), Hartley (1961), Heiss (1938) and Scott (1939). The surface of the spiracular plate of the posterior respiratory process show species differences in the form of the three pairs of spiracular openings, dorsal spur and circular plate (Heiss, 1938).

Scanning electron micrographs of the ventral surface and spiracular plate surface of the posterior respiratory processes (gold/palladium coated) of Betasyrphus species A, B and C, B. claripennis, I. aegyptius, M. cognatus and A. rotundicornis are given in figures 3 and 4. I. aegyptius and M. cognatus laid eggs in the laboratory, therefore, a comparison of scanning electron micrographs of a portion of the dorsal chorionic sculpturing of eggs (dried, Au/Pd coated) of the two species is illustrated in figure 3. The eggs of these two species are approximately 0.9 mm long and can be readily distinguished by the differences in the side branches of the axis that characterise the chorionic sculpturing (Chandler, 1968). The side branches of the M. cognatus chorion are more discrete (figure 3e) and not as interlaced as the longer side branches from the axes of the I. aegyptius chorion (figure 3d).

The posterior respiratory processes illustrated in figures 3 and 4 are characteristic for each species. The puparia of I. aegyptius and A. rotundicornis (figure 3a and c) do not have a dorsal spur on each posterior spiracular plate (compare with figure 4a). The three



x80 x80

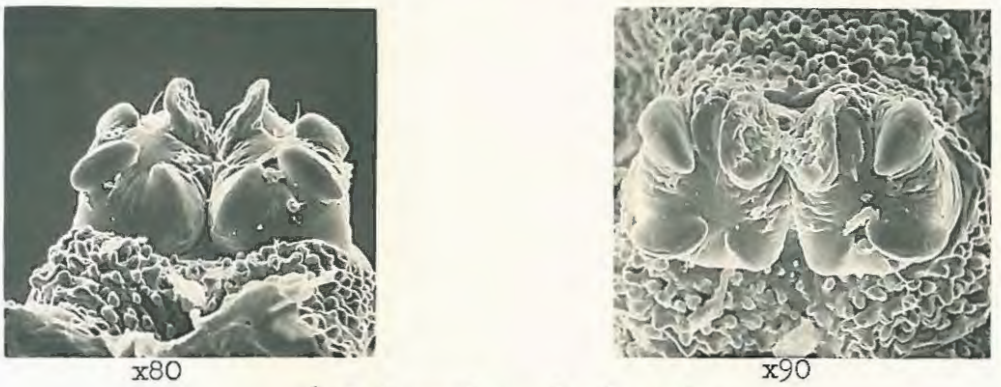
a) Betasyrphus species A.

x100 x100

b) Betasyrphus species B.

x100 x100

c) Betasyrphus species C.



x80 x90

d) Betasyrphus claripennis.

Figure 4. Scanning electron micrographs of the posterior respiratory process of the puparia of four Betasyrphus species. Ventral view of respiratory process on left & spiracular plate on right. (T = dorsal, median patch of tubercles; DS = dorsal spur).

spiracles of each spiracular plate of I. aegyptius are aligned approximately parallel and the circular plate is obvious (figure 3a). The circular plate of A. rotundicornis is indistinct and the spiracles are aligned radially (figure 3c). The whole respiratory process is much more produced in A. rotundicornis than the process of I. aegyptius which sits in a depression at the postero-dorsal tip of the puparium. The puparia of M. cognatus (figure 3b) are distinguished by the very large slits in the globular spiracles and the small dorsal spurs next to the circular plates of the respiratory process.

Each of the Betasyrphus species have large dorsal spurs (figure 4). The smooth dorsal spurs of Betasyrphus species C are the most produced and those of species B are the smallest (figure 4b and c). Betasyrphus species B is further characterised by the dorsal, median patch of tubercles on the posterior respiratory process (figure 4b). The spiracular plates of the puparia of B. claripennis (figure 4d) and Betasyrphus species A (figure 4a) are quite similar with very rough dorsal spurs. The two species can be distinguished as the spurs of species A are clearly separated and those of B. claripennis are contiguous.

The configuration of the posterior spiracular plates enabled larvae collected in the field to be identified readily. The differences in the dorsal spurs of the puparia support the division of the B. adligatus-like syrphids into three groups. The posterior respiratory process also further characterised I.aegyptius, A. rotundicornis, M. cognatus and B. claripennis.

It was unfortunate that both Dr. Vockeroth and Dr. Stuckenberg were unable to confirm the identities of the local aphidophagous Syrphini.

Nevertheless, the identities of I. aegyptius, A. rotundicornis and M. cognatus are in little doubt. However, there is doubt as to the identity of the Betasyrphus species, for at least two of the species are probably undescribed. The division into three B. adligatus-like species and B. claripennis for the most common local Betasyrphus species is as far as the identification could be taken without type comparison and species description.

3.A. BIOLOGY OF THE ASCLEPIAD-APHID-SYRPHID ASSOCIATION

Asclepiads - The climax order Apocynales is renowned for the large quantities of cardiac glycosides present in the roots, stems, leaves and seeds of most species (Paris, 1963). These toxic steroids are used as an effective defence strategy by the Apocynales against herbivorous animals (Culvenor, 1970; Fraenkel, 1959). The asclepiad/ Aphis nerii/Ischiodon aegyptius-association was chosen as cardiac glycosides of the apocynale family Asclepiadaceae may be available to the aphidophagous larvae of I. aegyptius via the phloem-feeding, host specific aphid, A. nerii.

Aphid - Aphis nerii was considered particularly suitable as the link between syrphid and asclepiad as it is restricted to feeding on Apocynales (Patch, 1938). Rothschild et al. (1970a) have shown that the bright yellow, possibly aposematic, A. nerii (see figure 1) ingests and stores some of the cardiac glycosides present in the host plants. This aphid therefore provides an interesting means for the transmission of cardiac glycosides from asclepiad to syrphid. A. nerii is also very widespread and may form a significant link between aphidophagous syrphids and toxic asclepiads in many parts of the world, for example, Brazil (Brown et al., 1969), Israel (Avidov and Harpaz, 1969; Rothschild et al., 1970a), India (Das, 1918), Egypt (Ismail and Swailem, 1971), East and West Africa (Eastop, 1958; 1961), South Africa (Müller and Schöll, 1958) and Australia (Eastop, 1966). Eastop (1966) also records A. nerii from North America and Pacific and Caribbean Islands.

The identity of the locally collected A. nerii was verified by Dr. V.F. Eastop of the British Museum (Natural History) in London, where specimens of A. nerii from five, local, named asclepiad species

have been deposited.

Syrphid - Despite the aposematism of A. nerii, aphidophagous syrphid larvae have been recorded as predators of the aphid on various Apocynales in Israel, Egypt and India (Avidov and Harpaz, 1969; Das, 1918; Ismail and Swailem, 1971). Interestingly I. scutellaris, which is similar to I. aegyptius, is recorded by Bhatia and Shaffi (1932) as a predator of 'aphis on Ak (Calotropus)' in India. Das (1918) describes A. nerii in the vernacular as 'Ak ka tela' which feeds on the asclepiad Calotropus gigantea. Therefore the 'aphis on Ak' of Bhatia and Shaffi is almost certainly A. nerii. In Kenya, Schmutterer (1974) lists larvae of the syrphid Betasyrphus claripennis as a predator of A. nerii on the asclepiad Gomphocarpus symphocarpus.

My study of the asclepiad-aphid-syrphid association began with field observations of the occurrence of A. nerii and aphidophagous syrphid eggs and larvae on the locally common, indigenous asclepiad Gomphocarpus physocarpus E. Meyer. This study developed in the laboratory after a successful technique for the culture of I. aegyptius on the asclepiad-A. nerii association was established.

a) Field Techniques and Observations.

The number of syrphids in a population of A. nerii on G. physocarpus was counted each month for a year (1974) and the presence of aphidophagous syrphid larvae and eggs in the fluctuating colonies was recorded. The plants were located in a cleared area of the Alexandria forest reserve (at 33°42,5'S; 26°21,8'E) 80 kilometres south of Grahamstown. The cleared area, one kilometre square and sheltered on all sides, was evenly covered with up to a thousand G. physocarpus plants of approximately

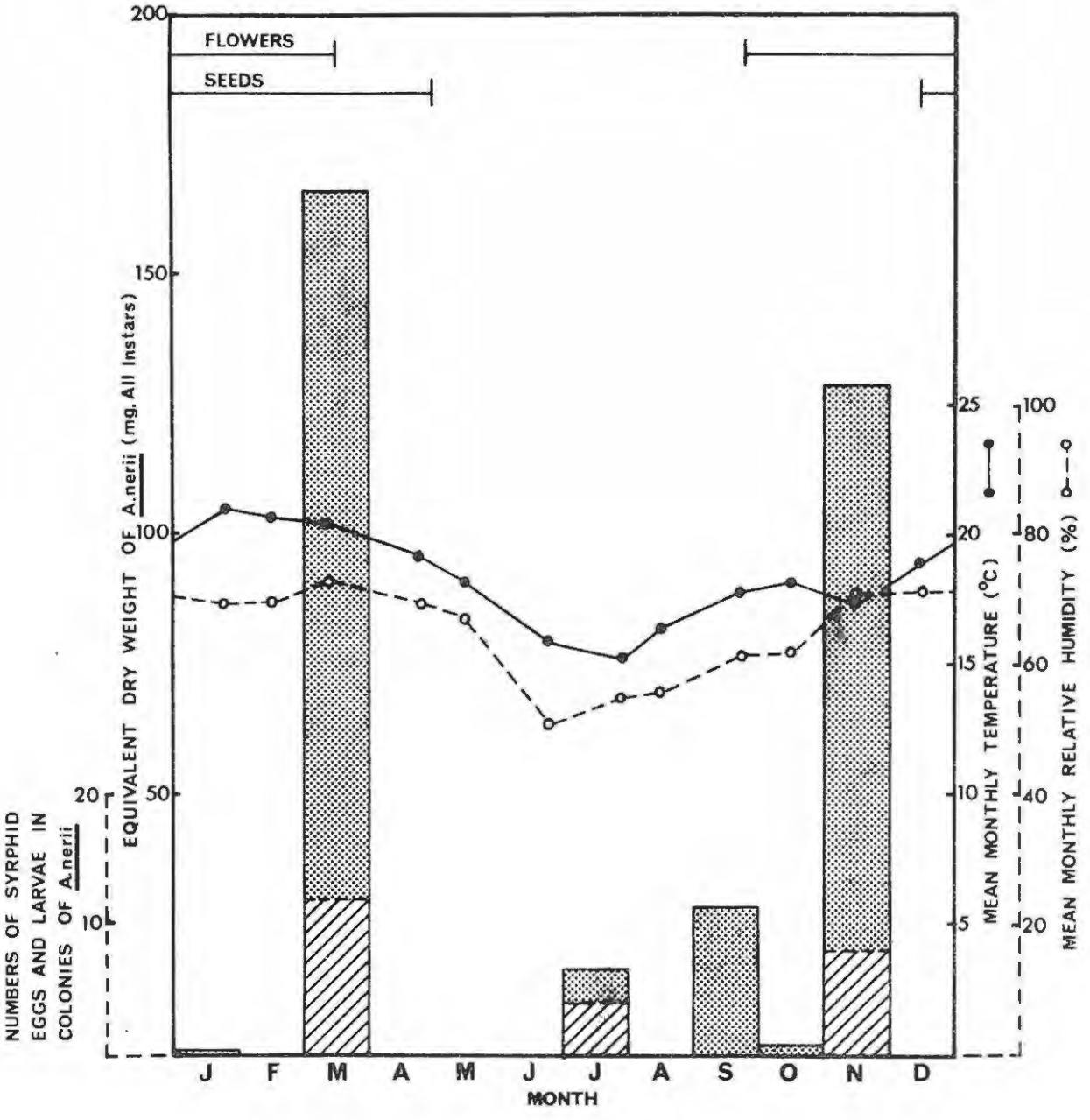


Figure 5. Monthly fluctuation (1974) in the size of Aphis nerii populations and numbers of syrphid eggs and larvae on the apical 100 mm of all shoots of Gomphocarpus physocarpus plants in the Alexandria forest. Aphid numbers are given in the stippled histogram as dry weight of aphids. Total numbers of syrphid larvae and eggs in the aphid colonies are represented in the hatched histogram. The mean monthly temperatures (°C) and mean monthly relative humidities (%) are plotted over the histograms along with the flowering and seeding period of G. physocarpus.

one metre in height. Eight numbered G. physocarpus plants from 0,45m to 1,36m in height were selected randomly and all aphids and syrphid eggs and larvae were counted on the apical 100mm of all branches. The same eight plants were recorded for the first seven months until destroyed by extensive clearing for conifer replanting. G. physocarpus plants surviving nearby were then recorded in the same manner. As the numbers of branches per plant did not vary and the aphids are restricted to the flush points of the branches, the aphids and syrphid eggs and larvae counted, approximate an absolute population estimate on the plants recorded (Southwood, 1966).

Total aphid numbers only gives an estimate of aphid populations regardless of instar. Therefore the aphid numbers were grouped according to instar, which prevents large numbers of first and second instar aphids prematurely indicating a large aphid infestation of little nutritional value to syrphid larvae. The colonies of A. nerii were recorded as grouped first with second and third with fourth instars, and the fifth instar alates and apterae were recorded separately. The dry weights of whole aphids in each group were multiplied by their numbers to give the monthly quantity of aphids shown graphically in figure 5.

Monthly temperature and humidity fluctuations were monitored for a week each month with thermohygrograph tracings recorded in a Stephenson screen next to the numbered asclepiads. The mean monthly temperature (^{o}C) and mean monthly relative humidity (%) are represented in figure 5.

The size of A. nerii colonies on G. physocarpus in the Alexandria forest showed two major peaks during the year in March and November, with two smaller peaks in July and September, and a few scattered aphids in

January and October. The principal aphid populations occurred at the beginning and end of flower and seed formation (figure 5).

Aphidophagous syrphid eggs and larvae were only found among colonies of A. nerii in the March, July and November populations of the aphid (see figure 5). A. nerii showed a markedly clumped distribution which produced localised aggregations of aphids and syrphid eggs and larvae. For example in November only 4 out of 42 G. physocarpus plants were infested with A. nerii colonies. Despite these localised aggregations of aphids, syrphid egg and larval numbers on G. physocarpus were very low in the Alexandria forest; 12 syrphid eggs and larvae in March, 4 in July and 8 in November (see figure 5).

The syrphid species collected as larvae in A. nerii colonies on G. physocarpus in the Alexandria forest were, Paragus longiventris, P. tibialis, Betasyrphus claripennis and Betasyrphus species B. Other localised groups of G. physocarpus plants by roadsides and in cleared areas around Grahamstown carried a similar syrphid fauna in the colonies of A. nerii but with the inclusion of larvae of I. aegyptius and Betasyrphus speciee A and species C, and occasionally larvae of Allograpta rotundicornis and P. minutus. Betasyrphus species C and I. aegyptius larvae were particularly numerous in localised A. nerii colonies near Grahamstown.

b) Laboratory Techniques and Observations.

The culture of all of the commonest local aphidophagous syrphide (Betasyrphus species A, B and C, B. claripennis, A. rotundicornis and M. cognatus) was successful in the laboratory. However, success was

eventually achieved in the laboratory culture of I. aegyptius. The culture of I. aegyptiue made possible the investigation of the presence of cardiac glycosides in the asclepiad-aphid-syrphid association (chapter 3B) and the effect of the asclepiad/aphid-reared syrphids on potential predators in the pharmacology of the syrphid extracts on frog and chamaeleon hearts (chapter 3C). The culture technique also allowed development data to be gathered for I. aegyptius which I considered would indicate any potential toxicity of different asclepiad-A. nerii associations to I. aegyptius. As the cardiac glycosides in asclepiads are a defensive strategy against herbivory, to which A. nerii is adapted, a range of defensive steroids in different asclepiad-A. nerii associations could be variably toxic to I. aegyptius.

Culture techniques have been described for aphidophagous syrphids by Adashkevich and Karelin (1972), Fluke (1937), Frazer (1972) and Schneider (1969). Fluke states that a diet of water and a paste of dried yeast and honey is necessary for the adults and suitable aphids are necessary for the larvae. Both Frazer and Schneider indicate that pollen is necessary as an adult protein source, with a provision of carbohydrate for the maturation of ovaries. Adashkevich and Karelin also provided sugar and pollen for adult syrphids. Frazer followed Schneider with the provision of a diet of dried hazel pollen and sucrose for adult aphidophagous syrphids. Lal and Haque (1955) found that the syrphid Sphaerophoria (=Ischiodon) scutellaris, a syrphid closely related to I. aegyptius, showed consistently greater longevity and fecundity when fed on sucrose than when fed other sugars. Sucrose was more effective in the maintenance of adult flies than the diet of 50% dried yeast and honey recommended by Fluke. Frazer also indicates that large culture cages with a centrally supported feeding and oviposition platform are essential features for the culture of aphidophagous syrphids.

The desirable features of a culture syrphid under laboratory conditions are that the adults should readily copulate, readily oviposit, be long-lived and simple to feed. It greatly helps manipulation of the culture if the larvae are tolerant of handling, will accept the aphid prey presented and in the form presented, show a short life cycle and do not diapause. I. aegyptius satisfies all these points admirably and has proven a very simple fly to maintain in culture. A. nerii has also proven particularly suitable as a prey aphid for the maintenance of I. aegyptius larvae as it is not easily dislodged from the plant. The aphid is also large and forms dense, closely packed, sedentary colonies if the plant is in good condition, which presents an easily accessible source of prey aphids for the larvae of I. aegyptius. The sedentary nature of A. nerii also eliminated the problem of oviposition by female syrphids on the sides of culture cages near wandering aphids.

Culture technique - I. aegyptius was reared on the aphid A. nerii which infested three plant species of the family Asclepiadaoeae. Obviously, as Schneider (1969) describes, a culture of aphids is a prerequisite for the maintenance of a population of an aphidophagous syrphid. The population of I. aegyptius larvae was maintained on separate cultures of A. nerii on the asclepiads, Gomphocarpus physocarpus E. Meyer (= Asclepias physocarpa (E. Mey.) Schltr.), Asclepias curassavica L., and Araujia sericofera Brotero. G. physocarpus is an indigenous asclepiad and As. curassavica and Ar. sericofera are exotics introduced from South America; all three species are common in Grahamstown. Dr. A. Jacot-Guillarmod of the Institute of Freshwater Studies, Rhodes University, suggested the identity of the plants, which were kindly verified by David Spellman at the Missouri Botanical Garden, U.S.A., where voucher specimens of the three asclepiad species have been deposited in

the herbarium. Dr. Spellman also pointed out that the name Ar. sericofera is the correct version of Ar. sericifera printed in Index Kewensis and the majority of works that describe the plant.

As a non-asclepiad/A. nerii-reared comparison in the chemistry and pharmacology tests described in the following two sections, I. aegyptius was also reared on two unrelated plant-aphid associations, which were chosen to exclude the presence of cardiac glycosides. Non-asclepiad/A. nerii-reared I. aegyptius were collected as larvae from honeysuckle, Lonicera caprifolium (L.), infested with the grey-green aphid Hyadaphis foeniculi (Passerini), and were also reared on a laboratory culture of the black aphid Macrosiphoniella sanborni (Gillette) on seedlings of Chrysanthemum morifolium (Ramat).

The aphid and syrphid cultures were maintained in a controlled environment room (C.E. room) to avoid the large variation in development times and quantities of aphids attacked by the syrphid larvae previously recorded by Lal and Haque (1955) at a series of different temperature and humidity régimes. The C.E. room conditions were controlled at a day length of 14 hours at an average light intensity of 3 800 LUX and at a temperature of 24°C and 65% relative humidity. The night régime was controlled at 17°C and 85% R.H. This diurnal change in conditions approximates the natural spring and autumn conditions of temperature and humidity recorded locally and produced good plant growth, large healthy aphid colonies and a thriving I. aegyptius culture. At this diurnal temperature and humidity régime the longevity and fecundity shown by I. aegyptius was far greater than that recorded by Lal and Haque (1955) for Sphaerophoria (Ischiodon) scutellaris maintained at the constant, optimal conditions of 20°C and 70% R.H. The light intensity was also well above the minimum of 500 LUX recommended by Schneider (1969) to

favour syrphid oviposition in aphid colonies.

To begin the asclepiad/*A. nerii*-reared *I. aegyptius* culture, one hundred each of the asclepiads *G. physocarpus*, *As. curassavica* and *Ar. sericofera* were grown from locally collected seeds. Large seedlings were potted individually in 125mm and 150mm diameter plastic flower pots, in an equal mixture of soil and sifted leaf and leaf and root fibre. The plants were watered daily and fertilised with "Supra Wonder 3:2:1(30) garden fertiliser" (15% N, 10% P, 5% K) once every four weeks. *G. physocarpus* was susceptible to fungal and nematode attacks and so diseased plants were supplemented with locally collected mature specimens up to one metre in height. Healthy plants ready for aphid infestation were placed in a C.E. room, six pots to a watering tray. The watering tray consisted of aluminium painted tin with a 20mm lip, which enabled six pots to be watered simultaneously and reduced stem decay by keeping water away from the stems of the plants.

The colonies of *A. nerii* were started with healthy alate and adult apterous aphids collected from local *G. physocarpus* plants and placed individually on the three asclepiad species in the C.E. room. Once the initial aphid colonies had been built up on each asclepiad species, rapid infestation of fresh plants was accomplished by placing heavily aphid-infested leaves on each new plant of the same species. The plants were changed to allow recovery once most of the aphid infested leaves had been cut off to feed syrphid larvae.

Destruction of *A. nerii* colonies wrought by the braconid parasite *Aphidius picipes* (Nees) (?) (determined by Mr. Gerhard Prinsloo and Mr. Huddlestone at the British Museum (Natural History) in London) was prevented as all asclepiads were fumigated in a closed room for seven

Figure 6. Wood-framed culture cage covered with white nylon 'sheer' for feeding, copulation and oviposition of I. aegyptius adults. Cut flowers as a pollen source are at the front of the cage with a potted A. nerii-infested G. physocarpus plant in the background. Sucrose on moistened filter paper is in the yellow painted petri dish on the left. The sealed perspex front door is hinged and the sealed glass top lifts off.

days before introduction to the C.E. room. A Shell insecticide in the form of strips commercially termed "Vapona" (=Dichlorvos or 2,2-dichlorovinyl dimethyl phosphate) proved effective in the destruction of the aphid parasites and potential pests of the plants, which included a very troublesome mite. The air intake into the C.E. room was also covered with fine nylon "sheer" (a fine synthetic, mesh-like organdie) to prevent accidental introduction of aphid parasites.

The culture of I. aegyptius was started with 50 third instar larvae of the syrphid collected from A. nerii-infested G. physocarpus plants located 15 kilometres south of Grahamstown. The flies which emerged from the pupae of these larvae were placed on emergence in white, wood frame cages which measured 350mm x 350mm x 550mm and were covered on three sides with white nylon "sheer". The bottom of the cage was of white plywood, with the top as a sealed lift-off sheet of glass and the front as a hinged, sealed perspex door (see figure 6). This cage is considerably smaller than that described by Frazer (1972) (in contrast to the "small cages" recommended by Fluke, 1937) for the culture of aphidophagous syrphids. However, this culture cage proved most effective as a container for the syrphid culture. Pollen was supplied in the cages as fresh flowers of Lobularia maritima Desv., and various composites, particularly the Shasta daisy, Chrysanthemum maximum Rarond. Freeze dried Port Jackson willow, Acacia cyanophylla Lindl., pollen was also tried as a protein source as it is readily available, however, this pollen was not as successful in the maintenance of the culture as pollen from fresh flowers. The cut flowers, watered in 100ml beakers, were changed once a week with flowers collected from the gardens of the Department of Nature Conservation by the University, with the generous permission of the supervisor, Mr. Cameron. The adult flies were also provided with sucrose on moistened filter paper in a yellow painted petri dish on the white floor

of the cage. The yellow petri dish attracted the syrphids to their source of carbohydrate, the sucrose. I. aegyptius readily feeds on the floor of the cage and at fresh flowers and uses any available substrate as a support in mating. The central platform recommended by Frazer (1972) for oviposition and feeding of syrphids was not found necessary. The adult flies readily copulated in the cages (see figure 1). Directly after copulation a single aphid infested plant, on which the females oviposited, was introduced for two days. Each oviposition period was separated by a day to allow further ovarian development. This oviposition cycle was carried out regularly as females lay eggs wherever aphid honey-dew has fallen - on the sides of culture cages and on flowers - if no aphid colonies are present. To prevent this non-productive oviposition the cages were cleaned frequently to remove deposits of aphid honey-dew. Clean cages were necessary for, according to Dixon (1959), gravid female aphidophagous syrphids lay eggs in response to olfactory stimuli provided by chemical evidence of aphid presence.

The eggs of I. aegyptius hatched on the aphid-infested plants and after a day the larvae were large enough to transfer easily without damage. Four first instar larvae were removed with a moist, fine (no. 0) sable brush to a washed plastic petri dish and supplied with freshly cut asclepiad stems and leaves infested with A. nerii. Larger numbers of intraspecifically competing syrphid larvae per petri dish results in mortality from cannibalism (particularly among 1st and 2nd instar larvae), starvation and infection (Hågvar, 1973). Hågvar (1973) also states that starvation of aphidophagous syrphid larvae produces an artificially lengthened development period and a reduction in pupal weight. A. nerii was provided as larval food on cut leaves and stems of G. physocarpus, As. curassavica and Ar. sericofera. The petri dishes were prepared with a 65mm diameter hole cut in the lid and covered with white nylon "sheer"

for ventilation. The larvae develop rapidly over eight days and were allowed to pupate in the petri dishes. The larvae and pupae in the petri dishes were stored in flat wooden trays, five dishes to a tray in the C.E. room. All trays were equally exposed to the same light intensity. Fresh larval food was supplied each day to avoid starvation and to prevent the sticky, waxy accumulation of asclepiad leaves, which, combined with aphid honey-dew effectively trapped I. aegyptius larvae and resulted in death from starvation or infection. The pupae of I. aegyptius were anchored to a small section of plant material to facilitate the successful emergence of adults. Once the adult has split open the puparium case, by expansion of the head from the ptilinal suture, the fly is able to pull itself out of the pupal case with the fore legs, which require a surface to cling to. The smooth surface of the petri dish does not allow sufficient anchorage for the fly to emerge, therefore the leaf is necessary to complete adult emergence. The adults emerged in the petri dishes where there is sufficient space for expansion and hardening of the wings and body of the four adult flies. Adults were then transferred to the breeding cages. The newly emerged adults were provided with the moist sucrose and pollen and after a day began to copulate. After a further two days the females began to oviposit on the aphid-infested plants introduced into the culture cages. Oviposition reached a peak after a week when individual females laid up to 50 fertile eggs a day and remained productive for a further two weeks.

Populations of I. aegyptius were divided after the first generation and were reared for a total of 12 generations on each of the G. physocarpus, As. curassavica-, Ar. sericofera-A. nerii associations. These populations showed no superficial differences in appearance or mortality. Excess asclepiad-derived syrphids, over those needed to maintain the breeding populations of approximately ten syrphids in each

(with Lonicera- and Chrysanthemum-derived flies), were collected and stored at -15°C immediately after emergence. These stored I. aegyptius were used for the analysis of cardiac glycoside content and pharmacological activity described in the following two sections of this dissertation.

Syrphid parasites - Of the third instar I. aegyptius larvae collected from local A. nerii-infested G. physocarpus that started the laboratory culture of I. aegyptius, 50% were parasitised. The parasites that emerged were determined by Gerhard Prinsloo whilst at the British Museum (Natural History) in London. A figitid, Anacharoides sp. and the ichneumonid, Diplazon laetatorius Fabr., equally accounted for the parasites that emerged from the pupae. An encyrtid parasite, Syrphophagus sp. and a ceraphronid, Trichosteresis sp., also emerged from I. aegyptius pupae, collected from Rosa sp. infested with the aphid Macrosiphum rosae. Isolation of the larvae in petri dishes prevented these parasites from attacking subsequent generations of syrphid larvae. The covered air intake into the C.E. room also prevented accidental introduction of syrphid parasites into the cultures. Syrphid parasites were therefore simple to eliminate and did not present a problem in the cultures.

Development of I. aegyptius - The development of I. aegyptius, reared in culture on two of the three asclepiad-A. nerii associations in relation to the quantity of aphid material ingested by the larvae, was recorded to determine any potential intraspecific differences in the development of the syrphid. Developmental differences could result from different degrees of toxicity of the two asclepiad-A. nerii associations to the syrphid. The development data were also used for a comparison with those of another polyphagous syrphid aphidophage, Metasyrphus cognatus

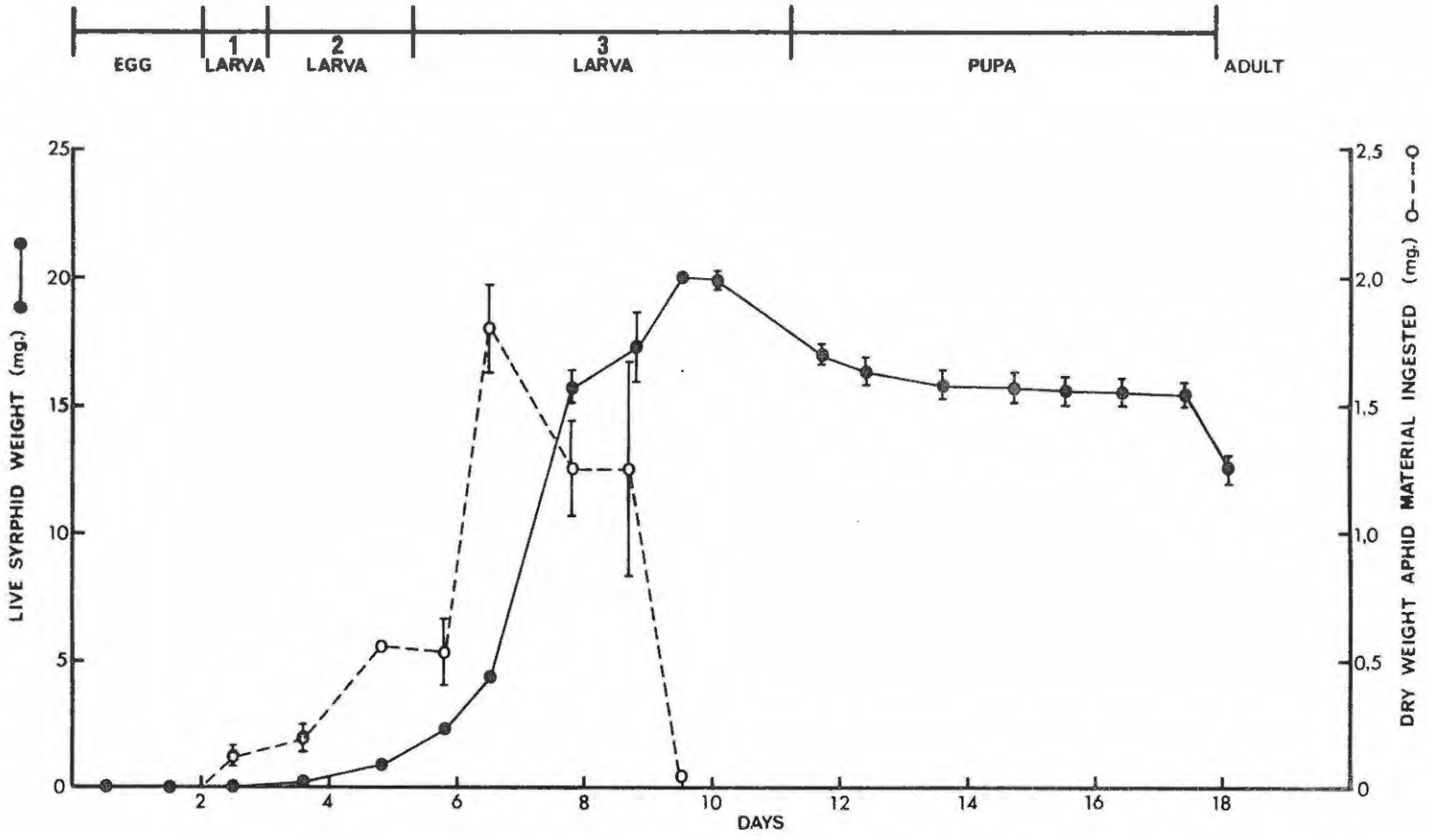


Figure 7. Development of I. aegyptius reared on A. nerii on G. physocarpus. Live weight changes of I. aegyptius are shown from oviposition to adult emergence, with the duration of egg, larval instar and pupal period indicated above the graphical representation of development. Dry weights (mg) of aphid material ingested by I. aegyptius larvae are also given when reared on the A. nerii-G. physocarpus association (means of values ±1 S.E. for 5 flies).

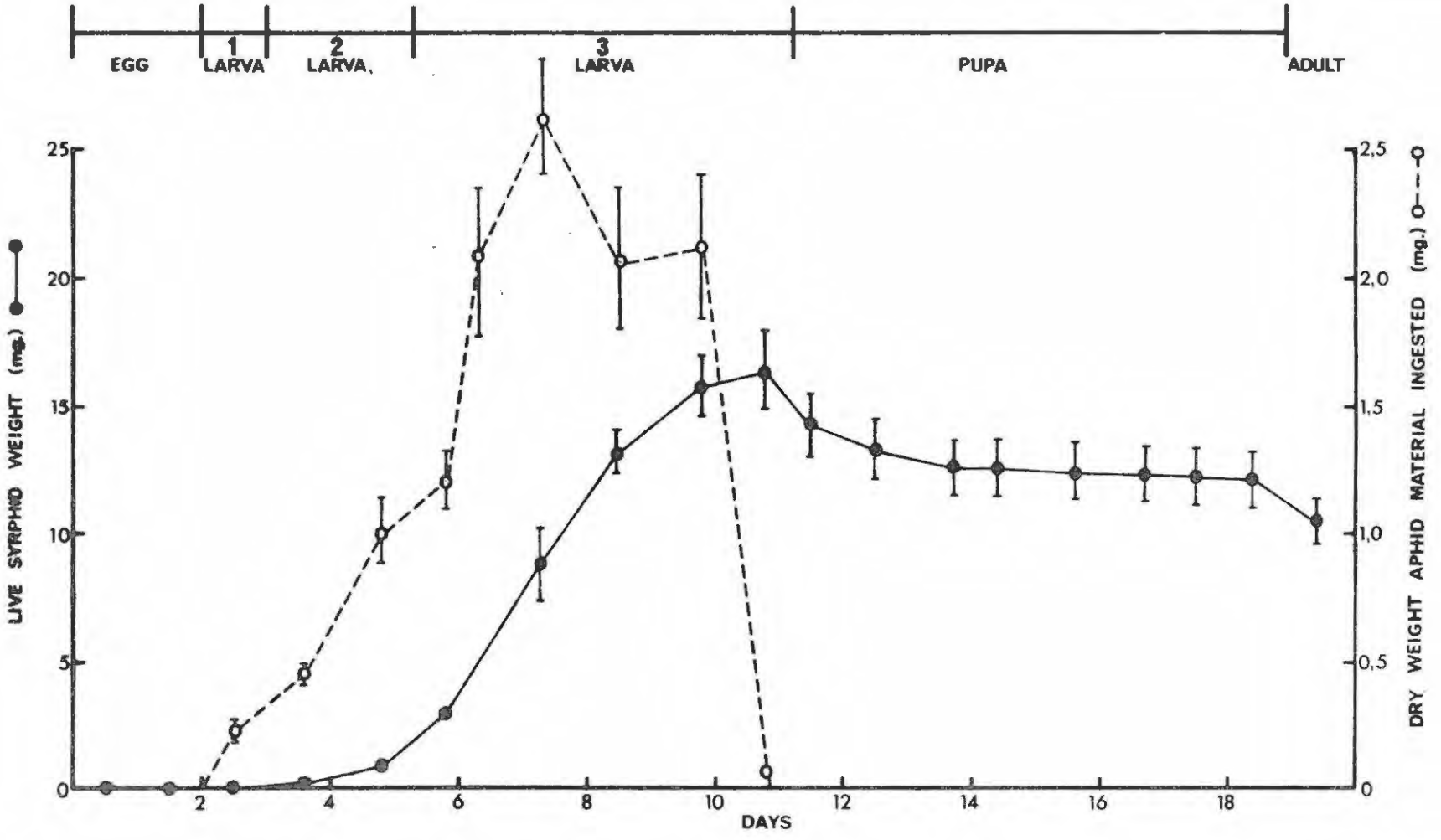


Figure 8. Development of I. aegyptius reared on A. nerii on Ar. sericofera. Live weight changes of I. aegyptius are shown from oviposition to adult emergence, with the duration of egg, larval instar and pupal period indicated above the graphical representation of development. Dry weights (mg) of aphid material ingested by I. aegyptius larvae are also given when reared on the A. nerii-G. physocarpus association (means of values ±1 S.E. for 5 flies).

to determine any interspecific differences in the development of another syrphid species on the asclepiad-A. nerii association.

The development of I. aegyptius reared on A. nerii on G. physocarpus and Ar. sericofera in the controlled environment room conditions is represented graphically in figures 7 and 8, as live weight change of eggs, larvae, puparia and adults. All stages of the syrphid from shortly after egg deposition to adult emergence were weighed daily on a Cahn G.2. Electrobalance (Ventron Instruments Corporation, Paramount, California, U.S.A.). The weights are given as milligrams (mg.) of live insect and are plotted in figures 7 and 8 as mean daily weights ($\pm$1 S.E.) for each of ten I. aegyptius; the developmental weight changes of five I. aegyptius were recorded on the G. physocarpus-A. nerii association (figure 7) and the other five were recorded on the Ar. sericofera-A. nerii association (figure 8).

The duration of the developmental stages of I. aegyptius were remarkably constant, eggs hatched two days after oviposition, larvae averaged nine days to puparium formation and the adult emerged eight days later. The first instar larvae cast their characteristically spined exuviae after one day and the second instar larvae ecdysed after a further two days. Six days are then left for the completion of larval development in the third instar. It is of interest to note that Barbosa (1953) records, for I. aegyptius reared on Aphis gossypii in Mozambique, longer development periods of three and ten to thirteen days for eggs and larvae and a shorter pupal duration of five to seven days.

The larvae of I. aegyptius have two colour forms, one dark to pale brown and the other a pale green. Both forms are otherwise developmentally and morphologically identical and are marked with a conspicuous white,

when the syrphid larvae were weighed and transferred to a clean petri dish with fresh aphid-infested leaves. The attacked aphids were preserved in the previous days' petri dish with 70% alcohol and subsequently counted and recorded. The quantity of A. nerii material ingested each day by I. aegyptius larvae was measured as ingested dry weights of 1st, 2nd, 3rd, 4th and 5th instar apterae and alatae (A. nerii has 5 instars). Entire and attacked aphids were simple to separate as I. aegyptius larvae of all three instars attack A. nerii in a similar manner. The syrphid larva pierces the aphid with anterior mouth hooks, lifts the aphid from the leaf substrate and supports the secured aphid in a cup formed by the withdrawal of the first two or three body segments within the immediately posterior segments. The aphid is held in this position until the rapid probing of the mouth hooks has removed the contents of the body, legs, head and antennae. After this attack procedure the aphid has a very characteristic collapsed appearance and a very large conspicuous puncture in the crumpled test. To obtain the dry weight of aphid material ingested daily by I. aegyptius larvae, the number of attacked aphids was multiplied by the difference between the dry weight of entire and the dry weight of attacked A. nerii, for each instar. The figure for dry weight of A. nerii material ingested by I. aegyptius larvae was recorded on each asclepiad by weighing 100 of each instar of both intact and attacked aphids. The difference in weight between intact and attacked aphids gives a figure for aphid material ingested by I. aegyptius larvae, per aphid of each instar. Alate and apterous fifth instar aphids were recorded separately as they have a considerable weight difference.

The total numbers of A. nerii attacked by I. aegyptius larvae were of interest in that under the same conditions the larvae attacked a mean number of 349 A. nerii on G. physocarpus, which represents 5,8mg

of dry aphid material ingested, but the larvae attacked a mean number of 483 A. nerii on Ar. sericofera, which represents 11,9mg ingested. These values are obviously very different (Student's t-test indicates probabilities of $0,01<p<0,025$ and $0,001<p<0,005$ of the compared total numbers and weights, respectively, being the same). This difference in the change in weight of aphid material ingested by I. aegyptius larvae fed on the two asclepiad-A. nerii associations is reflected by the differences in area under the curves for aphid material ingested on G. physocarpus and Ar. sericofera indicated in figures 7 and 8. Interestingly I. aegyptius reared on the Ar. sericofera association shows a higher level of aphid material ingested but a lower overall development weight, than I. aegyptius reared on the G. physocarpus-A. nerii association. I. aegyptius seems able to cope with toxins present in the asclepiad/A. nerii association. However, there is an indication of some difference in the two different asclepiad/A. nerii associations as larval food for I. aegyptius. The Ar. sericofera/A. nerii association is apparently less valuable nutritionally to I. aegyptius than the G. physocarpus/A. nerii association.

Comparison of the development of I. aegyptius and M. cognatus - I. aegyptius is a polyphagous aphidophage, as it was found and is recorded as a predator of many aphids on a wide range of plants. I. aegyptius is described by Schmutterer (1974) as the most polyphagous aphidophagous syrphid species in East Africa for the larvae prey on 21 aphid species. Schmutterer (1972) offered different aphid-plant associations to various syrphid species that did not occur naturally with the association. Schmutterer found that development times and mortality apparently varied according to the toxicity of the aphid-plant association that supported the larvae of the syrphid species. In East Africa

Table 1. Comparison of development period, mean number of aphids and equivalent mean dry weight of aphid material consumed, mean maximum larval weight and mean pupal weight one day before emergence, of I. aegyptius and M. cognatus reared on the A. nerii-As. curassavica association, under C.E. room conditions.

		I. aegyptius.	M. cognatus.
Mean development period in days.	Egg	2,0	1,0
	1st instar larva	1,1	1,2
	2nd instar larva	2,1	1,0
	3rd instar larva	5,7	4,8
	Pupa	7,9	6,3
Mean total development period (days)		18,8	14,3
Mean number of aphids attacked		349[1];483[2]	367
Mean dry wt. aphid material ingested (mg)		5,75[1];11,87[2]	12,25
Mean maximum larval wt. (mg)		18,0	25,3
Mean pupal wt. 1 day before emergence (mg)		13,5	15,0

([1,2] Numbers of aphids attacked by I. aegyptius larvae on As. curassavica were not determined. These are figures from G. physocarpus (1)/ A. nerii- and Ar. sericofera (2)/A. nerii-reared I. aegyptius.)

Schmutterer (1972) found that I. aegyptius suffered from high mortality when reared on aphids on the crucifers Brassica oleracea and Mathiola sp. Daiber (1971) however, records I. aegyptius larvae as the principal predator of three aphid species on cabbage (B. oleracea) in South Africa. Hodek (1966) and Okamoto (1966) also describe the toxicity of some aphid-plant associations to aphidophages, in this case to coccinellids. At the same symposium on aphidophagous insects Iperti (1966) recorded the toxicity of A. nerii on the apocynale Nerium oleander, which is rich in cardiac glycosides (Rothschild et al., 1970a), to most coccinellid species except Adonia variegata. It seems, therefore, that there is a measure of plant-aphid tolerance shown by aphidophagous insects and it was considered of value to briefly compare the development of the polyphagous I. aegyptius with the development of M. cognatus when reared on the A. nerii-asclepiad association. Schmutterer (1974) indicates that M. cognatus is a more prey specific syrphid than I. aegyptius as the larvae were recorded as a predator of only one aphid species in East Africa. M. cognatus is an interesting syrphid to compare with I. aegyptius as the larvae were found in some aphid colonies with I. aegyptius larvae. The adults of both syrphids are also similar in size and both have similar black and yellow colouring (see figure 23).

Hågvar (1973) considers minimum pupal weights of aphidophagous syrphids to be a good expression of the utilisation of the total amount of food ingested by the larvae. Pupal weights indicate the amount of aphid material used as the third instar larvae void their complete gut contents shortly before pupation. The development of I. aegyptius and M. cognatus on asclepiad derived A. nerii is given in Table 1. as a comparison of developmental weights of the two syrphids, which includes the minimum pupal weight one day before emergence.

The comparison of the development of I. aegyptius and M. cognatus was made possible by the capture of ten M. cognatus females at colonies of aphids on willow trees. One of these females laid twenty-four eggs among a colony of A. nerii on As. curassavica in the C.E. room. The eggs hatched on the second day after oviposition. Larvae of M. cognatus were treated in the same manner as the larvae of I. aegyptius - placed in petri dishes with excess aphid food and weighed daily. Of the twenty-four M. cognatus larvae which fed on As. curassavica-derived A. nerii three died as third instar larvae, five as poorly developed pupae, nine as pupae which contained well formed flies and seven adults actually emerged. Of these seven adults one male and one female expired during emergence and another two males and one female died immediately after emergence. Two females were the only adults to survive for more than one day out of the twenty-four original larvae. From the original twenty-four fertile eggs this lack of developmental success represents a mortality of 91,7%. Table 1. compares the mean development duration of I. aegyptius and M. cognatus eggs, larvae and pupae reared on the A. nerii/ As. curassavica association. Also compared is the quantity of aphid material attacked and ingested by the larvae and the maximum larval weight compared with the minimum pupal weights recommended by Hågvar (1973) as a measure of food utilisation. Despite the extremely high mortality of M. cognatus reared on the A. nerii/As. curassavica association the larvae did ingest large numbers of aphids (a mean of 367 attacked aphids) and grew rapidly over a period of 7 days compared with almost 9 days for the larval development of I. aegyptius. M. cognatus larvae attacked a similar number of aphids (367) to I. aegyptius larvae (349 or 483) but ingested a greater quantity of aphid material (12,25 mg dry weight compared with 5,75 mg or 11,87 mg) than I. aegyptius in a shorter period of time. M. cognatus larvae seem to have utilised similar

quantities of aphid material as I. aegyptius larvae, but more efficiently to produce third instar larvae and pupae that weigh considerably more (see Table 1.). The mean maximum larval weight of M. cognatus larvae is approximately 40% higher than that of I. aegyptius larvae (see Table 1.).

The suggestions inherent in the mortality of 91,7% and in the development data of M. cognatus reared on the A. nerii/As. curassavica association, presented in Table 1., is that the A. nerii/asclepiad association is toxic to M. cognatus but not to the more polyphagous I. aegyptius. The chemistry of cardiac glycosides in the asclepiad/ A. nerii/I. aegyptius association is studied chromatographically in the following section to indicate a possible chemical basis for aposematism in I. aegyptius and the possible nature of the toxicity of the aphid/ asclepiad association to M. cognatus. The tolerance of I. aegyptius to an aphid plant system that is toxic to at least one aphidophagous syrphid indicates the value of I. aegyptius in the study of an extrinsic basis for a chemical defence system. The incredible array of plant-synthesised, potentially toxic 'secondary plant substances' and primary defensive compounds of plants (Fraenkel, 1959; 1969) is made available to the tolerant syrphid via its aphid prey. The cardiac glycosides investigated in this study may be one or a part of a range of extrinsically derivable plant toxins that could contribute to the possible chemical defence of I. aegyptius.

3.B. CHEMISTRY OF THE ASCLEPIAD-APHID-SYRPHID ASSOCIATION

The detection, separation and tentative identification of the cardiac glycosides in each of the three asclepiad species was a necessary basis for the determination of possible ingestion, storage, alteration and selective absorption of cardiac glycosides by A. nerii and I. aegyptius reared on the three A. nerii/asclepiad associations. Solid-liquid and liquid-liquid phase, two-dimensional chromatographic techniques readily separate mixtures of compounds such as steroids, and also permit tentative identification of the compounds from a comparison of their Rf values with those of known standards (Applezweig and Lewis, 1969). The presence of cardiac glycosides in asclepiad, A. nerii and I. aegyptius extracts was investigated with two chromatographic techniques. Descending, partition paper chromatography (PC), based on the formamide impregnated paper method developed for the separation of steroids by Zaffaroni et al. (1950), was used. The technique was also used by von Euw et al. (1967); Parsons (1965); Reichstein (1967); Reichstein et al. (1968); Rothschild et al. (1973b), and Ulubelen (1962) for the separation of cardiac glycosides in extracts of plant and insect material. The second technique used was adsorption thin layer chromatography (TLC), based on the methods of Stahl and Kaltenbach (1961) and Duffey and Scudder (1972), for the separation of cardiac glycosides, also in extracts of plant and insect material. These two chromatographic techniques were used as they are convenient and their value in the clear separation of a wide range of steroids, particularly cardiac glycosides, is well documented. Rf values for many cardiac glycosides chromatographed in the PC system employed in this work are published, and this enabled a comparison of Rf values to be made and hence tentative suggestions as to their identity (Stahl, 1965). The

following Rf values for cardiac glycosides and aglycones, using the tetrahydrofuran:benzene solvent system employed in this work have been published by, von Euw et al. (1967); Reichstein (1967); Reichstein et al. (1968); Rothschild et al. (1973b; 1975) and Rothschild (personal communication, 1974).

syrioside	= ca. 0,05	syriogenin	= 0,30
calotropinic acid	= 0,11	afroside	= 0,48
calotropagenin	= 0,12	corotoxigenin	= 0,52
proceroside	= 0,14	calactin	= 0,54
calotoxin	= 0,15	gomphoside	= 0,68
Strospeside	= 0,18	voruscharin	= 0,70
syriobioside	= 0,23	uzarigenin	= 0,73
syriogenin	= 0,28	deacetyl oleandrin	= 0,76
calotropin	= 0,30	uscharin	= 0,76
coroglaucigenin	= 0,30	uscharidin	= long stripe

Slightly different Rf values are also given for calotropin = 0,28, calactin = 0,50 and Uzarigenin = 0,83 (Reichstein et al., 1968).

Asclepiad cardiac glycosides - Plant cardiac glycosides are derived from two aglycone (or genin) types, termed cardenolides and bufadienolides. Both aglycone forms have similar cardiac toxic activity but the cardenolides all occur as plant glycosides and the bufadienolides are products of both plants and animals, notably in toad skin secretions (Fieser and Fieser, 1959). Many cardenolide cardiac glycosides have been isolated and characterised in a number of apocynale plants.

Of the three asclepiad species employed in this work As. curassavica is the only species that has been worked on with regard to cardiac glycoside content. However, Watt and Breyer-Brandwijk (1962) indicate that several authors have recorded that both G. physocarpus and Ar. sericofera show some toxicity characteristic of cardiac glycosides. Steyn (1933) records the death of a sheep after an oral dose of 300 gm of G. physocarpus. The toxic effects of the latex of Ar. sericofera (as Arauya serisifera) on the ear of a rabbit are described by Steyn and

van der Walt (1941).

Tschesche et al. (1958; 1959) found seven cardenolide aglycones including calotropagenin in Brazilian As. curassavica leaves and stems. Aglycones only were isolated, because extracts were made under conditions of strong enzymatic hydrolysis, which splits the glycoside or sugar from the aglycone. Only one glycoside, uzarin, was present in the Brazilian material. Different results are reported by von Euw et al. (1967), Reichstein (1967) and Reichstein et al. (1968) for As. curassavica leaves from Trinidad. These workers found a series of calotropin glycosides, based on the aglycone calotropagenin, and smaller quantities of other cardenolides in extracts of the plant. Kupchan et al. (1964) isolated calotropin from leaves of As. curassavica grown in Costa Rica and Mexico. Seven other cardiac glycosides were also found. In 1969 Singh and Rastogi reported the isolation of three glycosides of calotropagenin, together with three other aglycones and four of their glycosides out of twenty-two cardiac glycoside-positive compounds detected in As. curassavica grown in India. Dr. Miriam Rothschild very kindly sent some seeds (0,47 gm) of her 'Oxford' strain of As. curassavica ('OXF'), which originally came from seeds collected in Trinidad by Professor L.P. Brower. As mentioned above Reichstein (1967) and Reichstein et al. (1968) investigated the same Trinidad material and found the calotropin glycosides uscharin, uscharidin, calactin and calotropin along with smaller quantities of other cardenolides. Large quantities of calotropin, uzarigenin and a little proceroside were also found in As. curassavica by Rothschild et al. (1975). Further information on the cardiac glycoside content of As. curassavica leaves was promised by von Euw et al. (1967), Reichstein (1967), Reichstein et al. (1968) and Rothschild et al. (1975) to be contained in a paper by Santavy, von Euw and Reichstein.

Unfortunately, this work is not recorded in Chemical or Biological Abstracts from 1966 to 1975. The seeds of As. curassavica sent by Dr. Rothschild were particularly useful as it was possible to compare their cardiac glycoside content with the published findings of Reichstein and his colleagues.

Gomphocarpus fruticosus (L.) R.Br., is very similar in appearance, habitat and distribution to G. physocarpus. Keller and Reichstein (1949) and Hunger and Reichstein (1952a; 1952b) isolated two cardiac glycosides in extracts of seeds of G. fruticosus (as G. fructicosus) grown in South Africa. Mitsuhashi and Kurumi (1968) also isolated cardenolides, as aglycones, in G. fruticosus (also as G. fructicosus). In Australia, Watson and Wright (1956; 1957) found a further two cardenolides in G. fruticosus. Eight cardiac glycosides were found in G. fruticosus by Rothschild et al. (1975). The aglycones of the glycosides found by Hunger and Reichstein were also found by Tschesche and co-workers (1958; 1959) in As. curassavica from Brazil. It therefore seems reasonable to suppose that similar aglycones would be isolated from Grahamstown grown species of As. curassavica and G. physocarpus. However, it is well known that the cardiac glycoside content of plants varies considerably. Fieser and Fieser (1959) state that the mixture of several cardiac glycosides in Digitalis purpurea varies in concentration and proportion according to locality and season. Hoch (1961) mentions that total amounts and relative proportions of cardiac glycosides may also vary according to the stage of development of the plant or even time of day collected. Some effort was made to control the variation in cardiac glycoside content by the preparation of aphid extracts at the same time as extracts of the leaves from which the aphids were taken. However, simultaneous sampling of plants, aphids and syrphids is clearly impossible as the development period of the syrphid precludes simultaneous sampling.

Cardiac glycosides in insects - It was hoped that the paper and thin-layer chromatograph methods used in this work to separate cardiac glycosides in locally grown G. physocarpus, As. curassavica, and Ar. sericofera and possible plant-derived cardiac glycosides in A. nerii and I. aegyptius would also reflect the similarities and differences in cardiac glycoside content between asclepiads and associated insects found by other workers. Rothschild et al. (1970a; 1972; 1973a) found three cardiac glycosides in A. nerii and the coccid Aspidiotus nerii fed on the apocynale Nerium oleander in Israel. However, they only found two cardiac glycosides in A. nerii fed on As. curassavica. The cardiac glycosides found in the aphid and coccid in Israel also occur in their host plants. Despite this similarity, the principal cardiac glycoside of N. oleander, oleandrin, was not found in the two homoptera. Neither was the cardiac glycoside, calactin, abundantly present in As. curassavica, found in the populations of A. nerii fed on this asclepiad. Interestingly Brown et al. (1969) did not find an accumulation of cardiac glycosides in A. nerii feeding on As. curassavica near Rio de Janeiro in Brazil. There seems therefore, to be a great deal of variation in the cardiac glycoside content of A. nerii fed on various apocynales, that may reflect the cardiac glycoside content of the feeding site of the aphid.

The cardiac glycoside content of various Hemiptera, Lepidoptera and Coleoptera has been shown by several workers to reflect the concentration of cardiac glycosides in the seeds and leaves of apocynales (Duffey, 1970; Duffey and Scudder, 1972; von Euw et al., 1971; Feir and Suen, 1971; Rothschild et al., 1972; 1973b; 1975; Scudder and Duffey, 1972). For example, eleven cardiac glycosides were found by von Euw et al. (1971) in adult Caenocoris nerii, an hemipteran which fed on oleander seeds. The most celebrated example of the similarity in cardiac glycoside content

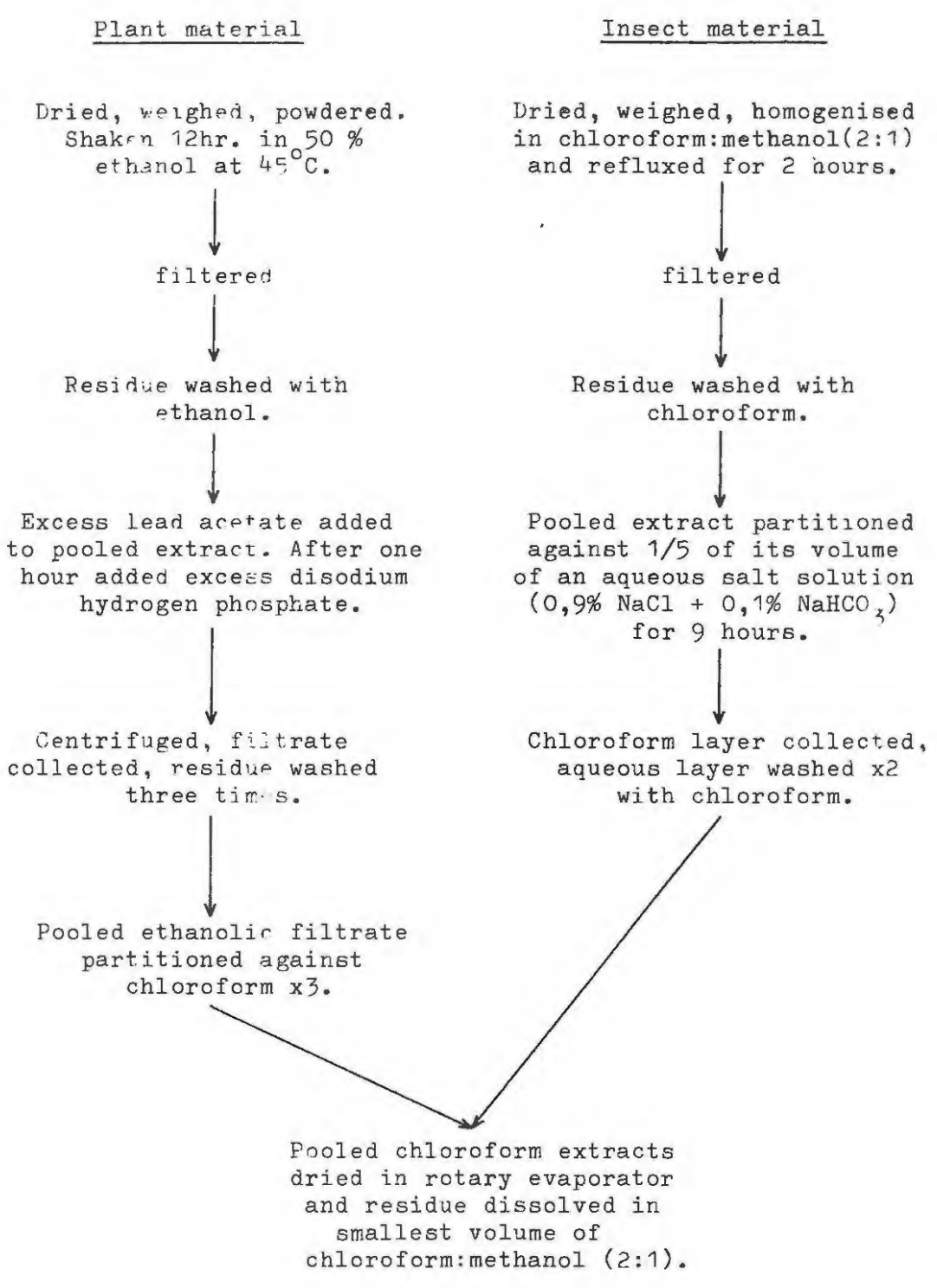


Figure 9. Extraction method 1. Extraction procedure for insect and plant material (after Duffey and Scudder, 1972; and Folch, et al., 1957).

of asclepiads and phytophagous insects is of course the monarch butterfly, Danaus plexippus which contains ten of the cardiac glycosides present in the larval food plants (Reichstein, 1967; Reichstein et al., 1968). Plant-derived cardiac glycosides were also found in aposematic Orthoptera by von Euw et al. (1967) and Reichstein (1967).

a) Extraction of plant and insect material

Plant extracts were made of leaf and stem material cut from potted asclepiads and of seeds collected from the three asclepiad species in and around Grahamstown. Insect extracts were made of aphids collected from heavily infested asclepiad leaves and stems placed in a Tullgren funnel (Kevan, 1962). The aphids were stored at -15°C until use or were dried and extracted immediately. Syrphid material was collected from the I. aegyptius culture and also stored at -15°C until use.

The plants, aphids and syrphids were dried at 100°C and weighed before extraction. This temperature was considered satisfactory as cardiac glycosides have melting points at, and in excess of 200°C (Fieser and Fieser, 1959).

Plant and insect extracts were initially prepared by method 1, shown in figure 9, which was taken from Du fey and Scudder (1972) after the lipid extraction technique of Folch et al. (1957). Method 1 results in the collection of plant and insect steroids in the chloroform fraction. The addition of lead acetate to the ethanol extract of plant material described in figure 9 precipitates plant pigments (Kedde, 1947; Rowson, 1952). This is necessary as pigments tend to obscure the visualisation of steroids in the chromatography techniques.

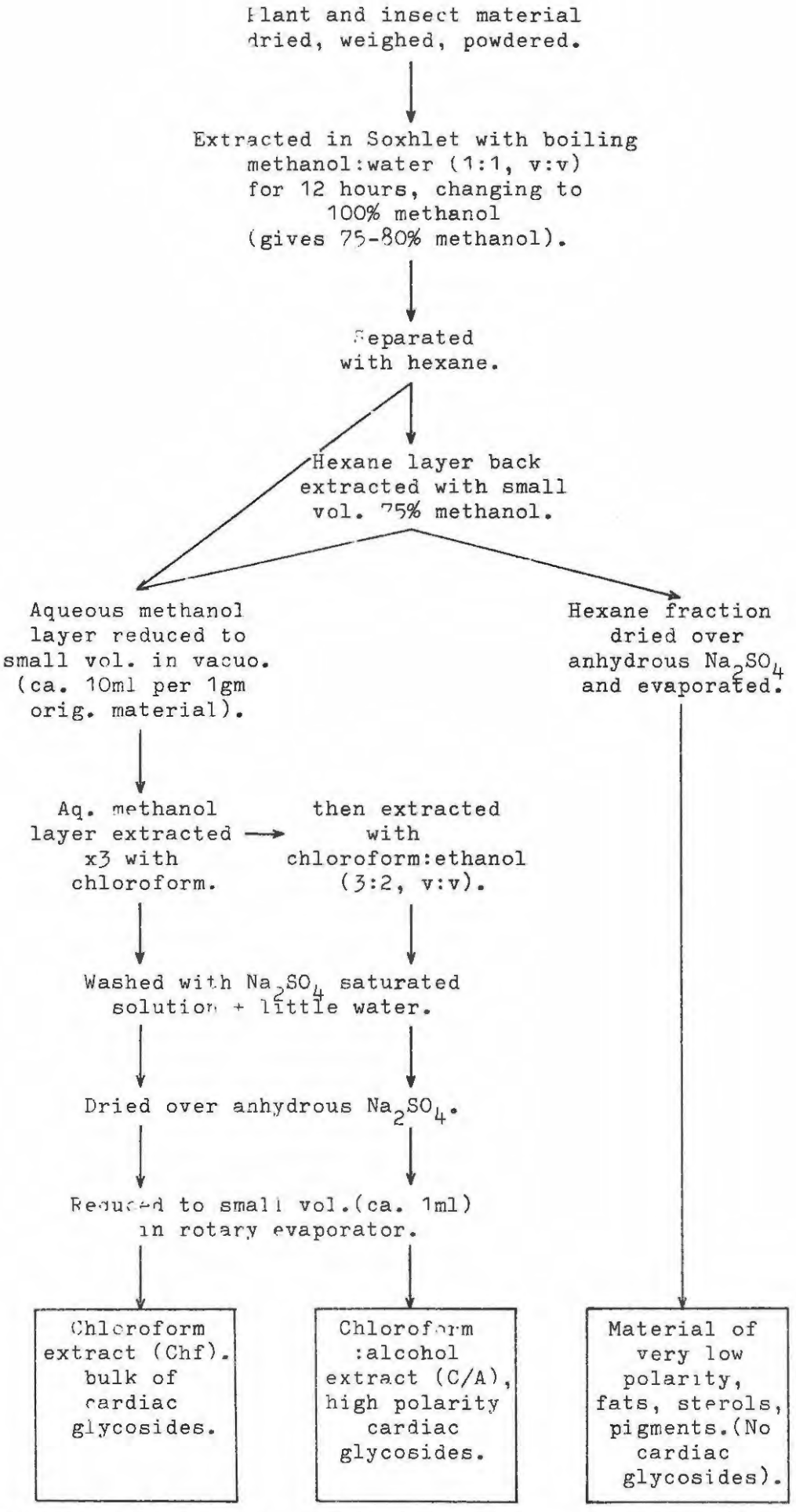


Figure 10. Extraction method 2. Extraction procedure for insect and plant material, all stages of the procedure are carried out under nitrogen (after Reichstein, et al., 1968; and Rothschil et al., 1970a).

Extraction method 2, kindly provided by Dr. Miriam Rothschild, shown in figure 10, was later used because it gave better separation of the components. This method, based on that of Reichstein et al. (1968) and Rothschild et al. (1970a), employs successive separations of a methanolic extract of plant or insect material with solvents of increasing polarity. The first gross alcoholic extract, of the dried, weighed and ground samples, was made in a Soxhlet apparatus, rather than just refluxing as in method 1. Soxhlet extraction resulted in a completely leached sample and precluded the need to filter the extract. Since calotropin and other cardiac glycosides, which contain an aldehyde group, deteriorate in solution by autoxidation (Reichstein et al., 1968), all reflux and solvent extractions of method 2 were performed in an atmosphere of nitrogen. Extraction method 2 was considered superior as the use of solvents of increasing polarity separated cardiac glycosides and other compounds, such as pigments, fats and sterols, into groups of different polarity. Generally speaking the polarity of cardiac glycosides increases as the number of hydroxyl groups on the steroid nucleus increases, and also depends on the nature of the attached sugar (Fieser and Fieser, 1959). Most of the cardiac glycosides are extracted by the chloroform (Chf.) and polar cardiac glycosides are concentrated in the chloroform+ethanol (C/A) fraction. Separation by method 2 was tested with a mixture of the cardiac glycosides ouabain and digitoxin (purchased from E. Merck, Darmstadt, Germany). Ouabain is highly polar with six hydroxyl groups on the aglycone and digitoxin is moderately polar, with only two hydroxyl groups attached to the aglycone (Fieser and Fieser, 1959). A methanolic mixture of ouabain and digitoxin was separated by extraction method 2 and the Chf. and C/A fractions were chromatographed on thin layer plates in a simple solvent system. Both ouabain and digitoxin appeared in the C/A fraction, but only digitoxin appeared in the Chf. fraction. This result was expected as the Chf. fraction is not

sufficiently polar to contain the polar cardiac glycosides. It was expected that the cardiac glycosides of high and moderate polarity would appear in the C/A fraction, with the moderate and low polarity cardiac glycosides separated in the Chf. fraction.

The details of all the extracts, the extraction method used and the results of tests for the presence of cardiac glycosides are given in appendix a.

b) Descending paper chromatographic separation of cardiac glycosides.

i) Materials and Methods.

Whatman no. 1. chromatography paper was prepared with the addition of 33% of the dry paper weight of the polar solvent formamide. Formamide in acetone was poured over the paper and left to dry for twelve hours in the laboratory atmosphere before use, instead of drying the papers between filter paper or in a wringer (Zaffaroni et al., 1950). Between 2 and 50 microlitres of chloroform and chloroform/alcohol extracts of asclepiad and asclepiad/A. nerii- reared insect material were spotted at 2cm intervals across the origin of the impregnated filter paper, with a 25 microlitre Terumo micro-syringe. The chromatograms were run by downward displacement in the solvent system tetrahydrofuran:benzene (1:1, vol:vol) at 16°C in a 340mm x 360mm x 170mm Shandon glass tank. To fully saturate the atmosphere of the tank, three sides were covered with solvent-saturated Whatman no. 1. filter paper. Under these conditions the solvent front moved approximately 300mm in two hours.

The chromatography technique for cardiac glycoside separation and the action and sensitivity of the visualisers was tested with the cardiac glycoside standards ouabain (g-strophanthin), digitoxin and the cardiac-active aglycone k-strophanthidin, purchased from E. Merck of Darmstadt, Germany. The cardiac glycoside standards were run on the paper chromatograms and their Rf. values were recorded. Developed and visualised paper chromatograms were stored with data sheets of the relevant details of time of run, solvent system, visualiser methods and Rf. values of separated cardiac glycoside-positive spots.

Cardiac glycoside detection methods - A number of reactions

specific for steroids and cardiac glycosides have been proposed for the detection of cardiac glycosides separated on chromatograms. The Legal reaction, for the detection of the α, β unsaturated lactone ring of cardenolides (Kedde, 1947 and Waldi, 1965) detects cardenolides with sodium nitroprusside in alkaline medium, but does not visualise bufadienolides. Stahl and Kaltenbach (1961) preferred to visualise Digitalis cardiac glycosides with chloramine-trichloroacetic acid. Matsumoto (1963) used antimony trichloride in chloroform (Carr-Price reagent) to detect a series of steroid sapogenins. Antimony trichloride also visualises vitamin A (Bolliger, 1965) and steroid glycosides (Waldi, 1965). Baljet's reaction uses alkaline picrate to detect cardiac glycosides in ethanolic solution (Kedde, 1947; Rowson, 1952). Rowson (1952) used sodium picrate to visualise Digitalis cardiac aglycones and also visualised the digitoxose glycosides of digitoxin with the Keller-Kiliani process, a mixture of acetic acid, ferric chloride and sulphuric acid. The most frequently used cardiac glycoside detection technique is some form of the Raymond process with 3,5-dinitrobenzene in alkaline solution. The Kedde (1947) modification of the Raymond process employs a 2% solution of 3,5-dinitrobenzoic acid in ethanol for the detection of Digitalis cardiac glycosides under alkaline conditions. Bush and Taylor (1952) used a more dilute solution of dinitrobenzoic acid to visualise the cardiac aglycones of the apocynale Strophanthus. Mauli et al. (1957) were the first to use a solution of 2,2'-4,4'-tetranitrodiphenyl in benzene for the detection of the lactones of cardiac glycosides in alkaline medium. Tetranitrodiphenyl has been used to T. Reichstein and co-workers to successfully visualise a wide range of cardiac glycosides (Brower et al., 1972; von Euw et al., 1967; Reichstein, 1967; Reichstein et al., 1968; Rothschild et al., 1973b). Duffey and Scudder (1972) and Scudder and Duffey (1972) used 1,2-Naphthoquinone-4-sulphonic acid to visualise cardiac glycosides in extracts of asclepiads and various insects

fed on the asclepiads.

Very little is known of the action of visualisers in chromatography and of their specificity to a known visualising reaction. Tan (1969) considers that the Kedde, Raymond, tetranitrodiphenyl reactions and also a picric acid reaction cannot be regarded as specific to the detection of cardiac glycosides. The dinitrobenzoic acid reaction is also used for the detection of the anhydride amino acid excretory product, creatinine (Kedde, 1947). Naphthoquinone-4-sulphonic acid is the principal ingredient of Folin's reagent for the detection of amino acids (Fruton and Simmonds, 1958; Waldi, 1959; 1965). Duffey and Scudder (1972) refer to Rowson (1952) and Wright(1960) as the source of the use of naphthoquinone-4-sulphonic acid as a cardiac glycoside visualiser. Rowson makes no mention of naphthoquinone and the reference, 'Wright S.E., 1960, "The metabolism of cardiac glycosides. A review of the absorption, metabolism and excretion of clinically important glycosides." Springfield, Ill., Chas. C. Thomas 94 pp.'. was not available as a photocopy due to copyright restrictions. My father, Mr. W.R. Malcolm and Dr. J. Walker, (Mill Hill, London) could not locate this reference in any of the major libraries and bookshops in London and Dr. Walker could find no further reference to the use of naphthoquinone-4-sulphonic acid as a cardiac glycoside visualiser. Since naphthoquinone-4-sulphonic acid was successfully used to visualise cardiac glycosides in the present work, it is unfortunate that the original reference to its use was not available.

Despite the paucity of information on the action and specificity of cardiac visualisers the colour reactions can be considered specific. Three methods, from those outlined above, were used for the detection

of cardiac glycosides in asclepiad and insect extracts on developed chromatograms.

1. Chromatograms were first sprayed with a 0,15% solution of 2,2'-4,4'-tetranitrodiphenyl (TNDP) in 95% ethanol (w/v). Spots were visualised in alkaline medium with an aqueous spray of 10% sodium hydroxide. Cardiac glycosides produced blue spots. (After Mauli et al., 1957 and Brower et al., 1972.)
2. Chromatograms were first sprayed with 70% ethanol saturated with 1,2-naphthoquinone-4-sulphonic acid (NAPQ) (as sodium salt), then sprayed with 10% NaOH to visualise cardiac glycosides as purple spots. (After Duffey and Scudder, 1972.)
3. Chromatograms were first sprayed with a 2% solution of 3,5-dinitrobenzoic acid (DNBZ) in 70% ethanol (w/v), then sprayed with 10% NaOH to visualise cardiac glycosides as purple spots. (After Kedde, 1947 and Rowson, 1952.)

Sodium hydroxide solution was added in each case after the first spray reagenthad dried. The purple or blue spots appeared rapidly after the addition of NaOH and faded over approximately five minutes.

The modified Kedde reagent of Bush and Taylor (1952), a 1% solution of 3,5-dinitrobenzoic acid on 0,5N KOH in 50% methanol, was also tried as a cardiac glycoside visualiser but proved less effective than method 3. Developed chromatograms were also immersed in the solution of benzene saturated at 20°C with 2,2'-4,4'-tetranitrodiphenyl (about 5%), recommended by Mauli et al. (1957). This proved no more effective in cardiac glycoside detection than the more dilute solution of 0,15% TNDP in ethanol of detection method 1. The technique of Mauli et al. (1957) had the disadvantage of the need for enormous quantities of TNDP. The reagents of detection methods 2 and 3 were purchased from

Table 2. Detection sensitivity in micrograms (µg) of three cardiac glycoside standards on formamide impregnated paper to three visualisers in alkaline medium.

cardiac glycoside		Visualiser TNDP	DNBZ	NAPQ
ouabain	µg.	0,96	0,19	0,96
digitoxin	µg.	1,01	0,30	1,27
k-strophanthidin	µg.	0,47	0,09	0,47

E. Merck, Darmstadt, Germany. TNDP was not available commercially, therefore, approximately 20 grams of 2,2'-4,4'-tetranitrodiphenyl were manufactured in the Chemistry department with the facilities and generous help of Professor D.E.A. Rivett. (Details of the synthesis of TNDP are given in appendix b.)

The sensitivity of the three cardiac glycoside detection methods was tested on Whatman no. 1 chromatography paper with the cardiac glycosides, ouabain, digitoxin and k-strophanthidin. Detection sensitivity in micrograms of cardiac glycoside standard is given in Table 2. The sensitivity of the detection methods is comparable with the minimum of 0,1 to 0,5µg of cardiac glycoside detected by Rothschild and fellow workers in their series of papers. (M. Rothschild, personal communication, 1974). The three cardiac glycoside detection methods were effective, with the DNBZ method the most sensitive.

ii) Results.

The only cardiac glycoside-positive spots visualised on the paper chromatograms were present in extracts of asclepiad seeds and leaves and stems. Neither aphid or syrphid extracts showed any definitely determinable cardiac glycoside-positive spots with any of the three visualisers.

The results of the detection of cardiac glycoside-positive spots on developed descending paper chromatograms were measured as Rf values of the purple or blue spots from both Chf. and C/A extracts of plant material. The Rf values of cardiac glycoside-positive spots (the results from Chf. and C/A extracts are combined) and the three cardiac glycosides are given in figure 11 and Table 3. Rf values were measured

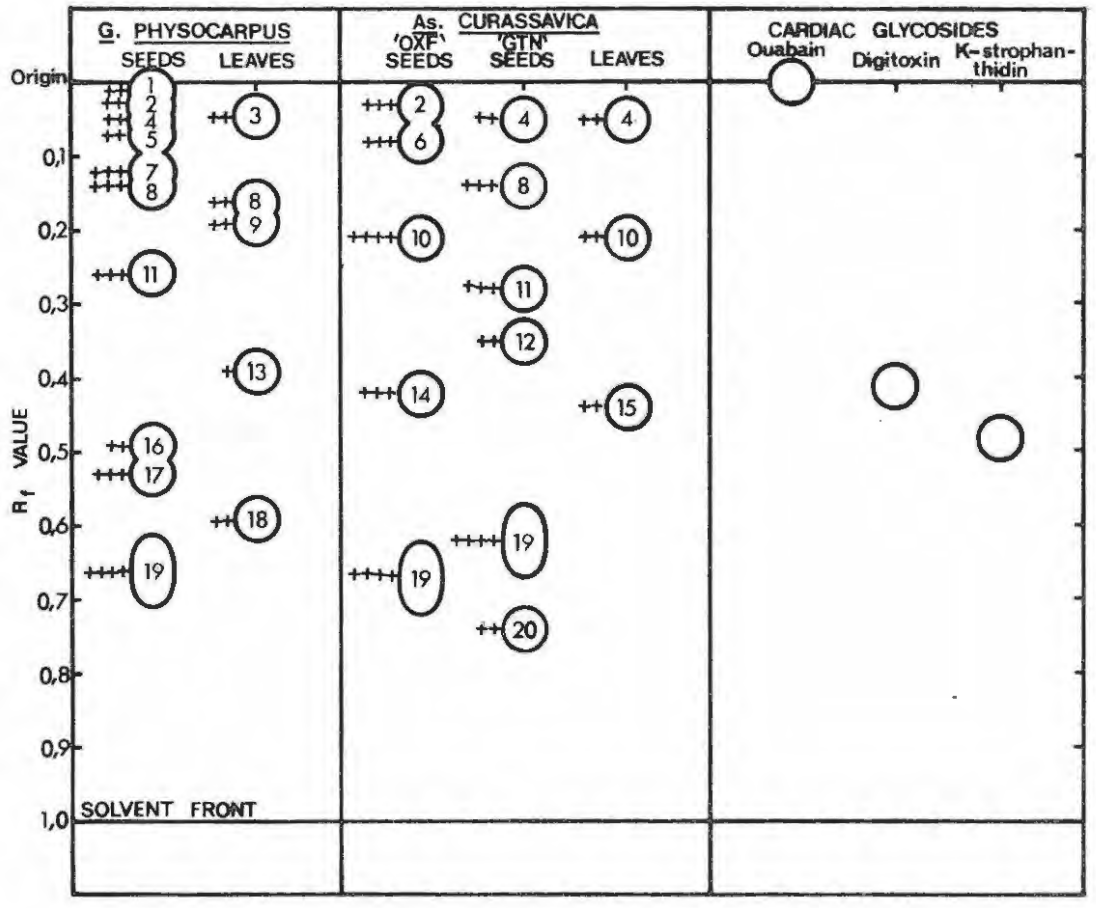


Figure 11. Paper chromatogram of G. physocarpus and As. curassavica seed and leaf extracts, with three cardiac glycoside standards. Descending, formamide-impregnated paper chromatogram of cardiac glycoside-positive spots in the solvent system tetrahydrofuran : benzene (1:1) at 16°C, run for circa 2 hours. Spot intensity is represented by crosses, ++++=v.intense, +++=intense, ++=visible, +=weakly visible. Spots visualised with TNDP, DNBZ, NAPQ. The spots are numbered according to their polarity in this solvent system.

Table 3. Rf. values of cardiac glycoside-positive spots from G. physocarpus and As. curassavica extracts and cardiac glycosides on descending, formamide-impregnated paper chromatograms. Solvent system, tetrahydrofuran:benzene (1:1) at 16°C; length of run, circa 2 hours. Spots visualised with TNDP, DNBZ and NAPQ. The spots are numbered according to their polarity in this solvent system.

			Rf value																		
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gomphocarpus physocarpus		seeds	0,012	0,031		0,052	0,066		0,12	0,14			0,26					0,49	0,53		0,66	
		leaves			0,043					0,16	0,19				0,39					0,59		
Asclepias curassavica	'OXF'	seeds		0,027				0,082				0,21				0,42					0,67	
	'GTN'	seeds				0,052				0,14			0,28	0,35							0,62	0,74
		leaves				0,051						0,21					0,44					
Cardiac glycoside standards		ouabain	ORIGIN																			
		digitoxin														0,41						
		k-strophanthidin																0,48				

directly from the chromatograms, run at 16°C. At this temperature, development times of the chromatograms ranged from 1hr. 44 min. to 2hr. 23min., which produced variation in Rf values recorded from subsequent sample separations. However, this variation was slight and enables detected spots to be compared. In figure 11 the cardiac glycoside-positive spots visualised with TNDP, NAPQ and DNBZ are numbered according to their polarity in the tetrahydrofuran:benzene solvent system. The positive spots are also marked according to their visualised intensity. The crosses in figure 11 indicate, very intense (++++), intense (+++), visible (++) and weakly visible (+) purple or blue spots. This intensity marker indicates the concentration of individual cardiac glycoside-positive spots in the separated sample.

Extracts of the 'OXF' As. curassavica seeds, sent by Dr. Rothschild, were compared with extracts of As. curassavica seeds collected in Grahamstown ('GTN'), and with extracts of G. physocarpus seeds, also collected locally. The results given in figure 11 indicate spot 19 (always as an elongate oval spot) as the only cardiac glycoside-positive spot common to all the seed extracts of G. physocarpus and As. curassavica. Seed and leaf extracts of Ar. sericofera produced no cardiac glycoside-positive spots on the paper chromatograms. Spots 4, 8 and 11 are also in the seed extracts of both G. physocarpus and 'GTN' As. curassavica. Spot 2 in G. physocarpus and 'OXF' As. curassavica seed extracts is also similar. The leaf and stem extracts of G. physocarpus have only spot 8 in common with their seed extracts and the leaves and stems of As. curassavica only have spot 4 in common with their seeds.

From the published Rf values of known cardiac glycosides, chromatographed in the same system employed in this work, the identity

of some of the cardiac glycoside-positive spots in the chromatograms may be suggested. Spots 4, 7, 8, 9, 10, 11, 16, 17, 19 and 20 in figure 11, with their corresponding Rf values in Table 3, may be respectively, syrioside (4), calotropagenin (7), calotoxin (8), strospeside (9), syriobioside (10), calotropin (11), afroside (16), calactin (17), gomphoside (19) and uzarigenin (20). As von Euw et al. (1967), Reichstein (1967) and Rothschild et al. (1975) indicate that As. curassavica is a very rich source of calotropis glycosides, spot 8 may be either proceroside or calotoxin with Rf values of 0,14 and 0,15 respectively. (Calotoxin and proceroside are two of a series of glycosides based on the aglycone calotropagenin (Brüschweiler et al., 1969; Fieser and Fieser, 1959).) Despite these tentative suggestions as to the identity of the cardiac glycoside-positive spots, none of the 'OXF' As. curassavica seed Rf values agree with the Rf values of uscharin, calactin and calotropin found by Reichstein (1967) and Reichstein et al. (1968) from the same material that originated in Trinidad. Despite the differences in the cardiac glycoside content of the same apocynale species reported by different chemists the published results (particularly Rothschild et al., 1975) do suggest that the cardiac glycosides calotropagenin (spot 7), calotoxin (spot 8), calotropin (spot 11), afroside (spot 16), calactin (spot 17), gomphoside (spot 19) and uzarigenin (spot 20) are present in either G. physocarpus or As. curassavica grown in Grahamstown. G. physocarpus seeds are particularly rich in a wide range of cardiac glycosides, four of which are highly polar in nature. The polar spots 2, 7 and 9 correspond with the Rf values of three unidentified cardiac glycosides detected in G. fruticosus by Rothschild et al. (1975). The seeds of G. physocarpus also seem to contain the two principal cardiac glycosides, afroside and gomphoside, isolated by Watson and Wright (1956; 1957) from Australian G. fruticosus.

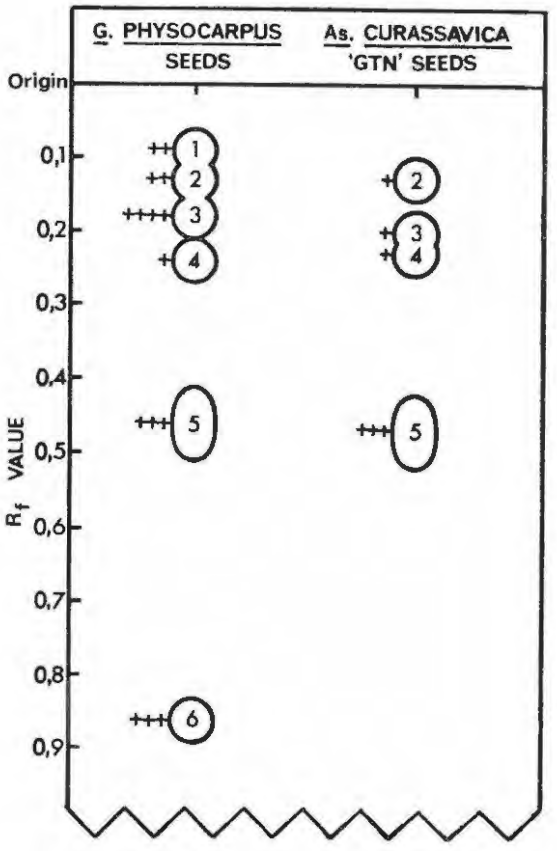


Figure 12. Paper chromatogram of G. physocarpus and 'GTN' As. curassavica seed extracts. Continuous elution (7-8 hours) of cardiac glycoside-positive spots on descending, formamide-impregnated (33%) paper chromatogram in the solvent system terahydrofuran : benzene (1:1) at 16°C. Spots visualised with TNDP; spot intensity is represented by the crosses (see figure 11). The numbers indicate the polarity sequence in this solvent system.

Table 4. Rf values of TNDP-positive spots of G. physocarpus and 'GTN' As. curassavica seed extracts in continuously eluted (7-8 hours), descending, formamide impregnated (33%) paper chromatography. (Solvent system, tetrahydrofuran:benzene, 1:1, at 16°C.) (The polarity sequence is indicated by the numbered Rf values, 1-6.)

	Rf value					
	1	2	3	4	5	6
G. physocarpus seeds	0,083	0,13	0,18	0,24	0,46	0,86
As. curassavica seeds		0,13	0,20	0,23	0,47	

Continuous elution - To separate the highly polar cardiac glycoside-positive spots in the G. physocarpus seed extracts, chromatograms were run with the continuous elution technique of Brüschweiler et al. (1969). Descending, formamide impregnated (33%) paper chromatograms were run for 7-8 hours in the same solvent system (tetrahydrofuran:benzene, 1:1) at 16°C. The TNDP visualised results are shown in figure 12 as a representative chromatogram of seed extracts of G. physocarpus, compared with seed extracts of 'GTN' As. curassavica. Rf values of the TNDP-positive spots are given in Table 4. Ouabain was also run in this system but remained at the origin as it did with the normal elution time of approximately 2 hours. Spots 5 and 6 on the continuously eluted chromatogram have similar Rf values to calotoxin and calotropin, respectively, separated by Reichstein (1967) under the same conditions over 7 hours. This similarity further reinforces the possibility that calotoxin and calotropin are present in local asclepiads, the former in both G. physocarpus and As. curassavica and the latter only in G. physocarpus. Calotoxin is also available in G. physocarpus leaves, but calotropin seems to be restricted to the seeds of both asclepiad species. The continuously eluted chromatogram in figure 12 also shows the four highly polar cardiac glycoside-positive spots of figure 11 more clearly. The three spots faintly visualised in extracts of As. curassavica ('GTN') seeds in figure 12 also indicate that spot 4 in figure 11 may be a mixture of cardiac glycosides.

All the extracts tested on the paper chromatograms are tested again in the following section on adsorption thin layer chromatography, to provide further information on the cardiac glycoside content of the asclepiads and to retest the asclepiad-reared insect extracts.

c) Adsorption thin layer chromatographic separation of cardiac glycosides.

i) Materials and Methods.

Silica gel-G was used as the adsorbent on glass plates as the gel forms a highly active, retentive layer which is designed to separate a wide range of compounds of different polarities with the appropriate solvents. Steroids, as secondary alcohols high in the eluotropic series (which indicates high polarity), are separated on an active gel layer with a moderately polar solvent mixture (Stahl, 1965). Stahl and Kaltenbach (1961) used the solvent system methylene chloride:methanol:formamide (80:19:1) on silica gel-G layers and obtained a good separation of cardiac glycoside mixtures. The technique used in the present work is the Duffey and Scudder (1972) modification of the Stahl and Kaltenbach separation.

Glass plates were prepared for ascending, adsorption thin layer chromatography with a 0,25mm layer of silica gel-G according to Waldi (1965). The layer was applied as an aqueous slurry with either a Desaga (Heidelberg) applicator (Stahl, 1965) or a Quickfit thin layer applicator and automatic plate leveller. Plates 200mm x 200mm or 200mm x 40mm were prepared and stored in a desiccator. The gel layer was finally dried and activated at 110°C for 30 minutes immediately before use. Plant and insect extracts were spotted on the activated plates with either 2 or 5 µl. Drummond 'microcaps' (Drummond Scientific Co., U.S.A.) or a 25 µl. Terumo microsyringe. Care was taken in sample addition to ensure that the gel layer of the TLC plates was not disturbed. Between 2 and 50µl. of each sample was tested. Extracts were spotted 15mm from one edge of the plate as quickly as possible to avoid more than 15 minutes exposure to the laboratory atmosphere. After sample addition the plates were

developed in a 210mm x 210mm x 90mm Desaga or a 280mm x 220mm x 100mm Shandon rectangular glass tank with ground glass lid. The atmosphere of the tanks was kept permanently saturated with solvent saturated Whatman no. 1. filter paper which covered three of the tank sides. Before each chromatogram run the filter paper was resaturated by gently shaking and tilting the tank to ensure an even saturation of the tank atmosphere. Chromatograms were run in the two solvent systems of Duffey and Scudder (1972).

1. Dichloroethane:methanol:formamide (80:25:1).
2. Dichloromethane:methanol:formamide (80:10:1).

The solvent systems were replaced frequently to ensure purity. The components of each solvent system were analytical grade reagents purchased from E. Merck, Darmstadt, Germany. All of the chromatograms were run at 15-16°C in a refrigerator or C.E. room maintained at 15°C.

The cardiac glycosides, ouabain (g-strophanthin), digitoxin and the cardiac aglycone k-strophanthidin were again used to indicate the approximate Rf range of the plant and insect cardiac glycosides. Thin layer plates were developed until the solvent front reached a line scored across the gel layer 170mm from the origin, which acted as an even barrier to further solvent movement.

The developed chromatograms were sprayed with the three detection reagents, as colour tests for the presence of cardiac glycosides, by the following procedure;

1. 70% ethanol saturated with 1,2-naphthoquinone-4-sulphonic acid (Na salt), then sprayed with 10% NaOH after heating the plate at 110°C for 1,5 minutes. Cardiac glycosides produced purple spots. (NAPQ)

Table 5. Detection sensitivity in micrograms (µg) of three cardiac glycoside standards on silica gel-G plates to three visualisers in alkaline medium.

Cardiac glycoside		Visualiser TNDP	DNBZ	NAPQ
ouabain	µg.	0,24	0,38	2,40
digitoxin	µg.	1,50	1,00	0,25
k-strophanthidin	µg.	0,93	0,28	0,19

2. 2% solution of 3,5-dinitrobenzoic acid in 70% ethanol (w/v), then sprayed with 10% NaOH after heating at 110°C for 1,5 minutes. Cardiac glycosides produced purple spots which faded more rapidly than those produced with NAPQ. (DNBZ)
3. 0,15% solution of 2,2'-4,4'-tetranitrodiphenyl in 95% ethanol (w/v) and then sprayed with 10% NaOH after allowing to dry. Cardiac glycosides produced blue spots which faced quickly over 5 minutes. (TNDP)

The reagents were used in the same form as for the detection of cardiac glycosides in paper chromatography. The only difference is that the plates were heated at 110°C for 1,5 minutes before the addition of NaOH. The visualisers were sprayed on the developed chromatograms with a Shandon spray gun and aerosol power pack.

The sensitivity of the visualisers for the detection of cardiac glycosides was tested on silica gel-G plates. The detection sensitivity of the three visualisers is recorded in Table 5. The sensitivity of between 0,19 and 2,4 µg of cardiac glycoside detected by the visualisers compares with the approximately 1 µg of various steroid sapogenins, which included cardiac active steroids, detected by Matsumoto (1963). Matsumoto used antimony trichloride (as "antimonous chloride") as the steroid visualiser on thin layer plates.

The samples from extraction method 1. were spotted as chloroform: methanol (2:1) extracts and those from extraction method 2., as chloroform or chloroform:ethanol (3:2) extracts. Low polarity ether and hexane fractions and methanol fractions were also collected from extraction method 2. and tested for cardiac glycoside content on TLC plates.

Developed and visualised thin layer chromatograms were traced onto transparent sheets of plastic and were filed with the appropriate details of time of run, visualiser application, sequence of visualiser application, colour of spots visualised, solvent system used, temperature, and comments on pigments in the chromatograms and unusual visualising. Rf values were measured from the original chromatograms and were stored with their respective chromatogram records on data sheets.

ii) Results.

The hexane and methanol fractions from extraction method 2. proved negative for cardiac glycoside content of all plant and insect extracts in both thin layer solvent systems. All the cardiac glycoside-positive spots appeared in the Chf. and C/A fractions of the extracts.

Ouabain and k-strophanthidin were visualised with all three visualisers; purple spots were produced with NAPQ and DNBZ and TNDP produced blue spots. Digitoxin was visualised most readily with DNBZ and produced an intense purple spot.

The results of ascending, adsorption chromatograms of seeds and leaf extracts of G. physocarpus, As. curassavica and Ar. sericofera, extracts of A. nerii fed on each of the asclepiads and extracts of I. aegyptius reared on each of the A. nerii-asclepiad associations are given in figures 13 and 14 as representative chromatograms. The results are also given as Rf values in Tables 6 and 7 for all the individual cardiac glycoside-positive spots detected by the three visualisers. Figures 13 and 14 are representative chromatograms of the cardiac glycoside-positive spots in both solvent systems. Only Rf values are

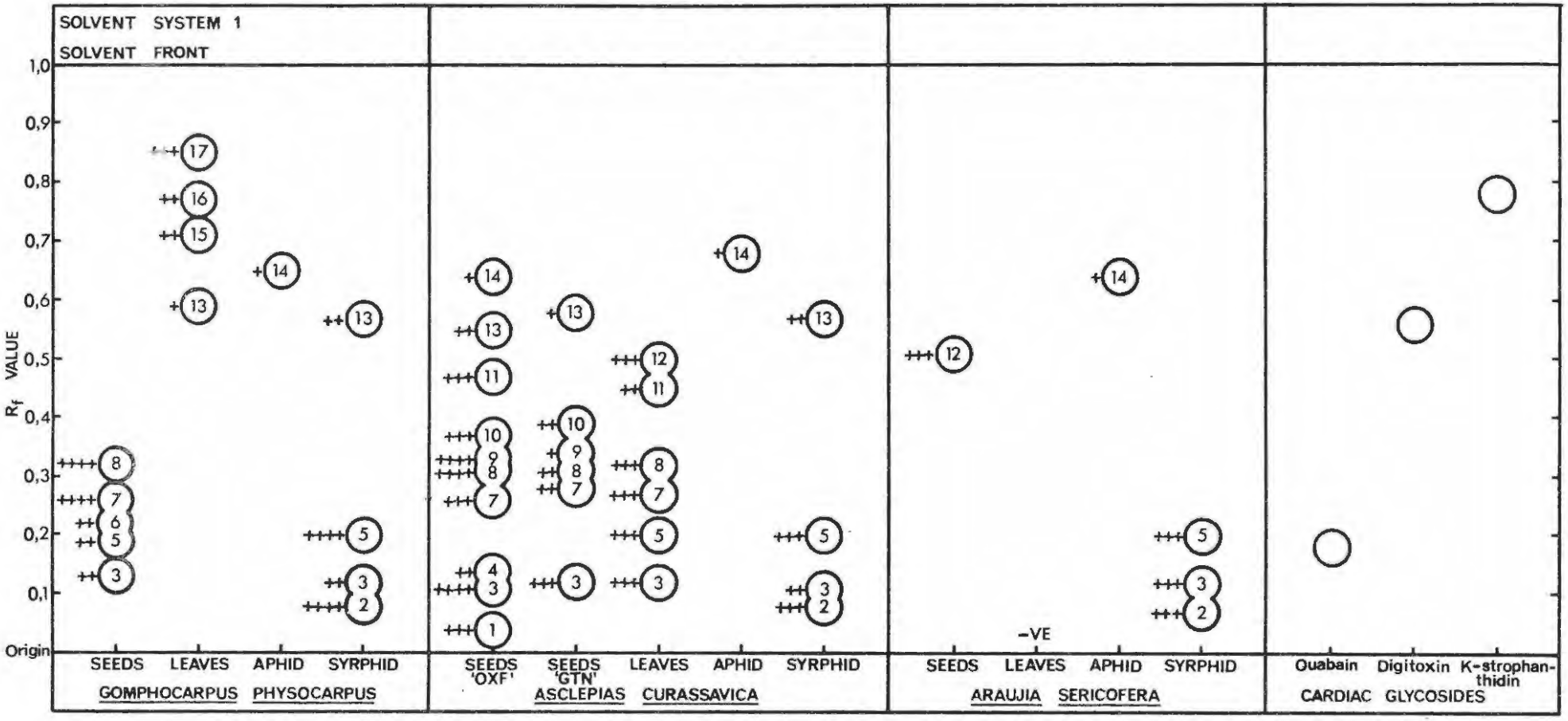


Figure 13. Thin layer chromatogram of cardiac glycoside-positive spots of asclepiad and asclepiad-derived insect extracts and cardiac glycoside standards in solvent system 1, on silica gel-G plates at 16°C. Crosses indicate intensity of spots (see figure 11); spots numbered according to polarity; spots visualised with TNDP, DNBZ, NAPQ.

Table 6. Rf values of cardiac glycoside-positive spots from asclepiad and asclepiad-derived insect extracts and cardiac glycoside standards chromatographed on silica gel-G plates in solvent system 1 at 15°C. Spots visualised with TNDP, DNBZ, NAPQ. The spots are numbered according to their polarity in this solvent system.

			Rf value																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Gomphocarpus physocarpus		seeds			0,125		0,19	0,22	0,26	0,32									
		leaves													0,59		0,71	0,77	0,85
		aphid														0,65?			
		syrphid		0,078	0,12		0,20								0,57				
Asclepias curassavica	'OXF'	seeds	0,04		0,11	0,14			0,26	0,31	0,33	0,37	0,47		0,55	0,64			
	'GTN'	seeds			0,12				0,28	0,31	0,34	0,39			0,58				
		leaves			0,12		0,20		0,27	0,32			0,45	0,50					
		aphid														0,68?			
		syrphid		0,078	0,11		0,20								0,57				
Araujia sericofera		seeds												0,51					
		leaves	-ve.																
		aphid														0,64?			
		syrphid		0,06	0,12		0,20												
Cardiac glycoside standards		ouabain					0,18												
		digitoxin													0,56				
		k-strophanthidin																0,78	

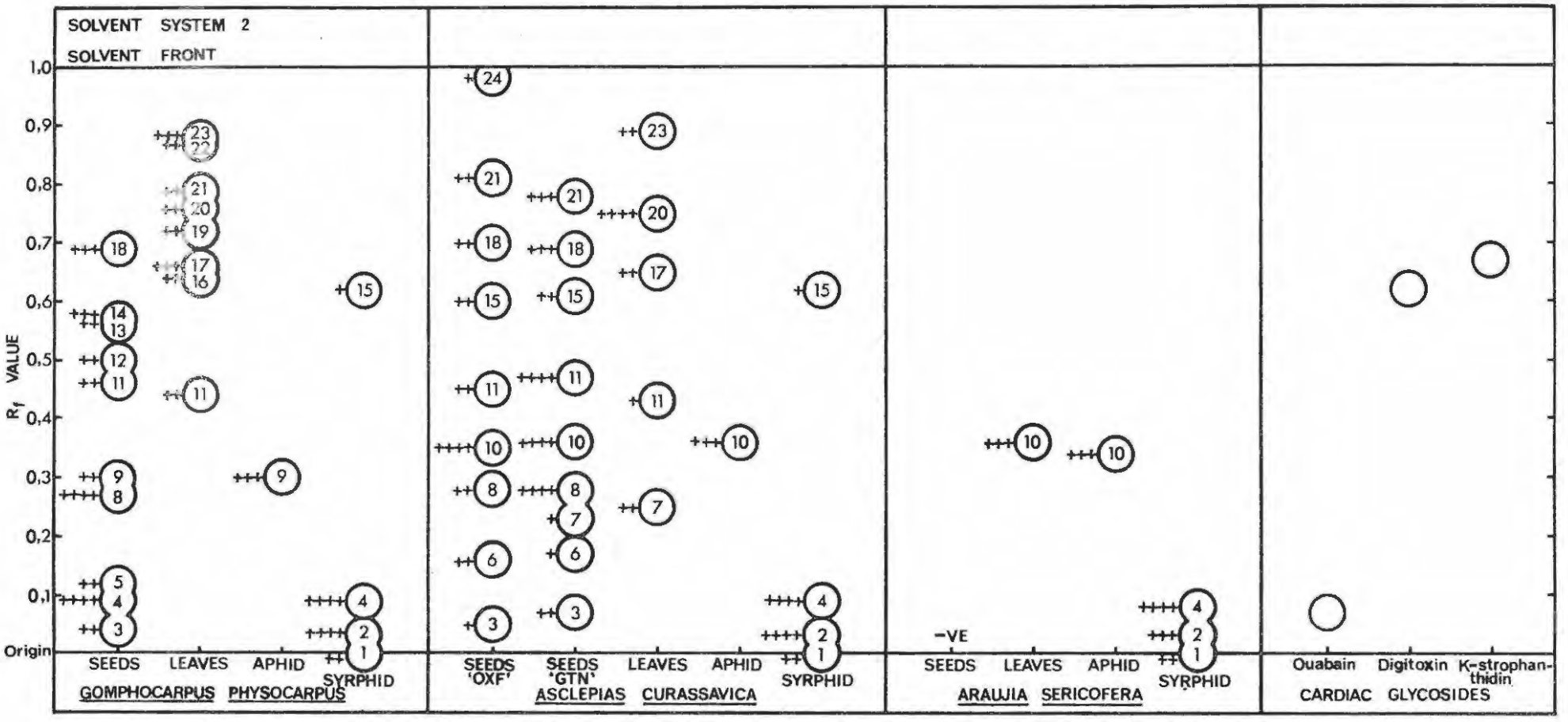


Figure 14. Thin layer chromatogram of cardiac glycoside-positive spots of asclepiad and asclepiad-derived insect extracts and cardiac glycoside standards in solvent system 2, on silica gel-G plates at 16°C. Crosses indicate intensity of spots (see figure 11); spots numbered according to polarity; spots visualised with PNP, DNBZ, NAPQ.

Table 7. Rf values of cardiac glycoside-positive spots from asclepiad and asclepiad-derived insect extracts and cardiac glycoside standards chromatographed on silica gel-G plates in solvent system 2 at 15°C. Spots visualised with TNDP, DNBZ, NAPQ. The spots are numbered according to their polarity in this solvent system.

	Rf value																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
G. physocarpus																								
seeds			0,04	0,08	0,12			0,27	0,30		0,46	0,50	0,56	0,57				0,69						
leaves											0,44					0,64	0,66		0,72	0,75	0,79	0,87	0,88	
aphid									0,30															
syrphid	ORIG.	0,034		0,09											0,62									
As. curassavica																								
'OXF' seeds			0,05			0,16		0,28		0,35	0,45				0,62			0,70			0,81			0,98
'GTN' seeds			0,065			0,17	0,23	0,28		0,36	0,47				0,61			0,69			0,78			
leaves							0,25				0,43						0,65			0,75			0,89	
aphid										0,36														
syrphid	ORIG.	0,034		0,09											0,62									
Ar. sericofera																								
seeds	-ve.																							
leaves										0,36														
aphid										0,34														
syrphid	ORIG.	0,034		0,08																				
Cardiac glycoside standards																								
ouabain			0,067																					
digitoxin															0,62									
k-strophan.																	0,67							

given as the cardiac glycoside standards were not run on every chromatogram and so Rst values, as the Rf of the sample spot relative to the Rf of one of the reference standards, cannot be given for each chromatogram.

The chromatograms in both solvent systems show a variation in Rf values of the detected spots despite the careful control of experimental conditions. Variations in Rf values were probably due to irregular gel layers, different concentrations of test samples, edge effects from differences in saturation of the chambers (Stahl, 1965) and differences in the laboratory atmosphere and time of exposure to the atmosphere during sample addition to the activated plates. The two sizes of plates also produced slightly different Rf values of detected spots from the same samples. Development times of silica gel-G plates in both solvent systems also varied considerably; the mean times were 65 minutes for the solvent front to travel 170mm in solvent system 1 and 60 minutes in solvent system 2. These times varied between 42 and 96 minutes in solvent 1 and 38 and 88 minutes in solvent 2. The time of development seemed to be associated with tank saturation. The cylindrical tanks, lined with solvent-saturated filter paper produced consistently faster developed chromatograms with lower Rf values of the detected spots. Digitoxin in solvent system 1 had Rf values that varied between 0,43 on fast development and 0,71 when the development was slow. Duffey and Scudder (1972) give Rf values of 0,08 for ouabain and 0,40 for digitoxin on silica gel plates in solvent system 1. However, the minimum Rf values obtained in the present work in the same solvent system were 0,11 and 0,43 for ouabain and digitoxin respectively.

Asclepiad extracts - The cardiac glycosides in the plant and insect extracts were visualised with successive applications of the three

visualisers. The spots are again marked in figures 13 and 14 according to intensity as ++++ = very intense, +++ = intense, ++ = visible and + = weakly visible. Despite testing quantities of cardiac glycoside-positive samples that varied from 2 to 50µl, (tested 10-40 times) to ensure as much sensitivity as possible, there was some variation in the number of cardiac glycosides visualised in the same sample, in each solvent system. Figure 14 shows 10 cardiac glycosides separated in samples of G. physocarpus seeds when chromatographed in solvent 2, but only 5 appeared from the same sample in solvent 1. The seeds of both G. physocarpus and As. curassavica contain more cardiac glycosides, and more cardiac glycosides of high polarity, than the leaves of both asclepiads. G. physocarpus, in particular, has a marked difference between the cardiac glycoside content of seeds and leaves.

Only one large, intense, NAPQ-positive spot was repeatedly visualised in extracts of Ar. sericofera seeds and leaves. Seed extracts of Ar. sericofera were NAPQ-positive in solvent system 1, whereas extracts of the leaves were only NAPQ-visualised in solvent system 2.

The Chf. and C/A extracts of the seeds of the 'OXF' As. curassavica were again compared with extracts of seeds of the 'GTN' As. curassavica. Extracts of the seeds of these two strains of As. curassavica have a similar cardiac glycoside content. The chromatograms indicate 10 cardiac glycosides from the combined results of the Chf. and C/A extracts of the 'OXF' seeds. 9 cardiac glycosides appeared in the extracts of 'GTN' seeds of As. curassavica. The polarities of the cardiac glycosides in the seeds of both As. curassavica and G. physocarpus are comparable (see figures 13 and 14 and Tables 6 and 7). These results agree with Duffey (1970), who noted, for three asclepiad species in North America, that the seeds are the

richest source of cardiac glycosides in the plant.

No identities have been suggested for any of the cardiac glycoside-positive spots as reference material was not available for comparison. The suggestions made in the paper chromatography section as to tentative cardiac glycoside identities cannot, therefore, be reinforced. Only the number and concentration of cardiac glycosides can be indicated.

Insect extracts - The plants readily show a significant difference in the cardiac glycoside content of the three species. G. physocarpus and As. curassavica have a similar concentration and number of cardio-active steroids, but Ar. sericofera apparently only contains one cardiac glycoside. The extracts of insects reared on the three asclepiads do not, however, reflect the differences in the cardiac glycoside content of the plants. The insect extracts have an almost completely independent cardiac glycoside content. It is important to point out that positive spots separated in the insect extracts were only visualised with the NAPQ detection method. All the described NAPQ-positive results only refer to detected spots with the purple colour characteristic of cardiac glycosides.

Only one NAPQ-positive spot appeared in the aphid extracts, which was clearly visualised in solvent system 2, but with some doubt in solvent system 1. The positive spot of the A. nerii extracts does correspond with spot 10 in Ar. sericofera and As. curassavica leaves and to the very similar spot 9 in extracts of G. physocarpus in solvent system 2 (see figure 14). A. nerii extracts AlJ (G. physocarpus-derived, C/A extract), AlL (As. curassavica-derived, Chf. extract) and AlO (Ar. sericofera-derived, Chf. extract) (see appendix a for details) produced the

consistenly NAPQ-positive spots at between Rf 0,30 and 0,36 on the chromatograms. In all aphid extracts, yellow, orange, brown and green pigments were strongly evident. A consistent NAPQ-positive spot in the aphid extracts was faintly detected at between Rf 0,65 and 0,68 in solvent system 1.

Pigments were a problem in some of the aphid and plant extracts, because cardiac glycoside-positive spots were occasionally obscured. The positive spot (10, in figure 14) in the Chf. extract FIX of Ar. sericofera always appeared at the base of a series of at least four pigments. The intense purple colour readily distinguished the NAPQ-positive Ar. sericofera spot from the green pigments. Some of the pigment that may have obscured a positive cardiac glycoside response in Chf. extract AlH of A. nerii from G. physocarpus was extracted with carbon tetrachloride. A portion of the extract was dried, resuspended in 3ml of chloroform:methanol (1:1) plus 1ml of water and shaken with carbon tetrachloride (Watson and Wright, 1956). The chloroform fraction was reduced in volume to approximately 0,5ml and retested. Less pigment was present in the extract but no cardiac glycoside-positive spots appeared after visualiser application. Pigments were largely removed in extraction method 2 with hexane. The hexane fraction was particularly rich in yellow carotenoid pigments from plant, aphid and syrphid extracts.

Extracts of I. aegyptius reared on A. nerii on both As. curassavica and G. physocarpus consistently produced four NAPQ-positive spots (figures 13 and 14). The fourth spot, number 14, in the syrphid extracts was considerably fainter than the first three. Spot 14 did not appear with spots 1, 2 and 4 in the extracts of I. aegyptius reared on A. nerii on Ar. sericofera. The four NAPQ-positive spots of all the asclepiad/

A. nerii-reared I. aegyptius extracts gave the same Rf values of 0,06 to 0,078, 0,11 to 0,12, 0,2 and 0,57 in solvent system 1. In solvent system 2 the Rf values were 0,034, 0,08 to 0,09 and 0,62 with a positive spot that remained at the origin (see figures 13 and 14, Tables 6 and 7).

It was anticipated that the syrphid extracts might show some similarity in cardiac glycoside content with both the aphid and leaf extracts. However, as can readily be seen from figures 13 and 14 this is not the case. The only syrphid extract that shows a similarity of cardiac glycoside content to that of a leaf extract is of I. aegyptius, reared on the A. nerii/As. curassavica association, chromatographed in solvent system 1. This syrphid extract has NAPQ-positive spots 3 and 5 in common with extracts of As. curassavica leaves (figure 13). Interestingly this similarity is not borne out in solvent system 2. There is also a similarity between the low polarity spot 13 in I. aegyptius, spot 14 of its aphid prey and spot 13 of the leaves of the asclepiad host, G. physocarpus, in figure 13. Again, the similarity is not confirmed for the same material in solvent system 2.

The detection of the syrphid, aphid and Ar. sericofera extracts, with NAPQ, was particularly interesting because the detection sequence differed slightly from the detection of the NAPQ-positive spots in the G. physocarpus and As. curassavica extracts. Chloroform extract P1X of Ar. sericofera leaves produced an intense purple spot at Rf 0,36 in solvent system 2 after the developed plate was sprayed with NAPQ and heated at 110°C for 1,5 minutes but before the addition of 10% NaOH. Addition of NaOH turned the purple spots a little greyer, but did not alter the intensity. Purple spots also appeared after the application of NAPQ and heat, before the addition of NaOH in all the NAPQ-positive

insect extracts. The positive spots were always purple and only appeared after heating. The DNBZ and TNDP visualisers did not detect the NAPQ-positive spots in the insect and Ar. sericofera extracts.

Further separation of the NAPQ-positive spots in extracts of I. aegyptius - As some of the NAPQ-positive syrphid material remained at the origin in solvent system 2, the polarity of the solvent was increased by an increase in the volume of methanol or with the addition of glacial acetic acid. As well as increase the polarity of the solvent system, glacial acetic acid produced an increased in the acidity of the plates. It was argued that the highly polar NAPQ-positive spots of the syrphid extracts and the less polar NAPQ-positive spots of the A. nerii and Ar. sericofera extracts were being detected under very weak alkaline conditions, without the need for the further addition of hydroxyl ions. All that was probably necessary for the colour detection of the NAPQ-positive spots was activation with heat after visualiser application. The addition of the acetic acid to the solvent created a more polar solvent system and an acid environment on the plates that would require the addition of alkali to visualise the spots. The following three solvent systems, as modifications of solvent system 2, were used to further separate the NAPQ-positive spots in the syrphid extracts.

3. Dichloromethane:methanol:formamide (80:25:1)
4. Dichloromethane:methanol:formamide:glacial acetic acid (80:10:1:1)
5. Dichloromethane:methanol:formamide:glacial acetic acid (80:10:1:5).

Thin layer chromatograms were run as previously but with these three solvent systems on 200mm x 40mm glass plates in the 240mm x 50mm glass cylinders.

The results of the separation of extract S2M (extract of 116

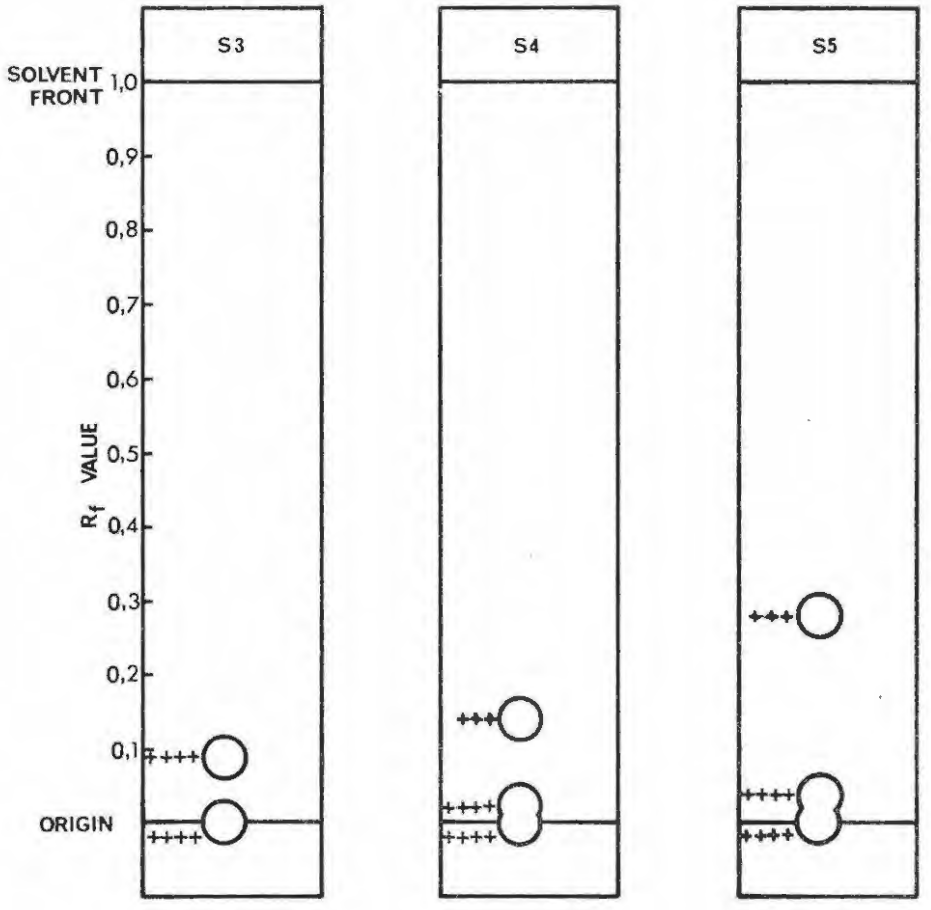


Figure 15. Thin layer chromatograms of NAPQ-positive spots from extract S2M of 116 I. aegyptius reared on A. nerii on G. physocarpus in solvent systems 3 (S3), 4 (S4) and 5 (S5) on silica gel-G plates at 15°C. Crosses indicate intensity of spots (see figure 11).

Table 8. Rf values of NAPQ-positive spots in extract S2M of 116 *I. aegyptius* reared on *A. nerii* on *G. physocarpus*, separated on silica gel-G plates in solvent systems 3, 4 and 5 at 15°C.

	Rf value of NAPQ-positive spots.		
	1	2	3
Solvent 3.	origin	0,087	-
Solvent 4.	origin	0,023	0,14
Solvent 5.	origin	0,04	0,28

I. aegyptius reared on A. nerii on G. physocarpus) on TLC plates in the three solvent systems are given in figure 15, as representative chromatograms, with the Rf values in Table 8. The increase in polarity of the solvents does 'push' another NAPQ-positive spot from the origin to give three NAPQ-positive spots. Spot 15 of the I. aegyptius extracts at Rf 0,62 in solvent system 2 (see figure 14) was not detected in solvent systems 3, 4 and 5.

All the spots in solvent systems 3, 4 and 5 were visualised with NAPQ. However, as anticipated, an interesting difference in spot detection was observed. In solvent system 3 the NAPQ-positive spots were visualised after heat only, as previously. In solvent systems 4 and 5 the NAPQ-positive spots only appeared slowly (over 5 minutes) after the addition of 10% NaOH, once the plates had been sprayed with NAPQ and heated at 110°C for 1,5 minutes. These NAPQ-positive spots lasted for at least 24 hours as bright purple, well defined spots.

Samples of I. aegyptius reared on A. nerii on the three asclepiads, prepared as ethanol extracts for the perfusion of frog and chamaeleon hearts described in the following section, also produced a series of four NAPQ-positive spots. The Rf values of these four spots were identical to those shown in figures 13 and 14 from I. aegyptius extracts prepared by extraction methods 1 and 2.

Non-asclepiad-derived I. aegyptius - Non-asclepiad/A. merii-reared extracts were prepared from flies reared on the black aphid Macrosiphoniella sanborni fed on Chrysanthemum morifolium, and on the grey-green aphid Hyadaphis foeniculi fed on honeysuckle, Lonicera caprifolium. The syrphid material was extracted with 75% ethanol. These syrphid extracts were prepared as non-asclepiad-derived

'control' flies for a comparison with asclepiad- derived I. aegyptius in the cardiac activity tests of the following section. Chromatograms of NAPQ-positive spots separated from extracts S3H (12 I. aegyptius reared on M. sanborni on C. morifolium) and S3I (62 I. aegyptius reared on H. foeniculi on L. caprifolium) in solvent systems 1 and 2 are represented in figure 16 and as Rf values of the spots in Table 9. The NAPQ-positive spots of extract S3I show a marked similarity to those from extracts of asclepiad/A. nerii reared I. aegyptius. The Rf values of the NAPQ-positive spots from both asclepiad- and non-asclepiad-derived I. aegyptius extracts are almost identical, particularly in solvent system 2. (Compare figures 13, 14 and 16 and Tables 6, 7 and 9.) Extract S3H of C. morifolium-derived I. aegyptius produced faintly visible (+ in figure 16) but obvious purple spots with NAPQ, with lower Rf values than those of the spots from extracts of asclepiad-derived flies.

Cardiac glycoside-positive results from non-asclepiad-derived I. aegyptius were completely unexpected as cardiac glycosides have not been recorded from the Compositae and Caprifoliaceae (Hoch, 1961; Paris, 1963), the families of C. morifolium and L. caprifolium, respectively. An extract (P20) of 5,5gm of dried, ground L. caprifolium leaves and stems was made by reflux with 75% ethanol. The ethanolic extract was filtered and the filtrate reduced to small volume to give a concentrated, gross plant extract. In solvent systems 1 and 2 the extract of L. caprifolium proved negative for cardiac glycoside content, when run on the silica gel-G plates. The extract only produced a streak of yellow pigment on the chromatograms. C. morifolium was not tested for cardiac glycoside content.

The chromatography results indicate that I. aegyptius adults contain compounds that may be cardiac glycosides. The nature of the

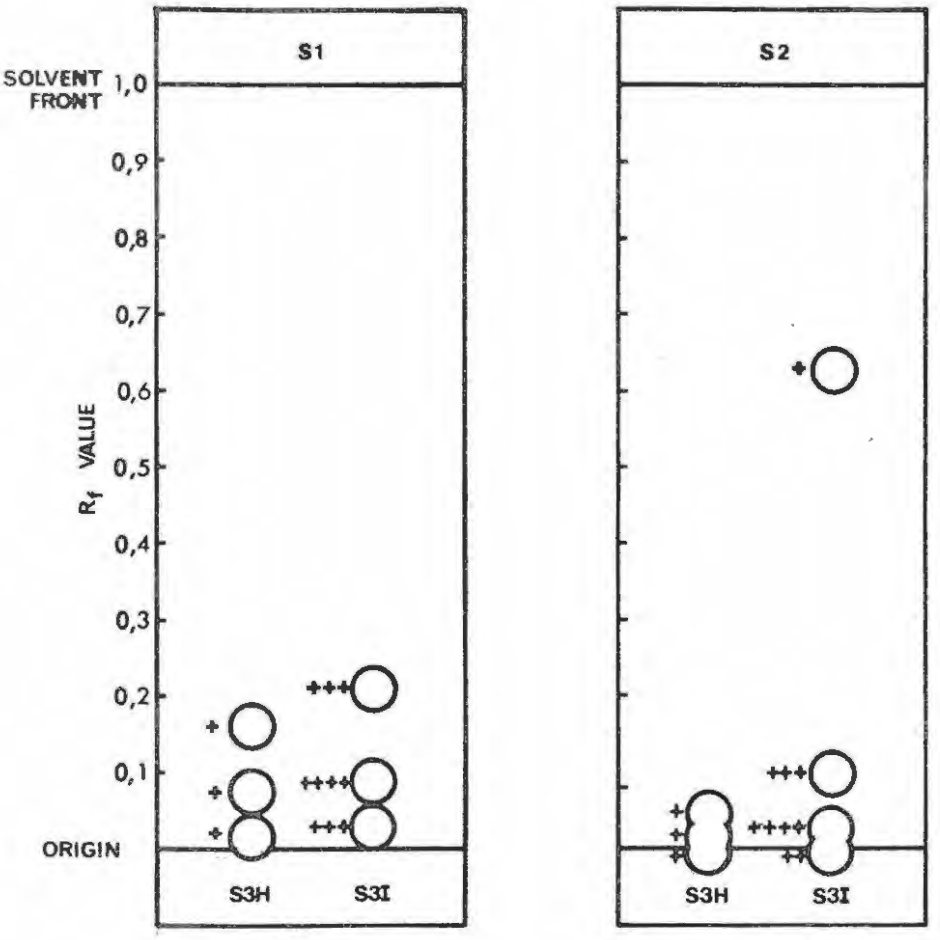


Figure 16. Thin layer chromatograms of NAPQ-positive spots from non asclepiad/A. nerii-reared I. aegyptius extracts S3H (reared on M. sanborni on C. morifolium) and S3I (reared on H. foeniculi on L. caprifolium) in solvent systems 1 (S1) and 2 (S2) on silica gel-G plates at 15°C. Crosses indicate intensity of spots (see figure 11).

Table 9. Rf values of NAPQ-positive spots from non-asclepiad-derived I. aegyptius extracts S3H (C. morifolium-derived) and S3I (L. caprifolium-derived) separated on silica gel-G plates in solvent systems 1 and 2 at 15°C.

	Rf value of NAPQ-positive spots.			
Solvent 1	1	2	3	4
S3H	0,026	0,068	0,16	-
S3I	0,039	0,084	0,21	-
Solvent 2.	1	2	3	4
S3H	origin	0,026	0,039	
S3I	origin	0,032	0,097	0,63

cardiac glycoside content is apparently independent of larval prey and host plant cardiac glycoside content. The cardiac glycoside content of the syrphid may however, be derived from the aphid-plant association. This result is surprising because, when this project was started, a series of extracts were made of 'wild caught' aphidophagous syrphids to test for cardiac glycoside content chromatographically. As can be seen from appendix a, all the original syrphid extracts from S1A to S2C were negative for cardiac glycoside content. These original extracts were all, however, of small quantities of material; for example, extract S1U was of 4 I. aegyptius females collected at Lake Sibaya in Kwa Zulu and proved negative for cardiac glycoside content. Extracts of other syrphid species of the genera Paragus and Betasyrphus also proved negative for cardiac glycoside content. Negative results were also obtained with extracts of larvae, pupae and third instar larval gut exudate of Ischiodon, Paragus and Betasyrphus species that were collected locally and transferred to A. nerii-infested asclepiads.

An extract of 6 B. claripennis females reared on A. nerii on Ar. sericofera did, however, produce an NAPQ-positive result. B. claripennis extract S2D was hydrolysed to separate any glycosides from their aglycones. The extract was stirred at pH 1 at 60°C in 95% ethanol for 1,5 hours. This solution was changed to pH 11 and then neutralised, extracted with chloroform, dried and resuspended in chloroform:methanol (2:1), (Duffey and Scudder, 1972). Only one NAPQ-positive spot appeared from the hydrolysed extract at Rf 0,5 on TLC plates in solvent system 2. The NAPQ-positive spot of extract S2D was also visualised with heat after the addition of NAPQ without NaOH. The Chf. (S2P) and C/A (S2Q) extracts of 80 Betasyrphus species A reared on A. nerii on G. physocarpus, produced a similar NAPQ-positive spot to spots 1 and 2 of the I. aegyptius extracts on TLC plates (Tables 6 and 7).

The Rf values of the NAPQ-positive spots from extracts S2P and S2Q were 0,065 to 0,071 in solvent system 1 and 0,034 to 0,038 and at the origin in solvent system 2.

Extracts of I. aegyptius reared on A. nerii on the three asclepiad species and on aphid infested Chrysanthemum morifolium are tested in the following section for their effect on two vertebrate heart preparations. These tests were made to determine any potential cardiac toxicity of asclepiad/A. nerii-reared I. aegyptius extracts to potential or actual syrphid predators. It was hoped that the response of two myogenic vertebrate hearts to I. aegyptius extracts would reinforce the chromatographic indication that the compounds separated and detected in the extracts of the syrphid are cardiac glycosides. Any recorded response in the myogenic hearts, from sample administration, that is characteristic of the cardio-toxic effects of cardiac glycosides would support the tentative suggestion that the NAPQ positive spots in syrphid extracts are cardiac glycosides.

3 C. Pharmacology of asclepiad/aphid-derived Ischiodon aegyptius extracts on exposed frog and chamaeleon hearts.

The response of syrphid predators to I. aegyptius reared on the three A. nerii-asclepiad associations is the most significant aspect of this thesis. Predator response, whether behavioural or physiological, determines the degree of selection pressure on the syrphid and hence the evolution and maintenance of possible aposematism. To maintain an effective selection pressure the syrphid predator or 'signal receiver' (Wickler, 1968) must be able to discriminate the colour pattern and respond to any chemical defence of I. aegyptius. Two vertebrates were chosen as experimental predators of I. aegyptius. The clawed toad, Xenopus laevis (Daudin) was conveniently available in a departmental culture, and the common dwarf chamaeleon, Chamaeleo pumilus ventralis (Gray) occurs abundantly in local gardens.

Some amphibia feed on flies as Cott (1932) describes Diptera as the principal food item of the tree frog, Megalixalus fornasinii. X. laevis has colour vision (Cronly-Dillon and Muntz, 1965), therefore at least some amphibia are able to discriminate the colour patterns of syrphids. However, as X. laevis is entirely equatic, syrphid predation is rather unlikely. X. laevis is simply a convenient experimental indicator of amphibian cardiac response to aphidophagous syrphids. Chamaeleo pumilus is recorded by Burrage (1973) as a syrphid predator. During the winter months of July and August at Stellenbosch in the Cape, 25-27% of the prey items of male C. pumilus were syrphids. Chaemaeleons are also sensitive to colour and have very advanced binocular vision (Bellairs, 1969). The dwarf chamaeleon is likely to be effective as a 'signal receiver' of an aposematic message from aphidophagous syrphids.

Saurian reptiles are able to detect unpalatable insects and probably respond to cardiac glycosides. Barnes (1971) records the rejection by Lizards of the asclepiad-feeding bug Oncopeltus fasciatus. O. fasciatus ingests and sequesters cardiac glycosides from asclepiads (Feir and Suen, 1971; Duffey, 1970; Duffey and Scudder, 1972; Scudder and Duffey, 1972). However, lizards may respond to the histamine-like activity of the body fluids of O. fasciatus, determined by Graham and Staddon (1974) and not to cardiac glycosides.

The toxic effects of cardiac glycosides to vertebrates is well documented. Cardiac glycosides are particularly toxic, in excess, to the vertebrate heart (Briggs and Brotherton, 1970). Cardiac glycosides also have a diuretic action (Briggs and Brotherton, 1970) and are neurotoxic (Detweiler, 1967). L.P. Brower used the initial, emetic response of birds to ingestion of the cardiac glycoside-rich monarch butterfly as an indicator of the unpalatibility or toxicity of asclepiad-reared butterflies (Brower, 1969; Brower and Glazier, 1975; Brower and Moffit, 1974; Brower et al., 1967; 1968; 1972; 1975).

The positive inotropic response of vertebrate cardiac muscle to cardiac glycosides is a qualitatively similar increase, in all vertebrates (Detweiler, 1967), of the systolic force of the heart (Koppanyi and Karczmar, 1964). In excess, cardiac glycosides result in arrest of the heart during systole (Heftmann, 1970). Koppanyi and Karczmar (1964) point out that a decrease in the heart beat rate is also a marked response of the heart muscle to cardiac glycosides, with a concomitant increase in the irritability of cardiac muscle. The rate of frog, and probably chamaeleon, heart beats is slowed by a reduction of the pacemaker effect of the sino-atrial node which allows undisturbed vagus nerve impulses to reach the heart. Vagal influence is therefore indirectly affected by

cardiac glycosides. The sino-atrial node is the regulator of the myogenic heart beat rate in reptiles and amphibians. Cardiac glycosides act on the ionic balance of cardiac muscle, particularly to bind Calcium ions to the contractile protein complexes which produce prolonged ATP-induced contraction (Farah and Witt, 1963; Lee, 1963; Waser, 1963). Cardiac glycosides also increase the time for excitation to spread from the pacemaker to the ventricle. There is no effective atrio-ventricular node in reptile and amphibian hearts, so an additional negative chronotropic (affecting the rate of action) effect from the depression of the a-v node will not occur. However, muscle fibres at the a-v junction of the frog heart run circularly and will increase the delay of the a-v conduction (Bell, Davidson and Scarborough, 1957).

Cardiac glycoside structure is important in the determination of their toxic effects to potential predators. Vertebrates have a wide variation of sensitivity and ability to transform or detoxify cardiac glycosides. For example, guinea pigs are extremely sensitive to digitoxin and have a high detoxification rate. Toads, however, are insensitive to digitalis and do not detoxify digitoxin (Detweiler, 1967; Repke, 1963). Repke (1963) describes the digitoxin sensitivities of man, dog, cat, rabbit and toad as varying over a range by a factor of one thousand, with man as the most sensitive. (See also Detweiler, 1967.) The aglycone or genin of the cardiac glycoside determines the physiological activity of the cardio-active steroids (Fieser and Fieser, 1959). However, the pharmacological tests of Tsuru et al. (1975) indicate that the steroidal skeleton (perhydrocyclopentanophenanthrene nucleus) of cardiac glycosides is not an essential structural requirement for cardiac activity. The isolated sugars of cardiac glycosides do not contribute to any cardio-active effect (Repke, 1963). But the glycoside does prolong the activity of the aglycone by binding the whole cardiac glycoside

molecule to myocardial tissue (Tamm, 1963). The specific affinity of cardiac glycosides for heart muscle tissue is determined by the glycosidic linkage with one or more sugars. Repke (1963) indicates that the number of hydroxyl groups in the steroid nucleus largely determines the biological effect of cardiac glycosides; the more hydroxyl groups there are the stronger and more rapid the effect. Repke also describes the differences in distribution of cardio-active steroid aglycones and their glycosides in rats. Glycosides are found at very high concentrations in the heart, and aglycones, at concentrations ten times higher than glycosides, in the brain. In most vertebrates glycosides are concentrated in the liver and specific toxicity to heart muscle is due to a selective response rather than selective concentration (also Detweiler, 1967). The transformation of cardio-active steroids is explained by Repke (1963) through detoxification to an inactive epimer, from enzymatic epimerization of the free hydroxyl group at C-3 in the aglycones (animal enzymes invert the configuration of the hydroxyl group). The enzymes that form epimers of cardio-active aglycones were mainly found in the liver, with almost no activity in cardiac muscle. However, the sugar chain of cardiac glycosides effectively blocks enzymatic alteration to inactive derivatives.

The highly polar nature of the syrphid, NAPQ-positive spots on the thin layer chromatograms, indicated by their low Rf values, is interesting as polar cardiac glycosides are more toxic to potential predators than cardiac glycosides of low polarity. Matsumoto (1963) observed that highly polar steroids are characterised by an increased number of hydroxyl groups. The polarity of cardiac glycosides also increases with the number of glycosidically linked sugars (Waldi, 1965). Fieser and Fieser (1959) tabulate the cat-lethal doses of 82 cardenolides and indicate that although hydroxyl groups increase polarity only hydroxyl groups in certain positions on the steroid nucleus enhance the toxicity of the steroid.

Tamm(1963) emphasises the same point. Generally speaking the increase in polarity results in a decrease in solubility that restricts the passage of polar cardiac glycosides across membranes, such as the gut lining of a predator. For example, the highly polar ouabain is very toxic when administered intravenously (cat LD = 0,116 mg/Kg (Fieser and Fieser, 1959) or 0,097 mg/Kg (Tamm, 1963)). However, when taken orally, the cardiac activity of ouabain is very slow to appear. The less polar digitoxin is very readily absorbed when taken orally, due to lipid solubility (Okita, 1967), and acts as quickly on the heart when taken orally as when administered intravenously (Fieser and Fieser, 1959). Digitoxin is also less toxic than the more polar ouabain as the cat lethal dose is increased approximately threefold to 0,32 mg/Kg (Tamm, 1963). Hoch (1961) lists digitoxin as even less toxic at a cat lethal dose of 0,390-0,420 mg/Kg.

The polar NAPQ-positive spots visualised in the syrphid extracts, if correctly interpreted as cardiac glycosides, are likely to have a high level of toxicity to vertebrate predators. The intestinal absorption of the polar NAPQ-positive compounds will be slow, but with long lasting effects due to slow permeability and accumulation. Parsons (1965) described systolic arrest after an increase in the amplitude of beat of the isolated heart of the frog Rana temporaria, when perfused with cardiac glycosides. Extracts of the wings of the monarch butterfly, Danaus plexippus reared on As. curassavica, produced a similar response in isolated frog hearts.

As vertebrate hearts have a specific response to cardiac glycosides, the cardiac glycoside content of I. aegyptius extracts was tested pharmacologically by perfusion of exposed hearts of the clawed toad and dwarf chamaeleon, with isotonic solutions of the extracts. The standard heart lever apparatus and smoked kymograph drum was considered a suitable

technique for recording heart beat response to sample administration.

a) Materials and Methods.

Extracts - Extracts of asclepiad- and non-asclepiad-derived I. aegyptius were prepared by a reflux of dried and ground adult flies with a mixture of 75% ethanol and methanol (3:2) for 12 hours (see appendix a for details of extracts S3E, S3F, S3G and S3H). The extracts were filtered, dried in a rotary evaporator under vacuum and resuspended in approximately 0,5ml of 75% ethanol. Extracts S3E, S3F and S3G were prepared of I. aegyptius reared on A. nerii fed on the three asclepiads G. physocarpus, As. curassavica and Ar. sericofera. Non-asclepiad-derived I. aegyptius (extract S3H) were reared on the black aphid, M. sanborni fed on the composite, C. morifolium. I. aegyptius was reared on the M. sanborni/C. morifolium association as a control, probably devoid of cardiac glycosides, for a comparison with asclepiad/A. nerii-reared I. aegyptius in the cardiac toxicity tests. To test the effects of cardiac glycosides on the heart preparations, ouabain, as a 10^{-4}M solution in 75% ethanol was used as a cardiac glycoside standard.

Experimental animals - The four syrphid extracts, ouabain and an ethanol control were diluted with frog- and chamaeleon-isotonic 0,6% sodium chloride solution. The diluted samples were tested by perfusion of the ventricle of exposed hearts of the clawed toad, X. laevis and the dwarf chamaeleon, C. p. ventralis. Specimens of X. laevis were taken from a permanent laboratory culture and the identity of the frog was verified in Poynton (1964). C. p. ventralis is the commonest dwarf chamaeleon in Grahamstown. The cryptic chamaeleon was collected from bushes on campus shortly before use. FitzSimons (1943) was used to determine the identity of the chamaeleon as Microsaura ventralis ventralis.

This identity was verified by Mr. L.R.G. Raw in Durban, South Africa. However, Burrage (1973) suggests it is probably advisable to consider the forms of dwarf chamaeleon in South Africa as subspecies of Chamaeleo pumilus until more information is available on the different groups of dward chamaeleon. The Grahamstown form is considered as C. pumilus ventralis (Gray).

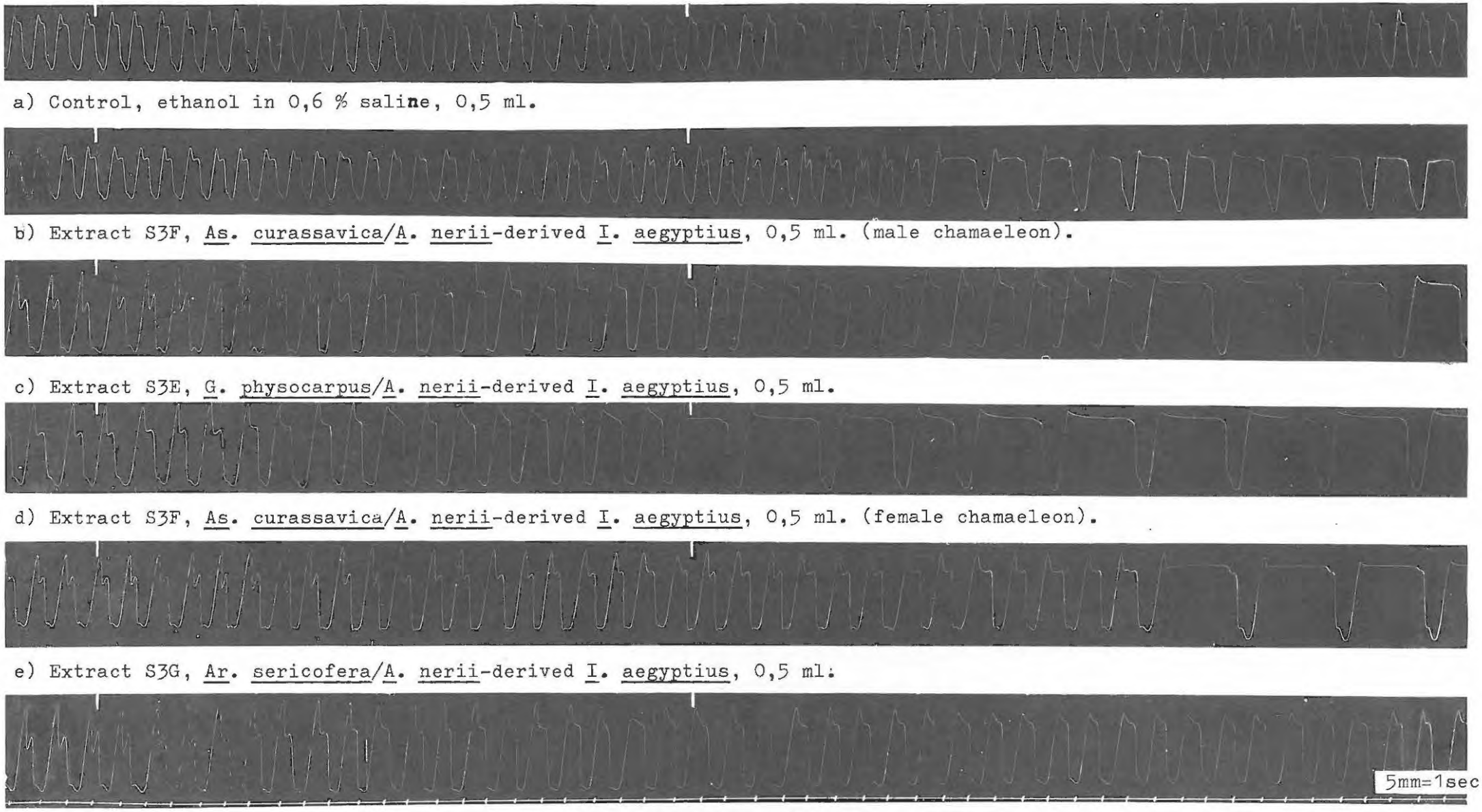
Heart preparations - The frogs were pithed to kill them, which ensured as little disturbance to the circulatory system as possible. Chamaeleons were lightly anaesthetised with chloroform because, inexplicably, the chamaeleons pithed unsuccessfully. The heart was exposed with as little disturbance to the animal as possible, with a ventral cut through the pectoral girdle. To expose the pericardium the fore limbs were pulled slightly apart. The pericardium was carefully cut away and the exposed heart was hooked with a fine pin through the tip of the ventricle to a Starling heart lever. The deflection of the beating heart, which was held vertically from the arterial arches, was recorded on a smoked Kymograph drum. To produce heart beats of large amplitude the pericardium was cut away as completely as possible. The period of sample administration was noted on each kymograph recording with a time marker to indicate the duration of heart perfusion.

Sample dilutions were made up by dilution of ethanolic syrphid extracts, ouabain in ethanol and ethanol controls, with 0,6% NaCl solution to a v/v concentration of 7,5% ethanol. The exposed heart preparations were perfused with ouabain as a 10^{-4}M solution in 7,5% ethanol diluted with 0,6% saline, to test the effect of a cardiac glycoside on the heart beat of X. laevis and C. p. ventralis. Perfusion of the heart preparations was performed by the manual addition of the sample volume, dropwise, to the tip of the ventricle, with a 1,0 ml plastic syringe.

To eliminate any effect on the heart due to ethanol in the test samples, each heart preparation was tested with the standard ethanol: saline (7,5% v/v) dilution. The 1973 British Pharmacopoeia (A112) recommends that the v/v concentration of ethanol in digitalis assay should not exceed 10% v/v. If the heart showed any response to the 7,5% ethanol dilution, the control was further diluted until no further response occurred. The heart was then perfused with the syrphid and cardiac glycoside samples at the new dilution. Individual tests of syrphid extracts and ouabain were alternated with control ethanol dilutions to ensure heart responses were not due to changes in sensitivity to ethanol. Heart preparations were kept moist and washed between each sample addition with frog Ringer solution (6,5 gm NaCl, 0,14 gm KCl, 0,12 gm $CaCl_2$, 0,10 gm $NaHCO_3$, 0,01 gm Na_2HPO_4 in 1L H_2O). Frog Ringer solution, heart preparations and test samples were maintained at a temperature of 21-22°C.

Five male and five female X. laevis that ranged in weight from a 38 gm male to a 122 gm female were sacrificed. Five female and four male C. p. ventralis from a 3,5 gm male to an 11,0 gm female were also tested for cardiac activity to sample perfusion. Both frog and chamaeleon heart preparations beat actively for at least ten hours under experimental conditions. Heart perfusion tests with syrphid extracts, ouabain and ethanol controls were each replicated ten times on both X. laevis and C. p. ventralis heart preparations.

Burn (1928) gives the 40% lethal dose of ouabain in injected frogs as 0,055mg ouabain/100gm of frog. As the weights of X. laevis varied from 38 gm to 122 gm, 0,029 mg of ouabain was used as the maximum dose tested by perfusion at any one time on frog and chamaeleon hearts.



a) Control, ethanol in 0,6 % saline, 0,5 ml.

b) Extract S3F, As. curassavica/A. nerii-derived I. aegyptius, 0,5 ml. (male chamaeleon).

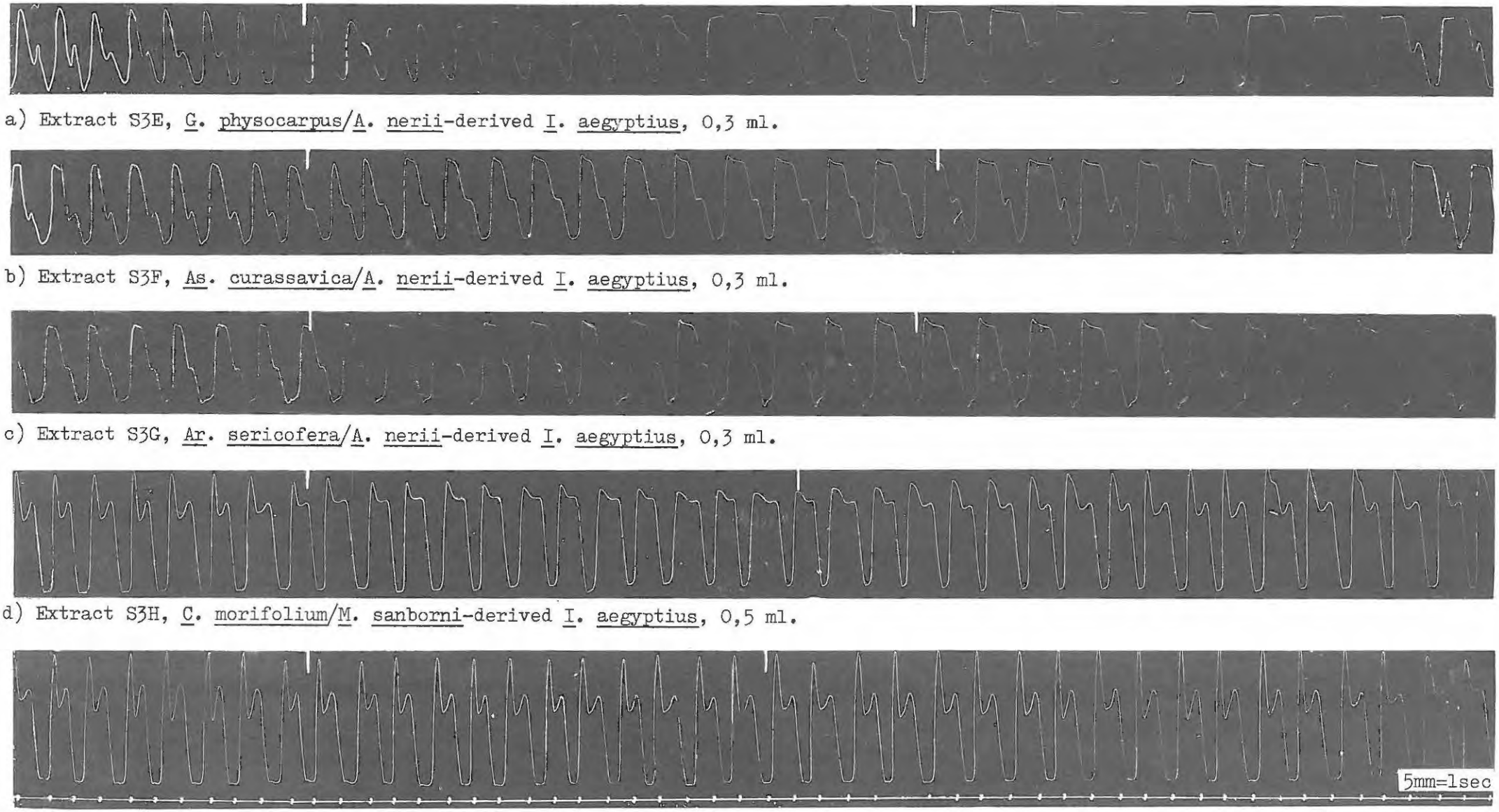
c) Extract S3E, G. physocarpus/A. nerii-derived I. aegyptius, 0,5 ml.

d) Extract S3F, As. curassavica/A. nerii-derived I. aegyptius, 0,5 ml. (female chamaeleon).

e) Extract S3G, Ar. sericofera/A. nerii-derived I. aegyptius, 0,5 ml.

f) Extract S3H, C. morifolium/M. sanborni-derived I. aegyptius, 0,5 ml.

Figure 17. Heart beat response of C.p. ventralis to perfusion with ethanol extracts of I. aegytpius adults and ethanol control diluted with 0,6 % saline (7,5 %, v/v), recorded on a smoked kymograph drum. Samples added between markers on trace.



a) Extract S3E, G. physocarpus/A. nerii-derived I. aegyptius, 0,3 ml.

b) Extract S3F, As. curassavica/A. nerii-derived I. aegyptius, 0,3 ml.

c) Extract S3G, Ar. sericofera/A. nerii-derived I. aegyptius, 0,3 ml.

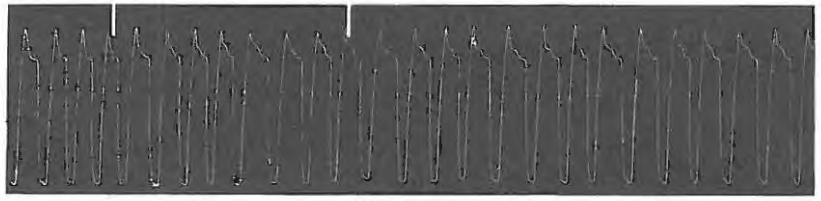
d) Extract S3H, C. morifolium/M. sanborni-derived I. aegyptius, 0,5 ml.

e) Control, ethanol in 0,6 % saline, 0,5 ml.

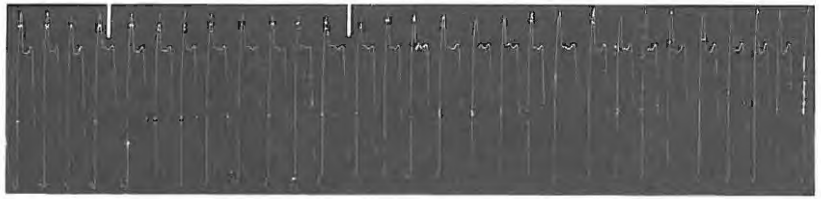
Figure 18. Heart beat response of X. laevis to perfusion of the ventricle with ethanol extracts of I. aegyptius adults and ethanol control diluted with 0,6 % saline (7,5 %, v/v), recorded on a smoked kymograph drum. Samples added between markers on trace.

a) Ouabain, 0,029 mg in 7,5 % ethanol on C.p. ventralis heart. 5mm=1sec.

b) Ouabain, 0,018 mg in 7,5 % ethanol on X. laevis heart.



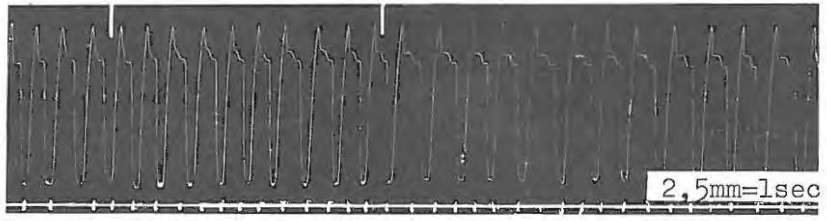
c) Ouabain, 0,0035mg in 1,5% ethanol on X. laevis heart.



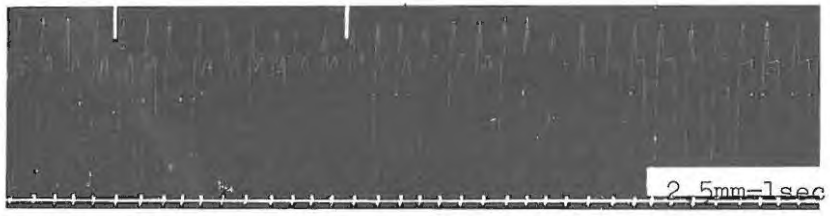
d) Control, 1,5% ethanol on X. laevis heart, 0,3ml.

e) Extract S3E in 1,5% ethanol on X. laevis heart, 0,3ml.

f) Extract S3F in 1,5% ethanol on X. laevis heart, 0,3ml.



g) Extract S3G in 1,5% ethanol on X. laevis heart, 0,3ml.



h) Extract S3H in 1,5% ethanol on X. laevis heart, 0,3ml.

Figure 19. Heart beat response of C.p. ventralis and X. laevis to perfusion of the ventricle with ouabain, ethanol control and ethanol extracts of I. aegyptius adults, diluted with 0,6 % saline, recorded on a smoked kymograph drum. Samples added between markers on trace.

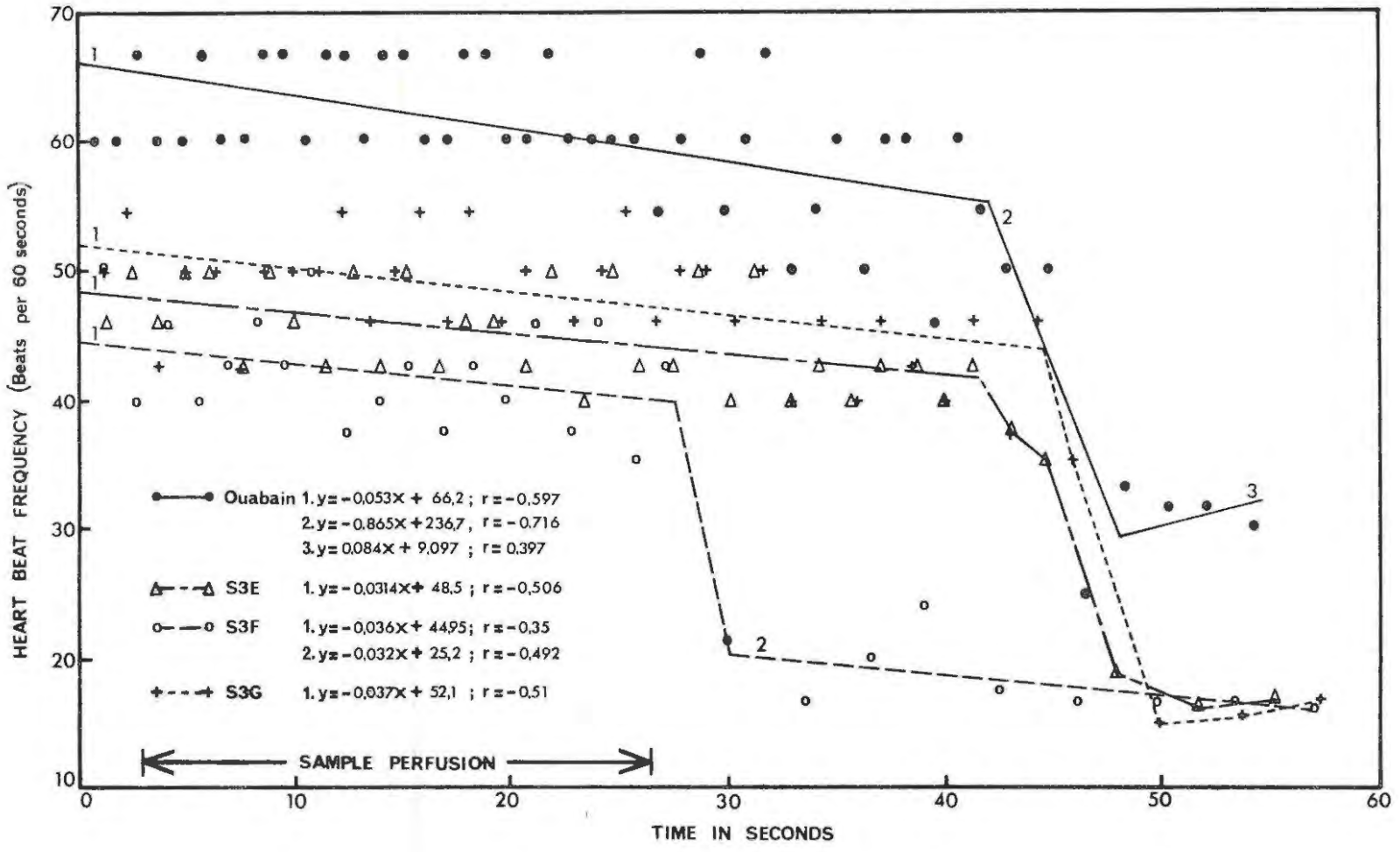


Figure 20. Change in heart beat frequency over 60 seconds after perfusion of the exposed ventricle of C.p. ventralis with three asclepiad/A. nerii-derived I. aegyptius extracts (S3E - G. physocarpus based; S3F - As. curassavica based; S3G - Ar. sericofera based) and ouabain (0,5 ml of ethanol samples in 0,6 % saline (7,5 % ethanol) tested).

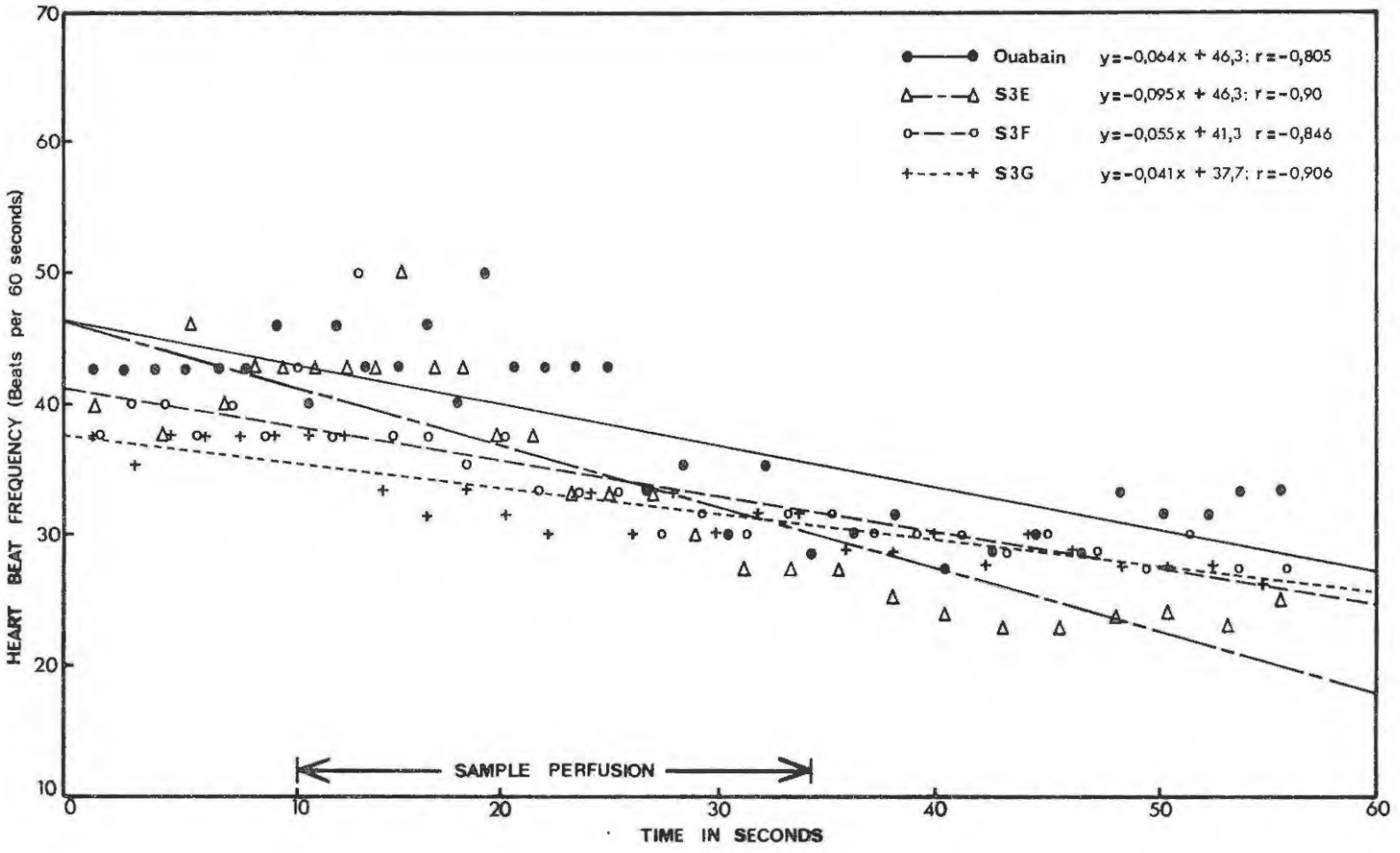


Figure 21. Change in heart beat frequency over 60 seconds after perfusion of the exposed ventricle of X. laevis with three asclepiad/A. nerii-derived I. aegyptius extracts (S3E - G. physocarpus based; S3F - As. curassavica based; S3G - Ar. sericofera based) and ouabain (0,3 ml of ethanol samples in 0,6 % saline (7,5 % ethanol) tested).

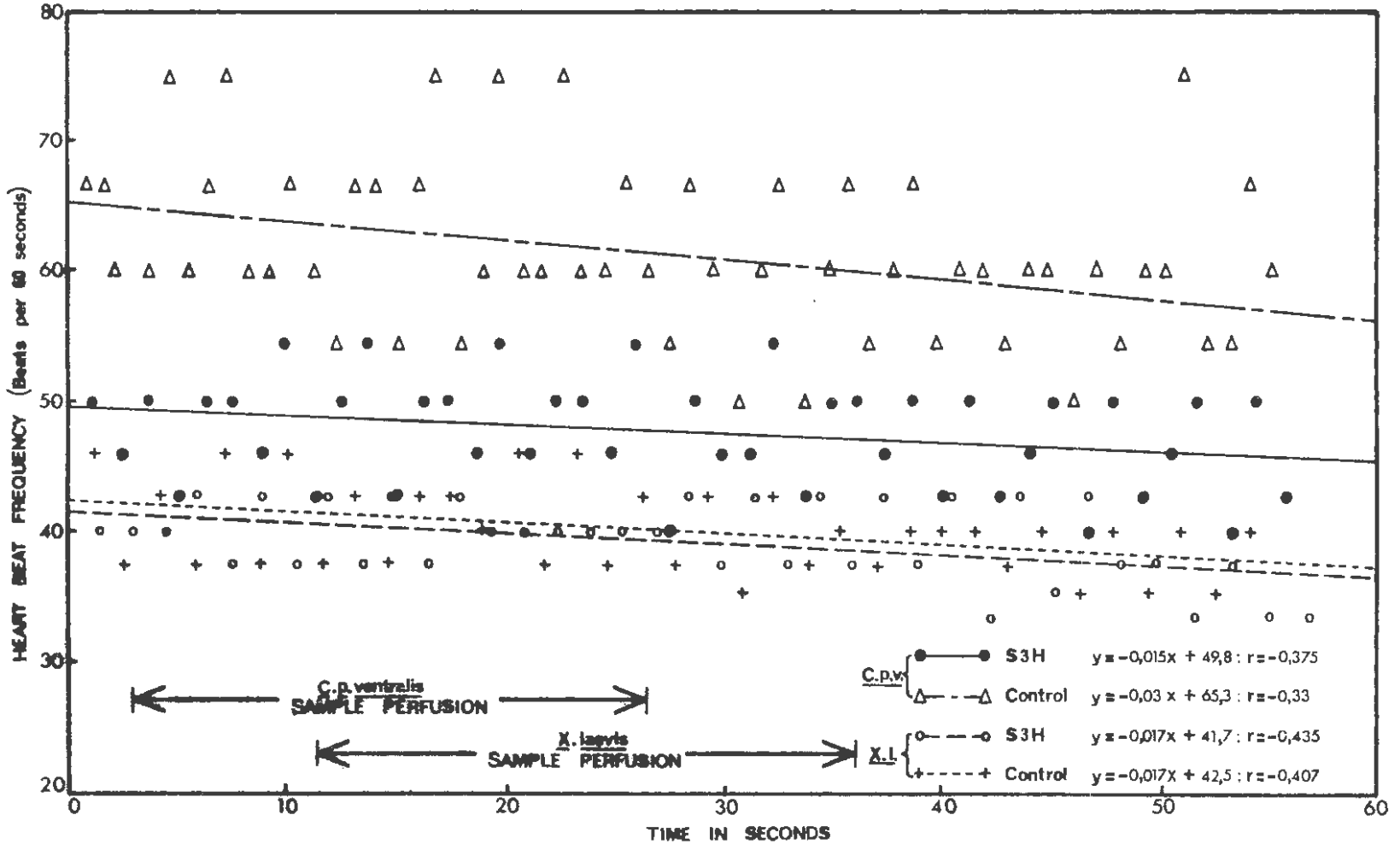


Figure 22. Change in heart beat frequency over 60 seconds after perfusion of the exposed ventricles of X. laevis and C.p. ventralis with non asclepiad/A. nerii-derived I. aegyptius extract S3H (C. morifolium/M. sanborni-derived) and ethanol controls (0,5 ml of ethanol samples in 0,6 % saline (7,5 % ethanol) tested).

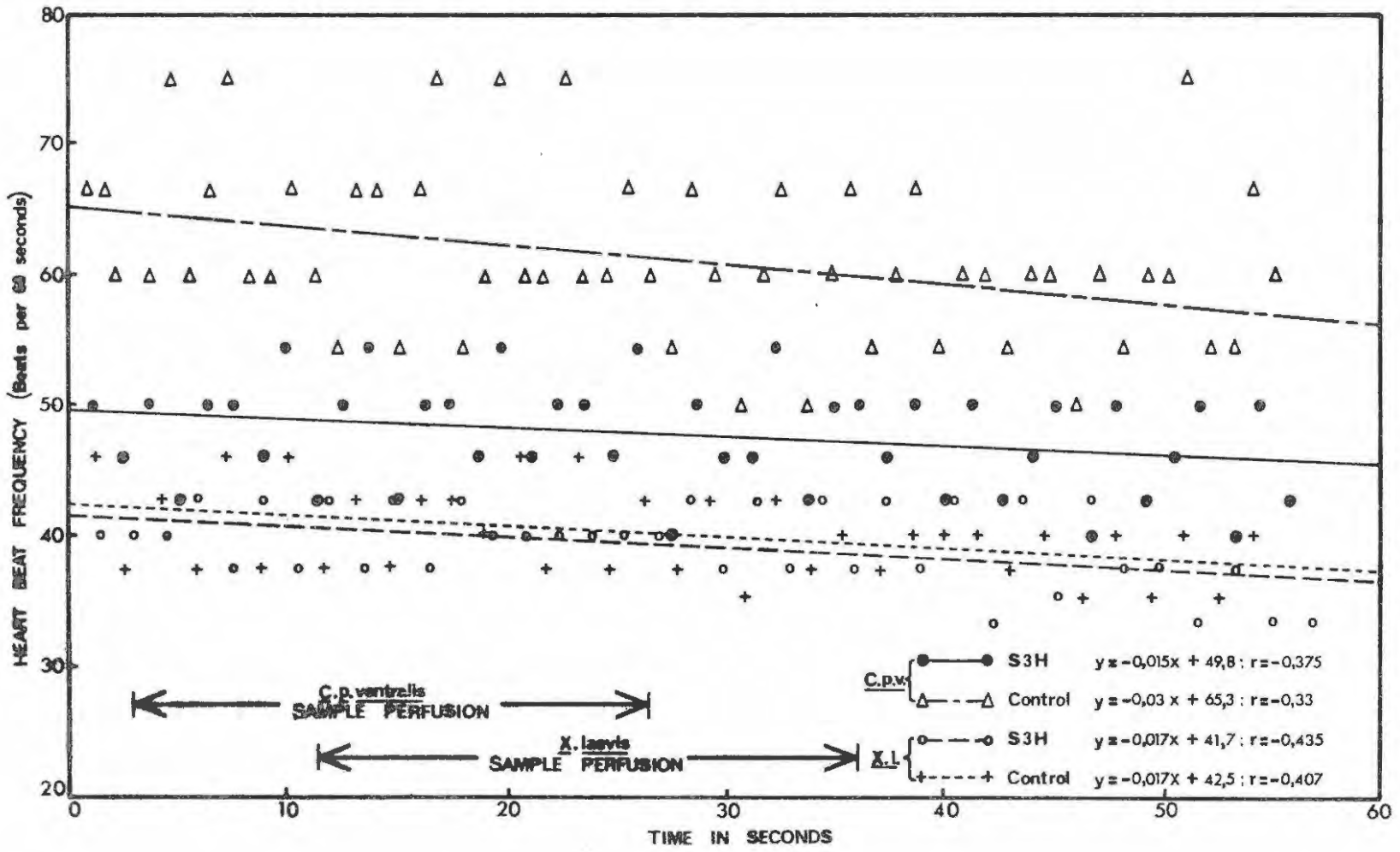


Figure 22. Change in heart beat frequency over 60 seconds after perfusion of the exposed ventricles of X. laevis and C.p. ventralis with non asclepiad/A. nerii-derived I. aegyptius extract S3H (C. morifolium/M. sanborni-derived) and ethanol controls (0,5 ml of ethanol samples in 0,6 % saline (7,5 % ethanol) tested).

b) Results.

The results of the perfusion of exposed ventricles of X. laevis and C. p. ventralis, with adult syrphid extracts, ouabain and ethanol controls, are given in figures 17, 18 and 19 as photographs of selected kymograph drum tracings of heart beat response. The results are also given graphically as linear regressions of heart beat frequency against time in figures 20, 21 and 22. The data of figures 20, 21 and 22 are measured from the series of kymograph recordings. The number of heart beats per 60 seconds is a convenient measure of heart beat frequency or rate.

Initially, the effect of ouabain on the heart of X. laevis was tested by injection into the sartorius muscle of the thigh with 0,012 mg ouabain in ethanol diluted with 0,6% saline (7,5% ethanol v/v). The response appeared as a gradual increase in amplitude and decrease in frequency of the heart beat rate. As the response was gradual (over several minutes) the cardiac glycoside standard was then tested with direct perfusion of the ventricle. Direct perfusion of the heart with ouabain produced a similar heart response almost immediately. All samples were tested by direct perfusion to speed up sample testing. This allowed a larger number of replicates to be performed on each experimental heart preparation. The recovery period between sample administration was rapid as the heart was washed with frog Ringer solution immediately after the response to sample perfusion was recorded.

The principal recorded response of the heart to perfusion with syrphid extracts and ouabain was a marked and sudden decrease in heart beat frequency (prolonged ventricular systole). This decrease was significantly greater than any change in heart beat frequency on perfusion

of the heart with the ethanol control. An increase in the amplitude of heart beat was also recorded in the response of frog hearts to asclepiad/A. nerii-reared I. aegyptius samples and to the cardiac glycoside standard ouabain. Graham and Staddon (1974) describe a similar increase in amplitude and decrease in the heart beat rate of isolated frog hearts perfused with ouabain. Amplitude was considered an unreliable statistic to indicate the heart response to sample perfusion, particularly at the recording speed used (see figures 17, 18 and 19). The inevitable irregularities of the surface of the smoked paper cylinder produced differences in heart beat amplitude from friction that restricted the movement of the heart lever.

As the hearts of X. laevis and C. p. ventralis gave such a marked response to ouabain and to the I. aegyptius extracts, by an increase in the duration of systole, the hearts were not made hypodynamic from reduction of calcium in the Ringer solution. Graham and Staddon (1974), Parsons (1965) and Tsuru et al. (1975) reduced the quantity of calcium in the Ringer solution that perfused isolated frog heart preparations. Reduction of Ca produced low amplitude heart beats after which the increase in amplitude produced by the effect of cardiac glycosides on the heart preparations was easily determined.

All the asclepiad/A. nerii-reared I. aegyptius extracts produced a marked decrease in the frequency of heart beat rate of both C. p. ventralis and X. laevis hearts. The response was particularly severe in chamaeleon hearts with a dramatic and sudden drop in heart beat rate which was maintained at a steady level for at least one minute after sample administration (see figure 17). The heart slowly recovered to the original heart beat rate. Hearts were washed with Ringer solution after each sample perfusion which restored the heart beat to the original rate

Table 10. Comparison of mean heart beat rate (beats per 60 seconds) of exposed C. p. ventralis and X. laevis hearts, 10 seconds before (A), and 25 seconds after (B), perfusion with 3 asclepiad/A. nerii-reared extracts (S3E, S3F, S3G), non-asclepiad/aphid reared extract (S3H), ouabain and ethanol control. (All in 0,6% saline at ethanol concentration of 7,5% v/v.)

	A		B		
C. p. ventralis Sample (plant basis)	Mean Beat Rate	Std. dev.	Mean Beat Rate	Std. dev.	p^1
S3E (G. physocarpus)	47,8	2,88	17,2	1,35	<0,001**
S3F (As. curassavica)	44,0	3,62	16,8	0,51	<0,001**
S3G (Ar. sericofera)	48,6	4,24	15,8	0,85	<0,001**
S3H (C. morifolium)	48,7	3,56	45,8	4,40	0,200<p<0,400n.s.
Ouabain	61,9	3,27	30,3	3,18	<0,001**
Control	66,3	6,22	60,7	7,14	0,100<p<0,200n.s.
X. laevis					
S3E (G. physocarpus)	41,7	2,78	12,7	0,41	<0,001**
S3F (As. curassavica)	39,3	2,01	28,1	1,20	<0,001**
S3G (Ar. sericofera)	37,2	0,83	27,8	1,26	<0,001**
S3H (C. morifolium)	40,6	2,06	35,0	2,30	0,001<p<0,005**
Ouabain	42,9	0,00	31,3	2,43	<0,001**
Control	41,9	4,37	37,7	2,57	0,050<p<0,100n.s.

p^1, probability of no significant difference between means A and B, Student's t-test, variances of both frog and chamaeleon mean heart beat rates before and after sample perfusion are homoscedastic - Fmax test of Sokal and Rohlf, (1969). The distribution of heart beat rates is normal. (** = highly significant probability of a difference between the means; n.s. = not significant.)

almost immediately. The three asclepiad/A. nerii-reared I. aegyptius extracts produced an almost identical response, on both chamaeleon and frog hearts, to the cardiac glycoside ouabain. The heart beat frequencies plotted in figures 20 and 21 show similar rates of decrease of heart beat frequency when the three asclepiad-derived I. aegyptius extracts and ouabain were tested on the hearts of both X. laevis and C. p. ventralis. The difference between the response of the hearts of X. laevis and C. p. ventralis to perfusion with the three I. aegyptius extracts and ouabain was a quicker drop to a steady, slower beat by the chamaeleon hearts than by the frog hearts.

A comparison of heart beat rates, as the mean number of beats per minute, ten seconds before and twenty-five seconds after sample application on both C. p. ventralis and X. laevis hearts is given in Table 10. Student's t-test indicates a highly significant probability of a change in mean heart beat rate after application of the three asclepiad/A. nerii-reared syrphid extracts and ouabain to both C. p. ventralis and X. laevis heart preparations. The ethanol control produced no significant change of heart beat rate in both 'predator' species. The C. morifolium/M. sanborni-derived I. aegyptius extract (S3H) also produced a significant decrease of mean heart rate when applied to the heart of X. laevis (99,5% to 99,9% confidence level), but not of the heart of C. p. ventralis when applied at the same concentration. It is readily apparent from Table 10 that the significant response of the heart to syrphid extract perfusion is due to a marked decrease in the heart beat rate.

The kymograph traces of figures 17, 18 and 19 show a maximum of four relaxations of cardiac muscle in each heart beat. These four relaxations are most evident in the traces of the chamaeleon heart beat. The primary vertical movement upward is due to ventricular systole. The

Table 11. Equivalent numbers of syrphids (A) and weight of ouabain (B) represented in the sample dilutions tested on hearts of *C. p. ventralis* and *X. laevis*.

	Equivalent no. of syrphids extracted in sample		
A. Sample (plant basis)	1,5% ethanol 0,3 ml	7,5% ethanol 0,3 ml	0,5 ml
S3E (*G. physocarpus*)	0,2	1,2	2,0
S3F (*As. curassavica*)	0,8	3,9	6,4
S3G (*Ar. sericofera*)	0,1	0,7	1,2
S3H (*C. morifolium*)	0,1	0,6	0,9
	Weight (mg) in sample.		
B. Sample	1,5% ethanol 0,3 ml	7,5% ethanol 0,3 ml	0,5 ml
Ouabain	0,0035	0,018	0,029

four downward steps are the relaxation of the sinus, atria, ventricle and truncus, probably in this order as this is the order of the rate of working of the parts of the heart (Bell, Davidson and Scarborough, 1957). The heart of C. p. ventralis does not have a truncus arteriosus, for the systemic arches and pulmonary artery separate immediately as they lead from the ventricle. As the heart was suspended from the systemic arch and the pulmonary arteries these large muscular vessels probably contributed to the shape of the heart beat trace. The most important aspect of these traces is that ouabain and asclepiad/A. nerii-derived I. aegyptius extracts slowed the heart beat rate with a markedly prolonged ventricular systole. Continued application of either ouabain or the asclepiad/A. nerii-derived I. aegyptius extracts resulted in systolic arrest of the hearts.

As can be seen from the traces the resting length or tone of the cardiac muscle never alters, as cardiac glycosides affect the systolic contraction of the heart. It should be noted that these in-situ heart preparations will show some regulatory effect from the pressure of returning venous blood to the heart. Due to increased systolic pressure from the effects of cardiac glycosides, the blood returning to the heart is at a higher pressure and so stimulates the sino-atrial node. The sino-atrial node is only inhibited by further addition of cardiac glycoside.

Sensitivity of the heart preparations - Heart preparations were perfused with samples as 7,5% and 1,5% ethanol solutions in 0,5ml or 0,3ml aliquots. The weight of ouabain and the numbers of adult flies represented by these dilutions are shown in Table 11. The most marked ouabain-like effect was given by extract S3F of 150 As. curassavica/A. nerii-reared I. aegyptius on the chamaeleon heart. Extract S3E of 49 G. physocarpus/A. nerii-reared I. aegyptius also produced a very marked

effect on the frog heart (see figures 17 and 18). All the asclepiad/ A. nerii-reared syrphid extracts produced a remarkably similar response in both frog and chamaeleon hearts. As can be seen from Table 11 the I. aegyptius extracts that produced prolonged systole of the hearts represented small quantities of syrphid material. The smallest quantity of syrphid material to produce prolonged systole was equivalent to 0,7 Ar. sericofera/A. nerii-reared I. aegyptius adults.

As a non-asclepiad-derived control extract S3H of 12 C. morifolium/ M. sanborni-reared I. aegyptius was tested for cardiac activity on the heart preparations. From the TLC results it was expected that extract S3H would also produce prolonged systole of frog and chamaeleon hearts. Table 10 supports this expectation with an indication of the significant change in the heart beat rate of X. laevis after administration of extract S3H. The heart of C. p. ventralis did not respond to perfusion with extract S3H at the same concentration. A slight increase in the amplitude of the heart beat on perfusion of the ventricle of X. leavis with extract S3H is also noticeable in figure 18 (trace d.) with two peaks that show a tendency to an increase in the duration of ventricular systole. Although extract S3H did show NAPQ-positive activity on TLC plates it must be concluded that the concentration of an extract of only 12 C. morifolium/ M. sanborni-reared syrphids was not sufficient to elicit a strong systolic response in all the heart preparations at the low sample concentrations employed. The TLC results indicate three faintly NAPQ-positive spots in extracts of C. morifolium/M. sanborni-derived I. aegyptius. Rf values of these spots approximate the Rf values of the NAPQ-positive spots in both asclepiad/A. nerii- and L. caprifolium/H. foeniculi-reared extracts of I. aegyptius. Cardiac glycoside-like activity is therefore apparently present in I. aegyptius, irrespective of the plant-aphid association on which it was reared.

NAPQ-positive spots were detected on TLC plates in extract S3H at a concentration equivalent to 0,2 flies. However, a mild heart response was produced by a minimum concentration, of perfused extract S3H, equivalent to 0,9 flies in 0,5ml of 7,5% ethanol extract. It seems that the chamaeleon and frog heart perfusion tests are approximately four to five times less sensitive than detection of cardiac glycoside-like activity in the same extract on silica gel-G adsorbent layers, with 1,2-naphthoquinone-4-sulphonic acid. This difference in sensitivity is to be expected as the perfused extracts were poured over the ventricle and a certain amount inevitably plays little rôle in the production of a heart response. The slightly more concentrated extract S3G of 23 Ar. sericofera/A. nerii-reared I. aegyptius did produce a more marked increase in the maintenance of a prolonged systole than the C. morifolium/M. sanborni-reared I. aegyptius extract S3H. The amount of syrphid material that corresponded with approximately one adult I. aegyptius is apparently the smallest quantity of syrphid material that will produce a response in frog and chamaeleon hearts on direct application of I. aegyptius extracts (see figures 17e and 18c, responses to extracts equivalent to between 1,2 and 0,7 I. aegyptius).

To conclude, the three ethanolic extracts of I. aegyptius reared on A. nerii on G. physocarpus (S3E), As. curassavica (S3F) and Ar. sericofera (S3G) have a very similar effect, on frog and chamaeleon hearts, to the cardiac glycoside, ouabain. The response of increased ventricular systole is characteristic of the response of the myogenic vertebrate heart to cardiac glycosides. These heart perfusion tests add further support to the conclusion that the NAPQ-positive compounds in extracts of I. aegyptius are cardiac glycosides. The toxicity of cardiac glycosides to vertebrate hearts is of secondary importance to the initial response of a predator to

an insect that contains and advertises the presence of cardiac glycosides. No attempt has been made to determine behavioural responses of predators to I. aegyptius. The behavioural response of a predator to its prey is obviously the key factor in the determination of the efficacy of aposematism in I. aegyptius. Cardiac glycosides are most important in the production of a non-lethal toxic reaction of a predator. Most predators are probably capable of prey discrimination and a learned response to advertised prey unpalatability. Brower and Glazier (1975) suggest that prey species may be sacrificed but it is advantageous to both predator and prey for the predator to survive and remember an encounter with an aposematic insect. As the gut lining of a predator is relatively impermeable to polar steroids I. aegyptius is more likely to produce an emetic response in a vertebrate predator, than death from systolic arrest. Cardiac glycosides of moderate or low polarity are readily absorbed through the gut wall and could result in serious cardiac toxicity and death of the predator. Cardiac glycosides of high polarity like those in I. aegyptius are likely to be advantageous, coupled with the aposematic appearance of the fly, as an anti-predation strategy.

DISCUSSION

Only two zoophagous insects have previously been shown to contain plant derived cardiac glycosides. These two insects were both aphidophagous predators and therefore support the concept of cardiac glycoside ingestion by an aphidophagous predator via its phloem feeding aphid prey. Rothschild *et al.* (1973a) detected cardiac glycosides in the coccinellid bettle, *Coccinella undecimpunctata* and in pupae of a chrsopid, probably *Chrysopa carnea*. Both of these insects were collected as predators of *A. nerii* on the cardiac glycoside-rich apocynale, *Nerium oleander*. A comparison between the number of plant, aphid and aphidophage cardiac glycosides was not made by Rothschild and her colleagues. Therefore, any similarity of cardiac glycoside content between the plant and insects was not established. A tachinid parasite, *Zenillia adamsoni*, which parasitises the asclepiad-reared african monarch butterfly, *Danaus chrysippus*, also has cardiac glycoside activity (Reichstein, *et al.*, 1968; Rothschild, 1973). Rothschild and her fellow workers have tested other aphidophagous insects for cardiac glycoside content when found as part of insect-plant associations that include cardiac glycoside-rich apocynales. Rothschild (1973) and Rothschild *et al.* (1972) examined larvae of an undetermined (personal communication, 1974) syrphid species, larvae of a *Cecidomyia* species, the cecidomyid *Aphidoletes aphidimyza*(?) and another coccinellid *Coccinella septempunctata*, for the presence of plant-derived cardiac glycosides. No cardiac glycosides were detected in each of these predators of *A. nerii* on *N. oleander*. Rothschild (1973) also reports that cardiac glycosides were not found in *Lespesia archippivora* a tachinid parasite of an asclepiad-reared danaid butterfly, *Danaus gilippus berenice*. A carnivorous lampyrid beetle also lacked cardiac glycosides.

In the light of the results of Rothschild and her colleagues the indications that the aphidophagous syrphid Ischiodon aegyptius contains cardiac glycosides which are present in its prey and prey-host plant are significant. The results presented in this dissertation suggest three facts that support the thesis that I. aegyptius is appsematic. Firstly, the asclepiad/A. nerii association is not toxic to I. aegyptius, although there are up to ten cardiac glycosides present in two of the three asclepiad species tested. An asclepiad/A. nerii association is however, toxic to another conspicuous, aphidophagous syrphid, Metasyrphus cognatus. Secondly, I. aegyptius has four cardiac glycoside like compounds in the adult fly. The cardiac glycoside nature of these compounds is suggested by their colour reaction with 1,2-naphthoquinone-4-sulphonic acid (NAPQ). The cardiac glycoside nature of the four compounds is also supported by the third fact. Extracts of I. aegyptius have a marked effect, like that of cardiac glycosides, on the myogenic heart of two vertebrate predators. The significance of these three facts may be indicated with a discussion of 1) the asclepiad/A. nerii/I. aegyptius association and 2) the response of predators to asclepiad/A. nerii-derived, I. aegyptius adults.

1) The asclepiad/A. nerii/I. aegyptius association.

Large quantities of cardiac glycosides are present in the three asclepiad species as the start of the plant-aphid-syrphid-predator food chain described in this dissertation. These cardiac glycosides are particularly evident in G. physocarpus and As. curassavica. Cardiac glycosides are used by asclepiads as a defensive strategy against grazing by herbivorous mammals. A. nerii and I. aegyptius are therefore also protected from accidental ingestion by large herbivores to which asclepiads are toxic (Trimen, 1887 p.54). The asclepiad cardiac glycosides do not appear to be toxic to A. nerii as the second link, or to I. aegyptius as

the third link in the food chain. Although the presence of numerous cardiac glycosides in the asclepiads is evident, the availability of these cardiac glycosides to A. nerii is not so apparent. Extracts of A. nerii from each of the asclepiads produced only one NAPQ-positive spot on the thin layer chromatograms (figures 13 and 14). The same or a similar NAPQ-positive compound is present in the aphid regardless of the cardiac glycoside content of its asclepiad host. This NAPQ-positive spot does approximate the Rf value of one cardiac glycoside in the leaves (the feeding site of the aphid) of Ar. sericofera and As. curassavica. Rothschild et al. (1970a) suggest that the presence of only three of the cardiac glycosides of N. oleander in A. nerii fed on the apocynale, or only two cardiac glycosides in As. curassavica-fed A. nerii, reflects the specific feeding site of the aphid. Alternatively, the other plant cardiac glycosides are structurally altered or metabolised and rapidly excreted by the aphid.

Botha et al, (1972; 1975a) have located the highly specific feeding site of A. nerii on G. physocarpus. The stylets of A. nerii bypass the external phloem of the stem and abaxial phloem of the leaf to terminate in the internal and adaxial phloem, respectively, of the bicollateral vascular bundles of G. physocarpus. The stylets of A. nerii on N. oleander also ended most frequently in internal and adaxial phloem (Botha et al., 1975b). Internal phloem is functionally more important as it translocates more plant metabolites than the external phloem of the bicollateral vascular bundles of G. physocarpus (discussion with Dr. Botha, 1975). We have also found that the stylets of A. nerii end preferentially in the internal phloem of the stems of As. curassavica, Ar. sericofera and two other common Grahamstown asclepiads, Cynanchum ellipticum and Sarcostemma viminale (Botha et al., in preparation). The reason for selective feeding by A. nerii is not clear. Preferential feeding may be associated with the cardiac glycoside content of internal phloem or with the greater nutritional

value of internal phloem sap.

Botha et al. (1975a) indicate that both the internal and external phloem of G. physocarpus are associated with laticifers. Laticifers contain the milky latex characteristic of apocynales. This latex may be considered as a reservoir of 'secondary plant substances', for example, terpenes, alkaloids, sterols and tannins (Esau, 1953), which could include cardiac glycosides. Phloem sap has been described by Kennedy and Fosbrooke (1973) as a "veritable 'widow's cruse' of water and pre-digested food (available to aphids) at a single feeding site". Auclair (1963) indicates that various amino acids and amides, sugars (particularly sucrose) and growth substances have been found as translocated constituents of sieve tube sap in many different plants. The chemical composition of aphids probably reflects this phloem sap composition. For example, the same sterol mixture was found by Forrest and Knights (1972) in the honey dew of the aphid Myzus persicae, as that in its radish seedling host.

Nothing is known of cardiac glycoside distribution in asclepiads except that high concentrations occur in the seeds (Feir and Suen, 1971). A. nerii was found at the highest population levels on G. physocarpus in the Alexandria forest in March and November, at the start and end of seed formation (see figure 5). The population peaks of A. nerii could therefore, be associated with a mobilisation of amino acids at seed formation and seed dispersal or senescence (van Emden, 1972; Kennedy, 1958). Steroids will also be mobilised in asclepiads at seed formation to produce the high seed concentrations of cardiac glycosides (see figures 11, 12 and 13). Cardiac glycosides should, therefore, be available to phloem feeding A. nerii and hence to I. aegyptius

Although cardiac glycosides of different polarity are likely to be available to A. nerii, there is no reason to expect that plant-derived cardiac glycosides should remain unchanged when ingested and used by aposematic insects. In fact, as was indicated at the end of chapter 3C the accumulation of highly polar cardiac glycosides by an insect is probably advantageous as an anti-predator strategy. This advantage must however be balanced against any physiological cost that may be incurred by a prey insect as it copes with storage of highly toxic cardiac glycosides (Brower and Glazier, 1975).

I deliberately limited the scope of this project to the possibility that plant-derived cardiac-active steroids could be the source of unpalatability of a conspicuous aphidophagous syrphid because insects are not capable of sterol synthesis (Robbins, et al., 1971). Sterols are ingested from an exogenous source by insects and are used as structural components of cells and as precursors for essential steroids (Clayton, 1964). Aphids are interesting as a link between a plant steroid source and a predatory insect because aphid endosymbionts are also capable of supplementing the sterol content of aphids (Ehrhardt, 1968).

Ingestion of aphid sterols by I. aegyptius larvae is necessary as predatory insects are unable to convert plant sterols to their dietary sterol requirements (Levinson, 1962). This observation does not preclude the possibility that I. aegyptius is able to convert low polarity plant-derived steroids to highly polar cardiac glycosides. I. aegyptius could also supplement its cholesterol and possibly cardiac active steroid requirements with steroids synthesised by endosymbionts in the gut of the larvae. Other zoophagous insects such as blood-feeding diptera have abundant symbionts at specific sites in the gut, or in mycetomes of the adult fly (Buchner, 1965).

I. aegyptius is particularly useful in this study of extrinsically derived defensive steroids because syrphids are holometabolous. Third instar I. aegyptius larvae exude their gut contents and gut lining shortly before pupation. The adult fly is therefore, on emergence, an effectively "pristine entity as far as food intake is concerned" (Brower et al., 1967). The presence of steroid toxins in the adult fly that resemble plant steroids ingested by the larvae, indicates assimilation and sequestration rather than the simple presence of ingested steroids in the gut of the insect.

I. aegyptius has up to four NAPQ-positive compounds in adults reared on the different plant-aphid associations. The purple NAPQ colour response is characteristic of cardiac glycosides, and the same I. aegyptius material also produced a cardiac glycoside-like response on two myogenic vertebrate hearts. However, these cardiac glycoside-like responses do not confirm the cardiac glycoside identity of the four compounds in I. aegyptius. Parsons (1965) has indicated that non-steroid lactones may mimic the action of cardiac glycosides on isolated frog hearts. Unfortunately the presence of the steroid mucleus in the NAPQ-positive compounds was not definitely confirmed, for example with the Liebermann-Burchard colour reaction (Fieser and Fieser, 1959) or with more sophisticated infra-red absorbance, nuclear magnetic resonance, or mass spectra techniques. Lactones are not known to produce a colour reaction with NAPQ. Conversely, amino acids turn red with NAPQ (Folin reaction, Waldi, 1959) but do not produce a response in cardiac muscle like cardiac glycosides.

The presence of the four cardiac glycoside-like compounds in I. aegyptius reared on plant-aphid associations that lack cardiac glycosides suggests three possibilities to explain their presence. Cardiac glycosides in I. aegyptius may be synthesised by the aphid, by aphid or syrphid endosymbionts or, finally, by the syrphid. This last possibility is

interesting as some researchers have identified defensive secretions synthesised by two other groups of aphid predators. Several alkaloids, and also skatole and tridecene have been identified as intrinsically derived defensive secretions in a number of coccinellid species (Pasteels, et al., 1973) and a chrysopid (Blum et al., 1973).

The origin of the four cardiac glycoside-like compounds in I. aegyptius is not clear, particularly as non-asclepiad-derived I. aegyptius also contains the same four compounds. Despite the uncertain origin of these four compounds their presence and cardiac glycoside nature is further reinforced by the effect of I. aegyptius extracts on the two vertebrate heart preparations.

2) Predator response to asclepiad/A. nerii-derived I. aegyptius adults.

The presence of plant-derived cardiac glycosides in both phytophagous and a few zoophagous insects has been firmly established by the efforts of Dr. Rothschild and her associates. The information gathered up to 1972/1973 has been clearly summarised by Rothschild (1973). However, it is primarily the work of L.P. Brower and his colleagues that has elegantly illustrated the significance of asclepiad-derived cardiac glycosides as a protective device in some conspicuously coloured danaid butterflies. The cardiac glycoside-based unpalatability of the monarch butterfly, (Danaus plexippus) to avian predators has been clearly established (Brower, 1958; Brower and Brower, 1964; Brower et al., 1967; 1968; Brower and Glazier, 1975; Brower, 1969). Brower and Glazier (1975) and Parsons (1965) have refined the study of monarch unpalatability to prove that the wings of the butterfly contain the most cardiac glycosides, but that the abdomen is the most toxic portion of the butterfly to avian predators.

The accumulated cardiac glycosides of the butterfly abdomen are probably highly polar to account for this toxicity difference.

A palatability spectrum of different quantities of asclepiad cardiac glycosides ingested by the larvae of D. plexippus has also been determined (Brower et al., 1967; 1968; Brower, 1969). The butterfly varies in palatability to birds according to the species of asclepiad, and hence the quantity of cardiac glycosides ingested by the larvae. This palatability spectrum has a geographical distribution associated with the distribution of different asclepiad species in North America (Brower et al., 1972; Brower and Moffitt, 1974). The extremely similar african monarch, D. chrysippus, has a parallel cardiac glycoside-based palatability spectrum (Brower et al., 1975; Reichstein et al., 1968; Rothschild et al., 1975).

From the highly polar nature of the NAPQ-positive spots in the extracts of I. aegyptius it seems that this syrphid parallels the high toxicity of the abdomen of D. plexippus. The significance of the polarity of the NAPQ-positive compounds becomes apparent in a consideration of predator response to syrphids. As with the emetic response of avian predators to the abdomen of D. plexippus, the highly polar cardiac glycosides of I. aegyptius would also produce a rapid emetic response. The gut lining of a predator is almost impermeable to highly polar cardiac glycosides and so an immediate emetic response is likely. For example, shortly after capture a single dwarf chamaeleon (C. p. ventralis) was placed temporarily in a C.E. room that contained A. nerii-infested G. physocarpus. An hour later the chamaeleon was found with its mouth crammed with A. nerii. The chamaeleon had started to retch violently and continued to do so up until its death approximately ten hours later. Rather than kill a predator it is obviously advantageous to both predator and prey for the predator to learn to associate

an unpleasant experience, such as emesis, with a particular conspicuously coloured, toxic prey insect. The moderate polarity NAPQ-positive compound detected in A. nerii could have been responsible for the chamaeleon's death after gut absorption. Highly polar cardiac glycosides would probably have been eliminated by the emetic response before their slow intestinal absorption produced a seriously toxic response in the chamaeleon. Unfortunately, this suggestion has not been borne out by an isolated observation. Ten asclepiad/A. nerii-reared I. aegyptius adults were offered to a single female dwarf chamaeleon. The chamaeleon accepted the flies readily and apparently suffered no ill effects. The chamaeleon is capable of an emetic response, and extracts of I. aegyptius have cardiac activity. Therefore, why the aphid should produce an emetic response, but the syrphid does not is not clear.

It is predation pressure that determines the response or defence of the prey. The 'signal receiver' (Wickler, 1968) may receive a prey signal that is either advantageous or disadvantageous to itself. Prey signals that are disadvantageous to the predator include cryptic and false warning (pseudaposematic) appearances (Poulton, 1890). The aposematic prey signal is advantageous to the predator as the predator does not waste time and energy in pursuit of unsuitable prey. The defence of any insect is never absolute. Jones (1932) pointed this out with a statement by Dr. Karl Jordan, "There is no absolute protection anywhere. Protection is relative like everything else. A steel helmet did not prevent soldiers from being killed, but far more would have been killed without it". It would be very surprising if all of the predators of I. aegyptius responded to the fly in the same manner!

Arthropod predators of syrphids include thomisiid, salticid and

web-building spiders (personal observations; Bankowska et al., 1975; Bristowe, 1958; Hobby, 1930; Myers, 1935; Turnbull. 1973), asilid, empid and cordylurid flied (personal observations; Marshall, 1902; Parmenter, 1968) and a crabronid wasp (Pickard, 1975). In contrast with Haase (1893), Marshall (1902) considers hymenopteran and dipteran parasites to be significant mortality agents of aposematic insects.

The dwarf chamaeleon, Chamaeleo pumilus, and the tree frog, Megalixalus fornasinii, have already been mentioned as vertebrate predators of syrphids (Burrage, 1973; Cott, 1932). In the Alexandria forest avain insectivores were common. Three flycatcher species and a sunbird were observed feeding on diptera. Bryant (1973) records syrphids as an important prey item of the house martin. In a fascinating paper Waldbauer and Sheldon (1971) have indicated the importance of insectivorous birds as signal receivers of syrphids.

Intuitively, the warning signal issued by I. aegyptius is primarily visual (see figures 1 and 23), but may also be partly olfactory, behavioural or acoustic. Thomisiid and salticid spiders have colour vision and are capable of accurate detection and identification of prey such as flies (De Voe, 1975; Land, 1969; Tyschenko, 1961). Asilid flies and mantids can discriminate shapes and movement (Dennis et al., 1975; Rilling et al., 1959). Gelperin (1968) has shown that mantids can also learn to avoid aposematic prey. Most insects have colour vision (Burkhardt, 1964) and are therefore capable of conspicuous-syrphid-pattern discrimination. Avain predators also have accurate colour vision (Sillman, 1973).

Both aphid and syrphid extracts contained several low polarity yellow pigments that moved with the solvent front on the TLC plates. Rothschild

(1971) and Rothschild et al., (1972) have suggested, on the basis of information published by Rosenberg et al., (1968), that yellow carotenoid pigments in aposematic insects enhance taste reception by predators. Most cardiac glycosides have a very bitter taste, however some polar cardiac glycosides, like ouabain, are not bitter. The three polar compounds in I. aegyptius need not be bitter tasting as the fly is unlikely to survive an encounter with a predator. Production of a rapid emetic response is all that is necessary for the predator to associate the experience with the aposematic syrphid. It is interesting that I. aegyptius contains a lower concentration of a moderate polarity cardiac glycoside-like compound. This compound is probably bitter tasting but is not present in sufficient quantities to be lethal to a predator.

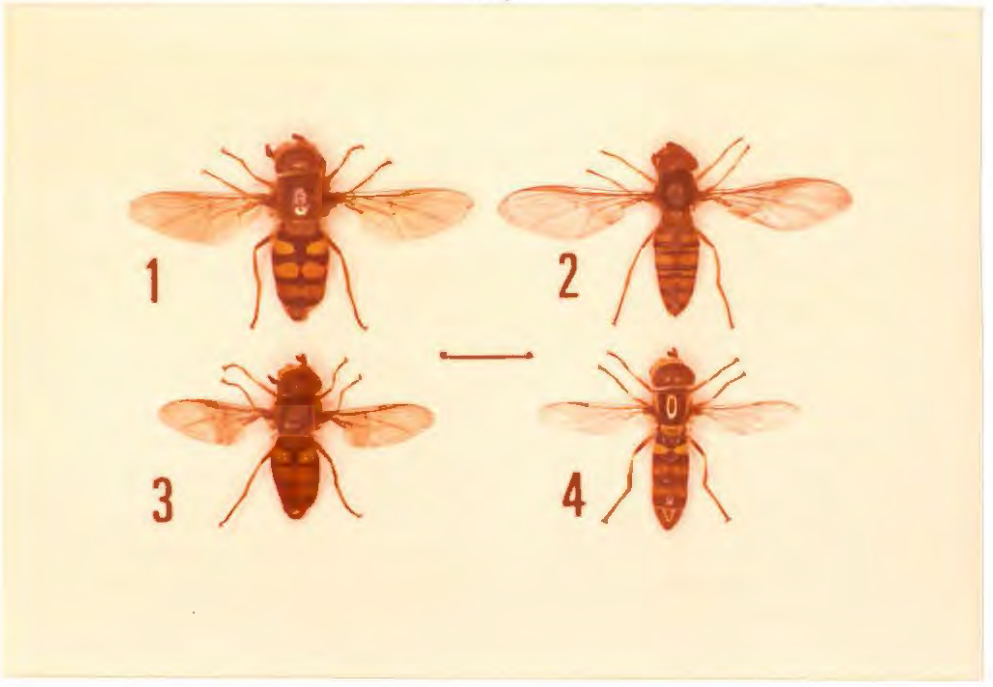
The toxicity of plant-derived cardiac glycosides in insects to a series of vertebrates is well established (see chapter 3C; Marsh and Rothschild, 1974; Rothschild, 1961; Rothschild and Kellett, 1972). Cardiac glycosides are also toxic to some insects, although little is recorded of their effect on the myogenic heart (Richter, 1973) of insect and arachnid predators (Krijgsman, 1952). Cardiac glycosides and other plant-derived steroids are toxic to, or have an effect on, ionic movement in drosophilid and tsetse flies, and three common orthoptera (Gooding, 1975; Harley and Thorsteinson, 1967; Mattson et al., 1971; Treherne, 1966; Weber-von Grotthuss et al., 1974).

Since the theories of warning colouration and mimicry were suggested by Bates (1862, in Wickler, 1968), Müller (1878; 1879) and Wallace (1867), many researchers have produced huge lists of predator responses to brightly coloured (both aposematic and pseudaposematic) and cryptic insects (for example, Carrick, 1936; Jones, 1932; Marshall, 1902). These early, mostly

haphazard, predator response experiments and general observations provided strong evidence in support of predator selection pressure. Predation pressure, particularly from birds, is responsible for the evolution and maintenance of protective appearances in insects. Carrick (1936) reports that four insectivorous bird species treated conspicuously coloured aphidophagous syrphids as aposematic and avoided them. The birds observed by Carrick readily fed on cryptic syrphids and other cryptic flies. Carrick points out that his experiments did not determine whether the birds' behaviour was in response to either an aposematic or a pseudaposematic signal from the syrphids.

Aposematism and pseudaposematism can be considered as the two extremes of conspicuous protection. Müller (1879) argued that the mutual advantage of aposematism to both predator and prey (Holling, 1965) results in convergence of the conspicuous appearance of unpalatable insects to produce "Müllerian mimics". Pseudaposematism, or "Batesian mimicry" is a direct corollary of aposematism. Bates considered that the accurate resemblance of a palatable insect to a conspicuous unpalatable model deceives the common predator and so protects the mimic (Rettenmeyer, 1970; Wickler, 1968). Batesian mimicry is particularly advantageous to a palatable insect as it takes advantage of the protected model species' metabolic expenditure on chemically based defence, without a similar expenditure (Whittacker and Feeny, 1971). The commonest aposematic appearance of insects is a bold pattern of black and yellow that advertises a complex spectrum of insects of different relative degrees of toxicity. Different predators may respond to the same insect as either a Batesian or a Müllerian mimic of an insect of similar appearance.

Syrphids are well known for their accurate Batesian mimicry of



Scale line = 5 mm.

Figure 23. Syrphidae, subfamily Syrphinae - typical appearance of hoverflies with aphidophagous larvae.
1. Metasyrphus corollae (Fabr.) (Herts., England).
2. Episyrphus balteatus (DeGeer) (Argyll, Scotland).
3. Metasyrphus cognatus (Loew) (Grahamstown, South Africa).
4. Ischiodon aegyptius (Wied.) (Grahamstown, South Africa).

Scale line = 10 mm.

Figure 24. Syrphidae, subfamily Milesiinae (except Chrysotoxum) - mimetic hoverflies with saprophagous or scavenging larvae. Batesian mimetic syrphids on left and Hymenopteran model on right of each pair. 1. Volucella bombylans (L) left; Bombus lapidarius (L) right (Glos., England). 2. Chrysotoxum veralli (Coll) left; Vespula vulgaris (L) right (Herts., England). 3. Ceriana caffra (Loew) left; Polistes sp. right (Grahamstown, South Africa). 4. Xylota segnis (L) left; Tenthredo livida (L) right (Argyll, Scotland).

hymenopteran models (see figure 24 and Brower and Brower, 1962; 1965). Waldbauer and Sheldon (1971) have described the phenological relationships of mimetic complexes of syrphids with their sting-protected hymenopteran models and bird predators.

It is interesting that almost all of the accurate syrphid Batesian mimics of hymenoptera, except Chrysotoxum, are restricted to the sub family Milesiinae (see figure 24). As indicated in Chapter 2 the larvae of the Milesiinae are primarily saprophagous and the larvae of the other syrphid sub family, the Syrphinae, are primarily zoophagous, as aphid or psyllid predators. The extremely accurate resemblance to hymenopteran models of four syrphids from different syrphid tribes with saprophagous (Ceriana, Chrysotoxum, Xylota) or scavenging (Volucella) larvae is shown in figure 24. Figure 23 illustrates four syrphid species typical of the world-wide appearance of the majority of the Syrphinae. A notable exception is the genus Chrysotoxum in the Syrphinae which are all apparently excellent Batesian mimics of wasps. However, Chrysotoxum mimics have saprophagous larvae which are derived from the aphidophagous type. The apparent restriction of aposematism and pseudaposematism to syrphid species with aphidophagous and saprophagous larvae respectively indicates that aphidophagous syrphids may commonly use an extrinsic source of chemical defence. This extrinsic source is available to aphidophagous syrphids from plants rich in defensive toxins, via the phloem feeding homopteran prey of larval Syrphinae.

CONCLUSION

Attractant or repellant properties of secondary plant substances are argued by Fraenkel (1958; 1959; 1969) to be almost wholly responsible

for the association of an insect with a plant. Fraenkel considered that the nutritional value of all plants is probably similar and that nutrients have little effect on host plant selection by insects. Culvenor (1970), Dethier (1970), Ehrlich and Raven (1964) and Whittacker and Feeny (1971) have developed Fraenkel's statement on the "raison d'être" of secondary plant substances with the consideration that these substances are paramount in the determination of patterns of plant use by phytophagous insects and of the more complex "allelochemic" interactions between plants, phytophages and predators. It is apparent, however, that all plants are not nutritionally similar as an insect food source and that the quantities and proportions of nutrients are also importants determinants of plant and insect associations (Dethier, 1970).

Plant-derived cyclic terpenoids have been identified as the basis of several steroidal defensive agents in plant and insect associations. For example, the cardiac glycosides described in this dissertation and insect or vetebrate hormone and pheromone mimics (Clayton, 1970; Miller and Mumma, 1974; Southwood, 1973). Clayton (1970) states that "terpenoids are of immense biologioal age" and reasons that their relatively simple synthetic pathway "should have been exploited throughout the biological continuum".

Aphids have been described as "examples of highly evolved plant parasites" that have become "intimately involved with the physiology of the host plant" (Southwood, 1973). Southwood concludes that "plants have responded to insect attack and thus insects and plants must be viewed as two co-evolving, competing and often mutually dependent biochemical systems" (see also Dethier, 1970). Aphidophagous syrphids are obligate predators, primarily of aphids, and so they too are part of co-evolving plant and

insect systems. The biological age of steroids and the close association between aphidophagous syrphids, aphids and plants indicates the value of exploiting established synthetic pathways to establish the chemical basis of aposematism in an aphidophagous syrphid.

SUMMARY

1. The thesis that predatory syrphids ingest and sequester plant toxins ingested via their herbivorous larval prey is investigated. These plant toxins are a possible extrinsic basis for aggressive chemical defence against predation in black and yellow aphidophagous syrphids.

2. This thesis is investigated as a) an account of the biology of a toxic plant (3 spp. of Asclepiadaceae) - aphid (Aphis nerii) - syrphid (Ischiodon aegyptius) association in the field and laboratory; b) an investigation into the presence of cardiac-active steroids (cardiac glycosides) in the asclepiad-aphid-syrphid association, as a likely defensive toxin; and c) recordings of the effect of aphid/asclepiad-reared syrphids on two myogenic vertebrate heart preparations, compared with the heart response to non asclepiad/aphid-derived syrphids.

3. These three sections are preceded by an account of the identity of the commonest aphidophagous syrphids in the Grahamstwon area. Characteristics of the male terminalia and developmental stages are compared for the seven dominant aphidophagous syrphids of the tribe Syrphini.

4. Each chapter is introduced with a selected account of the literature pertinent to the investigation.

5. Monthly population fluctuations in the field of A. nerii on an asclepiad and the small numbers of aphidophagous syrphid larvae and eggs in the A. nerii colonies are recorded for one years' observations.

6. A technique for the culture of I. aegyptius in the laboratory is

described. The development of I. aegyptius on A. nerii on two asclepiad species is also described.

7. The A. nerii/asclepiad association was found to be lethally toxic to the common oligophagous syrphid aphidophage, Metasyrphus cognatus, but not to the polyphagous aphidophage I. aegyptius. It is suggested that the tolerance of a polyphagous syrphid aphidophage is advantageous as a defensive strategy because a wide range of defensive plant toxins are available, via aphids, as an extrinsic basis for aposematism by the syrphid.

8. Paper and thin layer chromatography indicate the presence of cardiac glycosides in 3 asclepiad species, A. nerii from each of the asclepiad species and also I. aegyptius from each of the A. nerii/ asclepiad associations. Up to 10 cardiac glycosides have been detected by specific colour reactions in extracts of two of the asclepiad species. However, only one cardiac glycoside-like compound is present in A. nerii extracts but four cardiac glycoside-like compounds are detectable in extracts of I. aegyptius. The Rf values of these compounds do not reflect the Rf values of cardiac glycosides detected in the leaves as the feeding site of the aphid. The chromatographic activity of the cardiac glycoside-like compounds in I. aegyptius extracts is compared in different solvent systems.

9. One of the asclepiad species has only one cardiac glycoside-like compound which gives the same slightly atypical, cardiac glycoside detection response as the compounds in the insect extracts.

10. Although four cardiac glycoside-like compounds are repeatedly detected in I. aegyptius reared on the 3 A. nerii/asclepiad associations,

the same four compounds are also detected in I. aegyptius reared on two non-asclepiad based aphid/plant associations. It is suggested in the discussion that the four cardiac glycoside-like compounds in I. aegyptius may be synthesised by the aphid, by aphid or syrphid endosymbionts or by the syrphid.

11. The cardiac glycoside identity of the four compounds in I. aegyptius is supported by the response elicited in exposed hearts of the dwarf chamaeleon, Chamaeleo pumilus ventralis and the clawed toad, Xenopus laevis to perfusion with extracts of I. aegyptius. The heart beat response is recorded on smoked kymograph traces as an increase in the duration and amplitude of ventricular systole after perfusion with either the cardiac glycoside ouabain or extracts of I. aegyptius. Chapter 3C concludes with the suggestion that the high polarity of 3 of the 4 cardiac glycoside-like compounds detected in I. aegyptius is advantageous as production of emesis is a more likely predator response than death, due to low intestinal absorption.

12. The final chapter discusses selected literature relevant to the use by insects of plant-derived toxins as a defensive strategy against predation. The discussion briefly develops the topic of possible transmission of steroids from the internal phloem of the plant, as the specific feeding site of A. nerii, to the aphidophagous larvae of I. aegyptius. The response of predators to aposematic insects and the rôle of predators as selective agents of insect defensive strategies is also discussed. The hypothesis is proposed that many syrphids with aphidophagous larvae are typically aposematic and many syrphids with saprophagous-like larvae are typically pseudaposematic or Batesian mimics of hymenoptera.

13. It is concluded in the discussion that the biological age of steroids and the close association between aphidophagous syrphids, aphids and plants indicates the value of exploiting established synthetic pathways to establish the chemical basis of aposematism in an aphidophagous syrphid.

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APPENDIX a).

Details of plant, aphid and syrphid extracts tested for the presence of cardiac glycosides, with the visualising reagents TNDP, DNBZ and NAPQ. The extracts were tested on both thin layer and paper chromatograms. (C/M = chloroform:methanol (2:1), C/A = chloroform:ethanol (3:2), Chf = chloroform, E = ethanol, H = hexane, M = methanolic mother liquor; crosses denote intensity of cardiac glycoside detection; 1 or 2 refer to the extraction methods of chapter 3B section a).

Sample no.	Sample	dry wt. gm.	volume ml.	solv -ent	extr meth	card. glyc.
Gomphocarpus physocarpus based extracts.						
A. Plant - G. physocarpus.						
P1D	Leaves and stems.	3,69	1,87	C/M	1	+++
P1E	Leaves and stems.	1,00	1,05	C/M	1	+++
P1F	Leaves and stems.	-	-	C/M	1	+++
P1G	Leaves and stems.	5,93	1,69	C/M	1	++++
P1P	Leaves and stems infested with A1C.	0,52	2,20	C/M	1	+
P1V	Seed pods (plumose seeds + follicle)	8,60	2,10	Chf	2	++++
P1W	"	"	1,40	C/A	"	++++
P2F	Leaves and stems.	3,64	0,70	Chf	2	++++
P2G	"	"	0,67	C/A	"	++++
P2H	"	"	0,75	H	"	-
P2I	"	"	2,40	M	"	-
B. Aphid - A. nerii on G. physocarpus (collected in Tullgren funnel).						
A1C	aphids from P1P in C.E. room.	0,042	0,98	C/M	1	-
A1H	aphids from C.E. room.	1,09	0,92	Chf	2	-
A1I	"	"	1,54	E	"	-
A1J	"	"	2,68	C/A	"	++
A1K	"	"	2,40	M	"	-
C. Syrphid - I. aegyptius reared on A. nerii/G. physocarpus (plus 3 other syrphid species).						
S1D	6 female Betasyrphus claripennis.	0,029	1,03	C/M	1	-
S1E	4 male B. claripennis.	0,022	1,02	C/M	1	-
S1F	1 female Betasyrphus sp. A.	0,0057	0,87	C/M	1	-
S1G	3rd instar larval gut exudate, B. claripennis.	0,033	0,88	C/M	1	-
S1H	11 empty B. claripennis pupal cases.	0,051	1,03	C/M	1	-
S1I	3 female Betasyrphus sp. A.	0,0094	1,03	C/M	1	-
S1Y	2 female Paragus minutus.	0,0021	0,99	C/M	1	-

Sample no.	Sample	dry wt. gm.	volume ml.	solv -ent	extr meth	card. glyc.
S2E	50 female I. aegyptius.	0,141	1,14	C/A	2	++++
S2F	"	"	1,40	Chf	"	++++
S2G	"	"	1,00	E	"	-
S2H	50 male I. aegyptius.	0,128	0,30	Chf	2	++++
S2I	"	"	0,25	H	"	-
S2J	"	"	0,77	C/A	"	-
S2K	8 I. aegyptius pupae.	0,031	0,57	Chf	2	-
S2L	"	"	0,40	C/A	"	-
S2M	56 male & 60 female (116) I. aegyptius.	0,427	0,48	Chf	2	++++
S2N	"	"	0,39	C/A	"	++++
S2O	"	"	1,18	H	"	-
S2P	39 male & 41 female (80) Betasyrphus sp. A.	0,283	0,50	Chf	2	++++
S2Q	"	"	1,00	C/A	"	++++
S2R	"	"	0,75	H	"	-
S2Y	Methanol fraction of S2M-S2O.	-	1,75	M	2	-
S2Z	Methanol fraction of S2P-S2R.	-	1,19	M	2	-
S3C	Methanol fraction of S2K-S2L.	-	0,82	M	2	-
S3D	Methanol fraction of S2E-S2G.	-	0,45	M	2	-
S3E	27 male & 22 female (49) I. aegyptius.	0,134	1,22	E	Bio-assay	Heart activ.
	Asclepias curassavica based extracts.					
	A. Plant - As. curassavica.					
P1A	Leaves and stems (extract hydrolysed)	4,70	1,40	C/M	1	++++
P1B	Leaves (not weighed)	-	-	C/M	1	+
P1C	Leaves (not weighed)	-	-	C/M	1	++
P1Q	Leaves and stems infested with A1D.	0,25	1,90	C/M	1	+
P1R	Flowers (freshly opened).	0,29	2,40	C/M	1	-
P1S	Leaves (not weighed or dried), infested with A1G.	-	-	C/M	1	-
P1T	'GTN' Seed pods (plumose seeds + follicle).	3,30	1,14	Chf	2	++++
P1U	"	"	2,50	C/A	"	++++
P2B	Leaves and stems.	6,40	1,10	Chf	2	+++
P2C	"	"	1,70	C/A	"	+++
P2D	"	"	0,83	H	"	-
P2E	"	"	1,90	M	"	-
P2M	'OXF' Seed pod (plumose seeds + follicle) from M. Rothschild.	0,47	0,40	C/A	2	++
P2N	"	"	0,97	Chf	"	++++

Sample no.	Sample	dry wt. gm.	volume ml.	solv -ent	extr meth	card. glyc.
B. Aphid - *A. nerii* on *As. curassavica* (collected in Tullgren funnel).						
A1D	Aphids from P1Q in C.E. room.	0,0313	1,00	C/M	1	-
A1G	Aphids from P1S in C.E. room (not dried)	1,70 (wet)	3,13	C/M	1	-
A1L	Aphids from C.E. room	1,16	1,40	Chf	2	++
A1M	"	"	1,68	C/A	"	-
A1N	"	"	1,00	H	"	-
C. Syrphid - *I. aegyptius* reared on *A. nerii*/*As. curassavica* (plus one other syrphid species).						
S1A	3 *Betasyrphus* sp. *A.*	-	-	C/M	1	-
S1B	1 female *I. aegyptius.*	0,0035	-	C/M	1	-
S1C	Salticid spider preying on S1B.	0,0053	1,03	C/M	1	-
S2V	25 male & 27 female (52) *I. aegyptius.*	0,145	0,50	Chf	2	++++
S2W	"	"	1,07	C/A	"	++++
S2X	"	"	0,55	H	"	-
S3B	"	"	1,08	M	"	-
S3F	76 male & 74 female (150) *I. aegyptius.*	0,308	1,17	E	Bio-assay	Heart activ.
Araujia sericofera based extracts.						
A. Plant - *Ar. sericofera.*						
P1H	Leaves and stems.	2,21	1,43	C/M	1	+ ?
P1I	Leaves and stems infested with A1A.	0,66	2,37	C/M	1	-
P1O	Leaves and stems infested with A1B.	2,29	2,45	C/M	1	-
P1X	Leaves and stems.	8,70	1,17	Chf	2	-
P1Y	"	"	1,35	C/A	"	+++
P1Z	"	"	0,95	H	"	-
P2A	"	"	4,00	M	"	-
P2J	Seeds only.	3,25	1,22	Chf	2	-
P2K	"	"	1,60	C/A	"	+++
P2L	"	"	1,75	M	"	-
B. Aphid - *A. nerii* on *Ar. sericofera* (collected in Tullgren funnel).						
A1A	Aphids from P1I in C.E. room.	0,026	0,98	C/M	1	-
A1B	Aphids from P1O in C.E. room.	0,150	1,06	C/M	1	-
A1O	Aphids from C.E. room.	1,34	0,69	Chf	2	+++
A1P	"	"	3,05	C/A	"	-
A1Q	"	"	0,96	H	"	-

Sample no.	Sample	dry wt. gm.	volume ml.	solv -ent	extr meth	card. glyc.
C. Syrphid - I. aegyptius reared on A. nerii/Ar. sericofera (plus 1 other syrphid species).						
S2D	6 female Betasyrphus claripennis, hydrolysed.	0,019	0,93	C/M	1	++
S2S	7 female & 8 male (15) I. aegyptius.	0,041	0,83	Chf	2	+++
S2T	"	"	0,44	C/A	"	+++
S2U	"	"	0,59	H	"	-
S3A	"	"	1,00	M	"	-
S3G	16 female & 7 male (23) I. aegyptius.	0,0598	1,00	E	Bio-assay	Heart activ.
Miscellaneous extracts.						
A. Plants.						
P1J	Cynanchum ellipticum (Asclepiadaceae) leaves and stems.	1,035	0,90	C/M	1	- ?
P1K	C. ellipticum, leaves and stems.	0,57	1,60	C/M	1	-
P1L	Sarcostemma viminale (Asclepiadaceae) succulent stems infested with A1E.	1,59	0,78	C/M	1	-
P1M	Pergularia sp.(?)(Asclepiadaceae), leaves and stems.	7,18	3,35	C/M	1	++++
P1N	C. ellipticum, leaves and stems infested with A1F.	0,803	2,05	C/M	1	-
P20	Lonicera caprifolium (Caprifoliaceae) leaves and stems.	5,47	3,00	M	reflux	-
B. Aphids - A. nerii on other asclepiads.						
A1E	A. nerii on S. viminale stems (P1L).	0,004	1,00	C/M	1	-
A1F	A. nerii on C. ellipticum, leaves and stems (P1N).	0,033	0,97	C/M	1	-
C. Syrphids.						
S1J	2, II Betasyrphus claripennis larvae on C. ellipticum + A. nerii.	0,0014	0,94	C/M	1	-
S1K	1, III Betasyrphus sp. larva on C. ellipticum + A. nerii.	0,0053	0,88	C/M	1	-
S1L	I. aegyptius pupa + larval gut exudate, on C. ellipticum + A. nerii.	0,008	0,95	C/M	1	-
S1M	1, III Betasyrphus sp. larva on C. ellipticum + A. nerii.	0,007	0,89	C/M	1	-
S1N	1, III Betasyrphus sp. larva on C. ellipticum + A. nerii.	0,005	0,90	C/M	1	-
S1O	3 Betasyrphus sp. pupae + larval gut exudate, on C. ellipticum + A. nerii.	0,057	0,88	C/M	1	-
S1P	I. aegyptius pupa on C. ellipticum + A. nerii.	0,003	0,87	C/M	1	-

Sample no.	Sample	dry wt. gm.	volume ml.	solv -ent	extr meth	card. glyc.
S1Q	1, III Betasyrphus sp. larva on C. ellipticum + A. nerii	0,0057	0,97	C/M	1	-
S1R	I. aegyptius pupa on C. ellipticum + A. nerii.	0,0038	0,90	C/M	1	-
S1S	I. aegyptius pupa on C. ellipticum + A. nerii.	0,0057	0,87	C/M	1	-
S1T	Betasyrphus sp. pupal cases + larval gut exudate, on C. ellipticum + A. nerii.	0,0084	1,01	C/M	1	-
S1U	4 female I. aegyptius, adults collected at Lake Sibaya, Kwa Zulu.	0,0216	0,97	C/M	1	-
S1V	4 male Paragus borbonicus adults, collected at Lake Sibaya, Kwa Zulu.	0,0098	0,99	C/M	1	-
S1W	11 male P. borbonicus adults, collected at Lake Sibaya, Kwa Zulu.	0,020	0,95	C/M	1	-
S1X	2 male I. aegyptius adults, collected at Lake Sibaya, Kwa Zulu.	0,009	0,98	C/M	1	-
S1Z	14 pupal cases of B. claripennis (larvae transferred to A. nerii on S. viminale).	0,041	1,06	C/M	1	-
S2A	7 male B. claripennis (from S1Z)	0,036	1,03	C/M	1	-
S2B	7 female B. claripennis (from S1Z)	0,042	0,97	C/M	1	-
S2C	1 B. claripennis pupa + larval gut exudate (larvae transferred to A. nerii on S. viminale).	0,0074	0,97	C/M	1	-
S3H	5 male & 7 female (12) I. aegyptius reared on Macrosiphoniella sanborni on Chrysanthemum morifolium (Compositae).	0,0175	0,64	E	Bio-assay	Heart activ.
S3I	35 male & 27 female (62) I. aegyptius reared on Hyadaphis foeniculi on Lonicera caprifolium (Caprifoliaceae).	0,210	2,00	E	Bio-assay	Heart activ.

APPENDIX b).

Preparation of 2,2'- 4,4'-tetranitrodiphenyl (TNDP).

As 2,2'- 4,4'-tetranitrodiphenyl is not commercially available it was prepared from o-nitroaniline to 1) o-chloronitrobenzene to 2) 2,2'-dinitrodiphenyl to 3) 2,2'- 4,4'-tetranitrodiphenyl.

1) Preparation of o-chloronitrobenzene.
(Beilsteins Handbuch der Organischen Chemie, Isocyclische Reihe, Band V, 1922, Berlin, pp. 241-243.).

180 gm. of o-nitroaniline was mixed with 1,2L ofwater and 900 ml. of conc. HCl in a beaker until the o-nitroaniline dissolved to form a brown solution. 90 gm. of sodium nitrite in 300 ml. water was added dropwise to the solution. During addition the solution was kept at between 5° and 10°C. Sufficient sodium nitrite must be added to diazotise all of the aniline present. The solution of diazonium salt was added to 90 gm. of copper/bronze moistened with conc. HCl and then warmed slowly. The brownish-black solution turned green and then dark olive-green. The solution was maintained at 70°- 80°C until nitrogen was no longer given off. The solution was filtered through celite 545, cooled and the product separated in two litres of ether. The ether layer was washed with 500 ml. of sodium hydroxide solution. A brown emulsion formed which was washed with 750 ml. water. After filtration the ether was distilled off to leave approximately 200 ml. of a dark brown liquid. The dark brown liquid was distilled under vacuum (1,7 mm. Hg.) to produce approx. 120 gm. of a golden yellow, clear liquid that distilled over at 75°- 79°C. The liquid quickly crystallised to form bright yellow, needle-like crystals.

Melting point	= 32°- 33°C.
Yield	= 102 gm. o-chloronitrobenzene.
Theoretical yield	= 205,4 gm.
% yield	= 49,7 %

2) Preparation of 2,2'-dinitrodiphenyl.
(Fuson, R.C. & E.A. Cleveland. 1940. Organic Syntheses 20: 45-47.).

101 gm. of o-chloronitrobenzene and 100 gm. of clean dry sand were placed in a 500 ml. flask equipped with a mechanical stirrer. The mixture was heated in an oil bath to 215°- 225°C and 100 gm. of prepared copper/bronze was slowly added over 80 minutes.

The copper/bronze was prepared by washing it with a litre of 2 % iodine solution in acetone for 11 minutes. The washed copper/bronze was collected on a Büchner funnel and then resuspended in 500 ml. of a 1:1 solution of con. HCl in acetone. The copper/bronze was again collected in a Büchner funnel, washed with acetone and dried in a vacuum desiccator bfore using as above.

After adding the copper/bronze the temperature of the mixture was kept at 220°C for a further 90 minutes with continual stirring. The hot, almost solid, green-brown mass was removed to a beaker containing 200 gm. sand. This produced a khaki-brown powder after stirring. The mixture was boiled twice with successive 750 ml. volumes of ethanol and the filtrate was collected, pooled and the alcohol distilled off under vacuum. The concentrated product was filtered to collect large, dark olive-green crystals. These crystals were redissolved in 750 ml. of hot ethanol and 3 gm. of activated charcoal was added. The mixture was filtered rapidly through Whatman no. 40 filter paper in a water heated glass funnel. The filtrate crystallized on cooling. Crystals were collected on a Büchner funnel and dried in a vacuum desiccator. The product dried as fine, creamy-yellow crystals.

Melting point	= 123°- 124°C.
Yield	= 44,5 gm.
Theoretical yield	= 78,2 gm.
% yield	= 56,9 %

3) Preparation of 2,2'- 4,4'-tetranitrodiphenyl.
(Brower, L.P., P.B. McEvoy, K.L. Williamson & M.A. Flannery. 1972. Science, N.Y. 177: 429.).

44,5 gm. of 2,2'-dinitrodiphenyl were dissolved in 450 ml. of red, fuming nitric acid (density 1,512) and heated to 80°C. 450 ml. of conc. sulphuric acid and a further 450 ml. of red, fuming nitric acid were added and then refluxed for one hour. The mixture was then cooled and poured onto ice. The product was collected by suction filtration and appeared as a cream precipitate which was dissolved in 500 ml. of boiling glacial acetic acid. From this solution, fine, cream crystals formed. The product was recrystallized from glacial acetic acid seven times and dried in a vacuum desiccator.

The melting point of the purified product was partially at 149°C and partially at 164°- 168°C. The published melting point is 166,5° to 167°C. Continued recrystallization of TNDP did not improve the melting

point determination. Thin layer chromatography showed the product to be free of 2,2'-dinitrodiphenyl. An NMR spectrum of the product confirmed its identity as 2,2'- 4,4'-tetranitrodiphenyl.

Melting point	= 149°? - 164°- 168°C.
Yield	= 18,9 gm.
Theoretical yield	= 60,9 gm.
% yield	= 31 %

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