**Mutualism of *Stratiotes aloides* L. (Hydrocharitaceae) and *Hydrellia tarsata* Haliday (Diptera: Ephydridae): Tritrophic Interaction of Macrophyte, Leaf-Mining Dipteran Pollinator and Parasitoid Braconidae**

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**Abstract**—The aquatic macrophyte *Stratiotes aloides* L. is of conservation concern in central Europe due to its high importance for biodiversity in lowland floodplain and ditch ecosystems. However, over the last decades this species has shown population declines for instance in Germany or the Netherlands. *S. aloides* is dioecious with male and female individuals, in mixed or separated stands, often reproducing vegetatively. Generative reproduction is observed less frequently, but of great importance for declining plant populations facing threats of habitat destruction and eutrophication. Precisely which arthropods transfer *S. aloides* pollen was previously unknown. We examined flower visitors of *S. aloides* in the 2011 and 2014 flowering seasons in ditches of a wet grassland ecosystem in Bremen, Northwest Germany. *Hydrellia tarsata* Haliday (Diptera: Ephydridae) was found abundantly in male and female flowers of *S. aloides* in both years. Pollen of *S. aloides* was actively transferred by *H. tarsata* and reproduction of the fly in *S. aloides* leaves was detected by rearing *H. tarsata* from extracted puparia. The mining *Hydrellia* were parasitised by the braconid wasps *Chaenusa punctulata* Burghele and *Chorebus densepunctatus* Burghele, which also visited *S. aloides* flowers in 2011 and 2014. These results point to a mutualism between *S. aloides* and the ephyrid *H. tarsata*, with both partners benefiting with their own reproduction. This relationship between plant and dipteran pollinator is however complicated in a tritrophic interaction with the braconid parasitoids, which infest the mining stages of the ephyrid flies and could potentially also transfer *S. aloides* pollen.

*Keywords:* myiophily, pollination, Ephydridae, Braconidae, Dacnusini, plant-insect interaction

**INTRODUCTION**

Insect pollination is an essential part of the reproductive cycle in numerous angiosperm plant species. The most important pollinators are found among the Hymenoptera, which have a major impact on natural and anthropogenic ecosystems by transporting pollen between flowers of entomophilous plants and thereby facilitate fertilization and generative reproduction (Kevan & Baker 1983). The ubiquitous role of flies (Diptera) as flower visitors has long been noted (Larson et al. 2001), however their importance in plant pollination only recently became evident (Szymank et al. 2008; Woodcock et al. 2014). Examples of flower pollination by flies (miyophil) are manifold (Larson et al. 2001; Kevan 2002; Szymank et al. 2008) and come from both terrestrial and freshwater ecosystems (e.g. Mesler & Lu 1993; Lippok & Renner 1997). Biotic pollination commonly occurs in aquatic plant species, as these rely to a large part on insects for pollen transfer and less often on abiotic vectors like wind or water (Ackerman 2000). Although many aquatic plants have the ability to reproduce vegetatively, generative reproduction is an important aspect of their biology and has been retained throughout evolution (Philbrick & Les 1996).

The aquatic macrophyte *Stratiotes aloides* L. belongs to the fully aquatic family Hydrocharitaceae and is a perennial, dioecious plant species (Cook & Urmi-König 1983; Chen et al. 2012). It grows in sexually separated or mixed stands and often reproduces vegetatively, forming dense aggregations in slow-flowing to stagnant freshwater habitats (Cook & Urmi-König 1983). The generative reproduction of *S. aloides* relies on the close proximity of the two sexes (Smolders et al. 1995) but has not been studied comprehensively. Thus, no detailed account of the species’ pollination ecology exists and the only descriptions of flower-visiting insects come from early anthecology (Knuth 1899; Daumann 1931). From these early works Diptera emerge as likely pollination vectors (Daumann 1931). More recent observations of the fly *Hydrellia tarsata* Haliday (Diptera: Ephydridae) mining in leaves and visiting flowers of *S. aloides* suggest a possible interaction of the two species for reproduction (Przhiboro 2004; Stuke 2010; Katzenberger et al. 2013).

In general, *S. aloides* is seen as an indicator species for aquatic habitats with high biodiversity value in lowland floodplain and ditch ecosystems and it is therefore of concern to nature conservation in Europe (Suurtla et al. 2009; Kunze et al. 2012). *S. aloides* showed population...
declines over the last decades in central Europe, for instance in the Netherlands or Germany (Smolders et al. 1996; Sinning 2001; Sautari et al. 2009). The Bremen region in Northwest Germany remains one of the main distribution areas for *S. aloides* (Cordes et al. 2006) and the biology and ecology of the species is under extensive study here (Nüssel & Zacharias 2010; Zantout et al. 2011; Kunze et al. 2012; Katzenberger et al. 2013). Bremen is especially well-suited to studying the generative reproduction of the species as seed-set occurs plentifully (Kunze et al. 2012). The observed negative population trend is mainly attributed to eutrophication, leading to shading and degraded growth conditions (Smolders et al. 2003; Zantout et al. 2011). Another reason suspected to contribute to the decline is an overall lack of generative reproduction by the species and therefore less adaptability to changing environmental factors (Smolders et al. 1996, 2003).

In this study, we address the following questions: (1) Which insects visit flowers of *S. aloides*? (2) Do flower visitors transfer pollen and thereby indicate pollination activity? (3) Is there evidence for stable interactions between *S. aloides* and its flower visitors? With our results we aim to investigate the suggestion from early anthecology that *S. aloides* is likely an example of fly pollination (myiophily) and contribute towards to a detailed account of the pollination ecology for *Stratiotes aloides*.

**MATERIALS AND METHODS**

**Study species**

The water soldier, *Stratiotes aloides*, is distributed in Northern, Central and Eastern Europe and also in Central Asia (Cook & Urmi-König 1983). In Germany, *S. aloides* is listed as “vulnerable” in the national red list of flowering plants (Korneck et al. 1996) and considered an important indicator species for conservation management aiming to sustain lowland ditches rich in biodiversity (Kunze et al. 2012). The dense stands of *S. aloides* form a spatially complex and sheltered habitat for diverse generalist macroinvertebrate communities (Tarkowska-Kukuryk 2006) but they also foster specialised species like the dragonfly, *Aeshna viridis* L. (Rantala et al. 2004; Sautari et al. 2009).

The plant has a unique life form; during summer it is partially emergent but in autumn it sinks and over-winters under water as a rosette with green leaves (Cook & Urmi-König 1983). Then, in spring, *S. aloides* re-surfaces, old leaves are shed and fresh leaves develop, while during the whole cycle it is usually rooted below in loose substrate (Cook & Urmi-König 1983).

*S. aloides* is entomophilous and the flowers attract insects with large white petals, osmophores and nectaries (Cook 1998). The flowers emit a foul smell similar to that of rotting meat (Daumann 1931; Cook 1998). Male and female flowers of *S. aloides* look essentially alike only differing in the morphology of the regenerative organs. Male flowers have 5–17 stamens surrounded by 20–30 nectaries while female ones usually show 6 pistils likewise surrounded by 20–30 nectaries (Cook & Urmi-König 1983; Nüssel & Zacharias 2010). The first flowers appear in April and flowering continues until the end of August (Kunze et al. 2012). The only available field study on the generative reproduction of the species found that it required short distances (< 1 km) between male and female stands for successful fertilisation, but did not investigate pollinators (Smolders et al. 1995). Nevertheless, *S. aloides* is parthenocarpic and therefore produces sterile fruits when not pollinated which are distinctly smaller than fertile fruits and quickly start to rot (Cook & Urmi-König 1983; Smolders et al. 1995).

Flower visitation of *S. aloides* by the hoverfly *Eristalis tenax* L. (Diptera: Syrphidae) was already noted by Knuth (1899). In his detailed account on the floral morphology of *S. aloides*, Daumann (1931) also studied the mode of pollination and observed the dipterans *Calliphora erythrocephala* Meigen (Diptera: Calliphoridae), *Calliphora vomitoria* L. (Diptera: Calliphoridae), *Lucilia caesar* L. (Diptera: Calliphoridae), *Sarcophaga carnaria* L. (Diptera: Sarcophagidae), *Odontomyia tigrina* Fabricius (Diptera: Stratiomyidae) and especially abundant *Hydrellia cardamines* Haliday (Diptera: Ephryidae) as flower visitors.

The Braconidae are a diverse group of parasitoid wasps occurring worldwide, with over 40,000 species they form the second largest family among the Hymenoptera (Goulet & Huber 1993). They are often closely attuned to their hosts and highly efficient in controlling host populations and therefore used for biological control (Wharton 1993). Among the Braconidae the subfamilies Alysiinae and Opiinae are endoparasitoids of cyclorrhaphous Diptera, ovipositing in the larva or egg and emerging from the puparium (Wharton 1993). The biology and ecology of the braconid species belonging to the tribe Dacnusini in the subfamily Alysiinae is still largely unknown (Fischer 1999). In general Dacnusini are seen as very host specific and this is especially marked in *Chorebus*, where many species have only been obtained from a single host (Griffiths 1966).

**Sample collection and species identification**

Samples of flower visitors and mining fauna in leaves of *Stratiotes aloides* were taken in the 2011 and 2014 flowering season (April - July) in the nature reserve “Westliches Hollerland” (53°06′54.1″N, 8°51′30.8″E) in Bremen, Germany. The study area is an approximately 300 ha large and extensively managed agricultural grassland with a dense network of ditches for drainage in the marshland. The samples were obtained from ditches with compact stands of *S. aloides* (both mixed-sex and unsexed separated stands occurred).

Flower visitors were caught with a collapsible net (15 cm diameter, 2 × 2 mm mesh width) on a telescoprc pole. The net was lowered over randomly selected open flowers above the water line and was then collapsed, trapping flower visitors inside. Trapped insects were transferred into a vial and killed with ethyl acetate. All samples were taken during the middle of the day between 11 am and 4 pm. Dipteran puparia were collected by randomly gathering 7–10 leaves from several *S. aloides* plants on the water surface and dissecting these in the laboratory. The puparia were reared in petri-dishes on wet cotton wool, kept in a constant
temperature of 21 °C. Species identification of *Hydrellia tarsata* was achieved by dissection of the male genitalia which corresponded exactly to Collin (1966). Female *Hydrellia* in the samples could not confidently be identified to species level, however, they resembled male specimens of *H. tarsata* morphologically and in some instances copulation was observed on flowers before trapping. Insect dissections and identifications were performed under a Zeiss Stemi 2000-C dissection microscope. Species identification of braconid wasps was made by Dr. HCJ Godfray, Oxford. The samples were compared to museum specimens and will be deposited in the collection of the National Museum of Scotland for future research. Taxonomy and identification of Braconidae is a challenging field and little is known about the tribe *Dacnus* ini to which these wasps belong (Fischer 1999). The specimens collected in this study were determined with every possible care but, for the lack of a functional key, species identifications are termed “probably” or species names are put in quotation marks (HCJ Godfray, pers. comm.).

To determine pollen transfer of individuals, pollen grains were located on male *Hydrellia tarsata* specimens after collection from flowers. Single pollen grains from extremities and body setae, visible under the dissection microscope, were transferred with a dissection needle to a microscope slide coated with basic fuchsin gelatin and thereby stained (Beattie 1971). Under a Zeiss Axio Lab.A1 microscope, pollen grains were observed in 1000 × magnification (with oil) with a Ph1 filter and photographed with a Zeiss AxioCam ERC5s camera. Pollen morphology (exine structure and diameter) from the samples collected from *H. tarsata* individuals was compared to stained pollen from male *S. aloides* flowers under 1000 × magnification to ascertain pollen identity.

**RESULTS**

*Flower visitors of Stratiotes aloides*

In 21 samples from male and female flowers of *Stratiotes aloides* collected in 2011 and 2014 we found 42 male individuals of *Hydrellia tarsata* and 5 female *Hydrellia* specimens which could not be determined to species level (Tab. 1). *H. tarsata* was abundant in and around flowers of dense stands of *S. aloides* in small ditches (< 3 m) with almost stagnant water in the grassland of the study area (Fig. 1). Flower visiting *Hydrellia* were often observed deep within the flowers, moving actively inside around the flower nectaries and stamens or pistils, sometimes copulating. Even if we approached the flowers closely the insects would not fly away, only a strong movement of the flower would trigger escape.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Date</th>
<th>Flower</th>
<th>No.</th>
<th>ID</th>
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</thead>
<tbody>
<tr>
<td>2011-1</td>
<td>27.05.11</td>
<td>♂</td>
<td>1</td>
<td><em>Hydrellia tarsata</em></td>
</tr>
<tr>
<td>2011-2</td>
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<td>♀</td>
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<tr>
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<tr>
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<tr>
<td>2011-5</td>
<td>27.05.11</td>
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<td>4</td>
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<tr>
<td>2011-7</td>
<td>27.05.11</td>
<td>♀</td>
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<td>♂</td>
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<td>female <em>Hydrellia</em> sp.</td>
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<tr>
<td>2011-9</td>
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<td>28.06.11</td>
<td>♀</td>
<td>2</td>
<td><em>Hydrellia tarsata</em></td>
</tr>
<tr>
<td>2014-1</td>
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<td>♀</td>
<td>6</td>
<td><em>Hydrellia tarsata</em></td>
</tr>
<tr>
<td>2014-2</td>
<td>30.05.14</td>
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<tr>
<td>2014-7</td>
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<td>♀</td>
<td>1</td>
<td>female <em>Hydrellia</em> sp.</td>
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</table>

Table 1. Abundance of dipteran visitors to male and female *Stratiotes aloides* flowers in our samples from the study area in years 2011 and 2014.
In 8 samples from *S. aloides* flowers we observed 9 specimens of a braconid wasp belonging to the genus *Chaenusa*, probably *punctulata* Burghhele (Tab. 2). Another braconid wasp from the genus *Chorebus* (*uliginosus* group sensu Nixon), probably *densepunctatus* Burghhele was observed in 2 samples with 2 specimens (Tab. 2). Similar to the flower visiting *Hydrellia*, the braconid wasps were usually deep within the flower, only escaping upon flower movement. A one-time visitation of a *S. aloides* flower by a hoverfly was observed but no other Diptera or Hymenoptera were found. Other rarely observed arthropods in flower samples included three Coleoptera specimens, one type of Collembola, one Lepidoptera specimen and one Araneae specimen. Due to the low abundances those arthropods were not investigated further.

### Table 2. Abundance of hymenopteran visitors to male and female *Stratiotes aloides* flowers in our samples from the study area in years 2011 and 2014. Species identifications of braconid wasps are put in quotation marks due to the lack of a functional key; see also Material and Methods.

<table>
<thead>
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<tr>
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<td><em>Chorebus &quot;densepunctatus&quot;</em></td>
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<tr>
<td>2011-4</td>
<td>27.05.11</td>
<td>♀</td>
<td>1</td>
<td><em>Chaenusa &quot;punctulata&quot;</em></td>
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<tr>
<td>2011-7</td>
<td>27.05.11</td>
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<td>♀</td>
<td>2</td>
<td><em>Chaenusa &quot;punctulata&quot;</em></td>
</tr>
</tbody>
</table>

Transport of *Stratiotes aloides* pollen

*Stratiotes aloides* pollen could be detected on 4 male specimens of *Hydrellia tarsata*, found in two samples from male *S. aloides* flowers (Fig. 2). Pollen loads were always relatively low with never more than 10 pollen grains attached to body setae and legs of *H. tarsata* (Fig. 2). Exine structure of pollen grains found on *H. tarsata* corresponded to pollen from a male *S. aloides* flower, showing a granular structure and clearly visible bacula (Fig. 2). Pollen extracted from a male *S. aloides* flower had an average diameter of 42.3 µm (± SD 3.1, N = 5) while the average diameter of pollen grains found on *H. tarsata* specimens was 39.8 µm (± SD 1.6, N = 5).
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**Figure 2.** Male *Hydrellia tarsata* specimen (sample 2014-2, see Tab. 1) caught in a male flower of *Stratiotes aloides* carrying pollen grains of *S. aloides*. Part “a” shows adult fly with pollen (red arrows) on tarsus and tibia (25 × magnification) while part “b” shows dissected genital used for species identification (50 × magnification). Part “c” shows single stained *S. aloides* pollen grain (1000 × magnification, diameter 37 µm) extracted from legs of *H. tarsata* for comparison with stained pollen grain from anthers of *S. aloides* (1000 × magnification, diameter 38 µm) displayed in part “d”. Note the granular exine structure and bacula (circular spots) in *S. aloides* pollen grains.

**Puparia extracted from leaves of *Stratiotes aloides***

Dipteran puparia were collected three times in 2014 (29th April, 27th May, 29th June) from mines in *Stratiotes aloides* leaves and subsequently reared in the laboratory. During the first sampling in April *S. aloides* had just resurfaced, most of the leaves were still from the previous year and no flowers were present. In the two later samplings *S. aloides* stands were flowering and most of the old leaves had decayed. The puparia were subcylindrical and semi translucent with a variable degree of brown colour. The length of the puparia ranged between 2-3 mm. In total 10 *Hydrellia* specimens hatched from the puparia, of which two were male and could be identified as *Hydrellia tarsata*. From these puparia three braconid wasps also emerged; two were identified as *Chaenusa*, probably *punctulata*, and one as *Chorebus*, probably *densepunctatus*.

**DISCUSSION**

The results from our study clearly show that the ephrydrid *Hydrellia tarsata* reproduces in the leaves of *Stratiotes aloides*, but also that it is a main candidate to consider as pollination vector for the species. From earlier studies on *S. aloides* we know that fertile seeds are produced plentifully in our study area (Kunze et al. 2012) and thus pollination and fertilization occurs. We found *H. tarsata* abundantly in male and female flowers (Fig. 1) and revealed that it does in fact carry *S. aloides* pollen (Fig. 2). The results were consistent between the two study years and no other flower visitors were found regularly enough to consider relevant pollination activity. It has to be noted that our sample size was limited and the collections of flower visitors were taken from a small study area, chosen for easy accessibility. Also, we did not perform any experiments to demonstrate the pollination activity of *H. tarsata*. Nevertheless, the presented evidence strongly suggests an interaction between *S. aloides* and *H. tarsata*, where the fly reproduces in the plant tissue by means of its phytophagous larvae and the imagines pollinate the dioecious flowers of the macrophyte and thus facilitate its generative reproduction. However, our results indicate that the braconid parasitoids, visiting flowers of *S. aloides* and infesting immature *Hydrellia*, add another level of complexity to this plant-pollinator interaction and could mediate the strength of the relationship between *H. tarsata* and *S. aloides* in a set of tritrophic interactions.
Ecological relationships between flies and flowers are critical to each other’s survival in many habitats and flower visitation by small Diptera, including alcalectrys, warrants more investigation (Seymank et al. 2008; Woodcock et al. 2014). The Ephyridae in particular are already known as pollinators of aquatic plants such as Cabomba caroliniana Gray (Schneider & Jeter 1982) or several species of Nuphar (Lippok & Remmer 1997; Nagasaki 2007). In two of these studies Hydrellia species were confirmed as pollinators of an aquatic macrophyte, one in Europe (Lippok & Remmer 1997) and one in North America (Schneider & Jeter 1982). Small Diptera could compensate for their own supposed inefficiency as pollinators by their large numbers and their ubiquity (Seymank et al. 2008), which seems also to be the case in our study system. The pollen loads evident on Hydrellia tarsata were usually low, but flowers were visited by many individuals at a time and H. tarsata was abundant in the study area. Two of the only three known records for H. tarsata in Northwest Germany were made on, or next to, S. aloides stands (Stuke 2010; Katzenberger et al. 2013). Furthermore in the only systematic study on Statixletes aloides floral visitation, Daumann (1931) describes the common species Hydrellia cardamines as very abundant. But Hydrellia is a taxonomically challenging genus and species identifications are often only possible by dissection of the male genitalia (Deyrup & Deyrup 2008; Stuke 2010), thus it seems possible that old accounts in the literature contain misidentifications. H. tarsata probably has been overlooked on S. aloides for a long time.

The dipteran reproduction in plant tissues by mining phytophagous larvae has received some attention in the literature, for instance Deonier (1971) lists S. aloides as host for Hydrellia griseola Fallen and Hydrellia mutata Zetterstedt. That the rarer and less studied Hydrellia tarsata also mines in S. aloides was observed in Russia only recently (Przhbobo 2004) and our study confirmed that the relationship occurs in Germany.

Considering all of this information, the interaction between S. aloides and H. tarsata is likely a mutualistic one, with both partners benefiting in reproduction. Insect pollination is usually based on a mutual benefit and flowers receive primary (nectar/pollen feeding) or secondary (e.g. brood and/or mating places) rewards from flowers, while the plants get cross-pollinated for generative reproduction (Woodcock et al. 2014). Our results clearly show that H. tarsata utilises a secondary reward by brooding in leaves, but the high ratio of male to female flies we found on flowers and the observation of copulation suggest that S. aloides could also be a mating site for H. tarsata. Feeding on nectar as a form of primary reward in flowers is known for Hydrellia species as well (Deonier 1971; Schneider & Jeter 1982; Lippok & Remmer 1997). Nevertheless the mining H. tarsata damage and consume plant tissue during their reproduction in the leaves of S. aloides, but the interaction between plant and pollinator will persist if the plant has a benefit in propagules produced (Woodcock et al. 2014). More research on the pollination efficiency of H. tarsata could help to understand how much S. aloides plants actually benefit from this interaction.

In a tritrophic interaction plants interact with carnivores to indirectly defend against herbivory, a phenomenon also described for braconid wasps attracted by plant volatiles (Heil 2008). The braconids Chaenusa “punctulata” and Chorebus “densepunctatus” found in our samples from S. aloides flowers and infesting Hydrellia puparia belong to the very host specific tribe Dacnusini in the subfamily Alysiinae. This suggests that the parasitoids we found are the main limiting factor for Hydrellia tarsata in our study area. Thus, the dacnusine wasps could function as natural enemies for H. tarsata and thereby also affect the interaction with S. aloides. The two species Chaenusa punctulata and Chorebus densepunctatus were both described in Romania, from material collected in extensive swamps and wetlands near Bucharest (Burghel 1959, 1960). Chorebus densepunctatus was, in fact, reared by Burghel (1960) in large numbers from Hydrellia puparia, with the host identified as Hydrellia griseola. Even earlier, dacnusine wasps determined as Chorebus uliginosus Haliday were reared by Ruschka & Thienemann (1913) in West Germany, also from puparia extracted from S. aloides leaf mines. Similarly, the host was identified as Hydrellia griseola (Ruschka & Thienemann 1913). Despite being scattered and disputable, the literature still reflects that S. aloides is a great study object to increase the knowledge about ecological relations between Hydrellia and its parasitoids.

The reason why the Dacnusini visit S. aloides flowers seems to be primarily to locate their Hydrellia hosts and to perform oviposition. Such a strategy has already been supposed by Schneider & Jeter (1982), who similarly found Chaenusa sp. together with a Hydrellia species on flowers of the aquatic macrophyte Cabomba caroliniana. The authors also showed that the braconid parasitoids actively transfer pollen (Schneider & Jeter 1982). Nectar feeding and pollen transfer by parasitoid hymenopterans also occurs in the High Arctic, but their contribution to plant pollination is likely minimal (Kevan 1973). To ascertain which role the Dacnusini play in our study system further study of their ecological relationships is required, especially of pollination activity and induced mortality.

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