REPRODUCTIVE BIOLOGY AND NECTARY STRUCTURE OF *LYTHRUM* IN CENTRAL SASKATCHEWAN

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By

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ABSTRACT

This project examined multiple aspects of the reproductive biology of the wetland invasive species, purple loosestrife (*Lythrum salicaria* L.), in central Saskatchewan. An examination of insect taxa visiting the three floral morphs of tristylous *L. salicaria*, as well as a ranking of the pollination efficiency of individual insect species, an apparent first for *L salicaria*, was undertaken. Surface features of the floral nectary of *L. salicaria*, as well as floral nectar secretion dynamics, were also investigated. This project also re-visited some of the previous work done on this invasive species, including various floral organ morphometrics in relation to heterostyly, and aspects of the tristylous breeding system including self-fertilization, and fertilization potential of both "illegitimate" pollination and "legitimate" pollination.

The trimorphic nature of the sexual floral organs of *L. salicaria* were well defined in Saskatchewan. Significant differences in length (long-, intermediate- and short-style lengths) exist between all three floral morphs. Lengths of the staminal filaments (long, intermediate, and short) were also significantly different. Also the floral nectary in *L. salicaria* is located in a depression formed at the interface of the hypanthium and the gynoecium. Several stomata are located at regular intervals along the nectary surface, and may constitute the escape route for floral nectar. No morphological differences in nectary structure were apparent among the three floral morphs.

Nectar secretion dynamics of *L. salicaria* were examined between the three floral morphs throughout two summer days in 2006. Peak average nectar volumes and nectar sugar quantities were detected at 3:00 pm, and, interestingly, no significant differences were detected between floral morphs, in accordance with nectary morphology. The estimated secretion rates for *L. salicaria* ranged from 61 – 83 µg of nectar sugar per flower per hour.

Hand-pollination experiments carried out over the summers of 2006 and 2007 at three field sites in and around Saskatoon have verified the strong self-incompatibility in the breeding system of this tristylous species. Intramorph pollination, using illegitimate pollen, did not result in fertilisation, whereas legitimate hand-pollination experiments yielded multiple pollen tubes at the style base, without exception.

Lythrum salicaria in central Saskatchewan was visited by several bee taxa including honeybees (*Apis mellifera* L.), bumblebees (*Bombus* spp.), leafcutter bees (*Megachile* spp.), and sweat bees (*Lasioglossum* spp.). A single visit by *Anthophora furcata* (Panzer) was also recorded in 2007. Generally, bee visits led to high levels of pollination success as determined by fluorescence microscopy of pollen tubes following single insect visits to previously-unvisited flowers. However, most visits by hoverflies (Syrphidae) were non-pollinating. Visits by *Pieris rapae* (L.), yellowjacket wasps (Vespidae) and some non-syrphid flies (Diptera) also yielded no pollen tubes at the style base.

A study of the ultrastructure and development of the floral nectary of the purple loosestrife cultivar 'Morden Gleam' (*Lythrum virgatum* L. x *L. alatum* Pursh.) showed that starch build up in pre-secretory nectary tissues declined throughout secretion, and is virtually absent in post-secretory nectary tissues. The lack of a direct vascular supply to the floral nectary suggests that the starch breakdown products likely make up most of the floral nectar carbohydrates. Surface features of the floral nectary in 'Morden Gleam' closely resembled those of *L. salicaria*, located in the valley formed between the hypanthium and gynoecium. Nectary stomata, occasionally in pairs, likely serve as outlets for nectar in this cultivar.

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1. INTRODUCTION TO *LYTHRUM* SPP.

1.1 Classification and distribution

The Lythraceae is one of 14 families of the order Myrtales of the subclass Rosidae. This dicotyledonous family is widely distributed, though most species are tropical and many occupy aquatic or semi-aquatic habitats. The Lythraceae consists of 30 to 32 genera and approximately 600 species (Morris 2007; Judd et al. 2008). Within the Lythraceae, the genus *Lythrum* L. contains 35 species (Judd et al. 2008).

Of the 35 *Lythrum* species, only 12 are present in North America (USDA Plant Database 2008). However, only half of these species are native to North America: *L. alatum* Pursh; *L. californicum* Torr. & A. Gray; *L. curtissii* Fernald; *L. flagellare* Shuttlw. ex Chapm.; *L. lineare* L.; and *L. ovalifolium* Koenhe. The six non-native *Lythrum* species in North America are *L. hyssopifolium* L.; *L. portula* (L.) D.A. Webb; *L. salicaria* L.; *L. thymifolia* L.; *L. tribracteatum* Salzm. ex Spreng.; and *L. virgatum* L.

In Canada, specifically, the only native *Lythrum* species is *L. alatum*, which occurs in Ontario. There are three introduced species of *Lythrum* in Canada: *L. hyssopifolium* in British Columbia and Ontario, *L. portula* in British Columbia, and *L. salicaria*, which is most widely distributed, occurring in all ten Canadian provinces (USDA Plant Database 2008).

1.2 Biology of *Lythrum salicaria* (purple loosestrife)

1.2.1 History and environmental impact in North America

Lythrum salicaria (purple loosestrife) is a perennial herb of Eurasian origin that has become an invasive species in North America. Although thought to have been introduced to North America in the early 1800's (Mal et al. 1992; Houghton-Thompson et al. 2005), *L.*

salicaria was not recognized as invasive until the 1930's (Levin 2003). This species was known from wet meadows of Canada from about 1814 onward, and had become established as a serious weed in Quebec wetlands by the early 1930's (Mal et al. 1992). By the latter half of the 20th century, it was known from all Canadian provinces except Saskatchewan, where specimens were identified by the end of the 1970's (Cody 1978).

Levin (2003) regarded this spread of *L. salicaria* throughout North America as a "spectacular expansion" and suggested that the mechanism responsible may have been fostered by crossing between genotypes originating in different areas of Europe, giving *L. salicaria* a source of extra genetic variation with which to adapt to new environments. There is some morphological and molecular evidence to suggest that hybridization between *L. salicaria,* and the native *L. alatum,* may be the driving force that has allowed the rapid spread throughout the wetlands of North America. Thirty *L. salicaria* populations across eastern North America had individuals that showed morphological traits of *L. alatum* including fewer than four flowers per axil and alternate leaf arrangements. Four sympatric *L. salicaria* populations also showed mean heights more in line with *L. alatum* (Houghton-Thompson et al. 2005). The molecular evidence is based around analysis screens of variation patterns using 279 AFLP markers. Out of the 279 markers, only two showed clear evidence for introgression from *L. alatum* to *L. salicaria* (Houghton-Thompson et al. 2005). However, if hybridization did occur, most North American stands of *L. salicaria* appear to have retained almost all traits of the Eurasian form of *L. salicaria,* and none of *L. alatum* (Houghton-Thompson et al. 2005).

Whatever the mechanism, *L. salicaria* now represents a threat to wetland environments in North America through its ability to become established and then expand into monospecific stands, displacing much of the native diversity (Mal et al. 1992; Diehl et al. 1997). This

reduction of native plant diversity in turn creates a threat to the native fauna of these ecosystems, which may rely on aspects of habitat like food and shelter which are changed by the presence of *L. salicaria.*

1.2.2 Plant morphology, growth, and vegetative reproduction

Lythrum salicaria is a perennial herb. The flowering shoots are annual, regenerating from a perennating rootstock every spring. There are no reports in the literature on the longevity of a single specimen (Mal et al. 1992). Seed germination occurs in late spring or early summer, a long taproot develops, and eight to ten weeks pass before flowering begins. Flowering starts in early July and continues through September.

The above ground features of *L. salicaria* include stems each terminating in a long inflorescence. Stems can reach 2.7 metres high. Many stems can be present on a single plant, and they completely die back every winter (Shamsi and Whitehead 1974; Mal et al. 1992). Leaves of purple loosestrife are sessile, lanceolate to ovate, and 3-10 cm long. Lower leaves can be opposite, decussate, or in whorls of three; however, upper leaves are generally alternate (Mal et al. 1992).

Various physiological studies on plant growth have been undertaken with purple loosestrife. Shamsi and Whitehead (1974) indicate that at least half of the assimilate of a single specimen is allocated to root development. Purple loosestrife has a 13 hour photoperiod threshold for spike elongation and flowering, whereas a nine hour photoperiod leads to dwarfism. Reduced growth was noted in nutrient deficient soils; deficiencies in N, P, and K led to an increased root: shoot ratio and reduction in flowering and fruit set (Shamsi and Whitehead

1974). By the end of the first year of growth, the upper parts of the main root and lateral roots become woody (Shamsi and Whitehead 1974; Mal et al. 1992).

Mal et al. (1992) reported that there are no known mycorrhizal associations with *L. salicaria* in Ontario. However, Philip et al. (2001) reported that such associations have indeed been found in wild *L. salicaria.* Mycorrhizal colonization reduced both above and below ground biomass of the plant. No significant changes were found in the number or length of inflorescences among plants with or without mycorrhizae. However, pollen production per anther and per flower was increased when *L. salicaria* had mycorrhizae (Philip et al. 2001).

There is controversy over the ability of *Lythrum salicaria* to spread vegetatively. Pearsall (1918) described *L. salicaria* as being "rhizomatous", but Shamsi and Whitehead (1974) indicated that while *L. salicaria* may persist in one spot for several years, it is "incapable of much vegetative spread". Localized clonal growth does occur, as shoots are produced annually from root buds (after overwintering), but clonal growth by means of rhizomes is insignificant (Mal et al. 1992). *L. salicaria* can be propagated by transplanting cuttings into soil. This ability suggests that *L. salicaria* may be more capable of vegetative reproduction than Mal et al. (1992) suggested.

1.2.3 Sexual reproduction involving heterostyly

Heterostyly is a genetic polymorphism where populations are composed of either two (distyly) or three (tristyly) floral morphs which differ in the relative heights of stigmas (length of styles) and anthers in the flower (Barrett 1992). It is represented by about 25 families in the angiosperms, including both monocotyledons and dicotyledons (Figure 1.1). The relationship of style length polymorphism and many other traits (such as pollen, anther, and stigma

polymorphism) to heterostyly was documented by Darwin (1877) and is supported throughout the modern literature (Barrett 1992; Hermann et al. 1999; Mal and Hermann 2000).

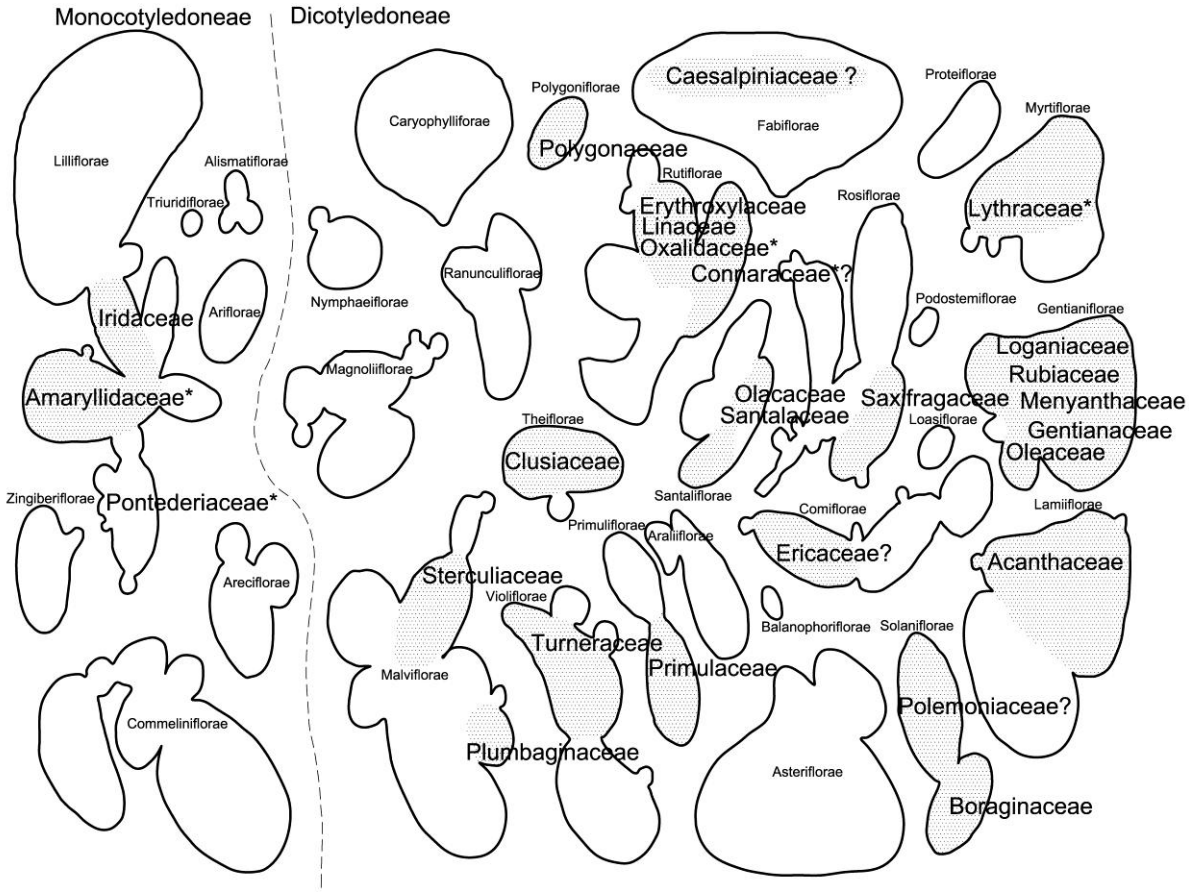


Figure 1.1 Diagram of angiosperm families which display the heterostylous syndrome. The occurrence of distyly is indicated by the shading, whereas families with tristyly are indicated by an asterisk (reproduced from Barrett 1992).

Distyly is considerably more common than tristyly (Barrett 1992). The distylous condition is represented by two floral morphs: "pin" flowers with long style and short stamens, and "thrum" flowers with short style and long stamens. Pollen size polymorphism in most distylous species has been demonstrated, thrum pollen normally being the larger of the two morphs (Darwin 1877; Ganders 1979; Bahadur et al. 1984). In addition to size, other types of pollen dimorphism occur in distylous species. Differences in pollen production between anther levels is also very common, and less frequently, differences in pollen viability, colour, exine

sculpturing, or shape also occur (Ganders 1979; Bahadur et al. 1984; Buchmann 1983). There are some distylous species of *Lythrum,* such as *L. californicum,* which have differences in pollen colour between the floral morphs, as well as differences in style length and in pollen size at both anther levels, typical of distyly. In *L. californicum*, pin pollen is yellow and thrum is green (Ganders 1979).

Tristyly, on the other hand, occurs in only three (Lythraceae, Oxalidaceae, Pontederiaceae) of the approximately 25 families known to exhibit heterostyly (Ganders 1979). *Lythrum salicaria* possesses this relatively rare self-incompatibility system (Darwin 1877; Stout 1925; Eckert and Barrett 1992; Ågren 1996; O'Neil 1997; Ture et al. 2004).

1.2.4 Floral attractiveness and pollination biology

The flower of *Lythrum salicaria* is epigynous, with six alternating whorls of appendages. There are two whorls of sepals (the outer whorl of sepals is termed 'epicalyx', whereas the inner whorl is called 'calyx'), one whorl of petals, two whorls of stamens and one whorl of two fused carpels per flower (Cheung and Sattler 1967; Mal et al. 1992). The flowers of *L. salicaria* are herkogamous (i.e., they possess a spatial separation of stigma and anther) and the three floral morphs present in this species show remarkable consistency in floral morphology (Mal et al. 1992). Floral primordial initiation was determined by Cheung and Sattler (1967) to be as follows: a) inner sepals, b) outer sepals, c) outer stamens, d) gynoecium, e) inner stamens, and f) petals. This sequence of initiation represents a situation where the normal centripetal or acropetal inception of floral primordia is reversed twice; once when the calyx is initiated before the epicalyx, and a second time when the inner stamens are initiated after the inception of the

gynoecium. This sequence of initiation was said to be unique to *L. salicaria* (Cheung and Sattler 1967; Mal et al. 1992).

In tristyly, there are three floral morphs present within a species (Figure 1.2), with only one morph per plant. This type of heterostyly features flowers with short, intermediate, or long styles. Three levels of stamens also occur in these three morphs, with two staminal whorls present in each flower. The short-styled flowers have mid- and long-staminal whorls; the intermediate-styled flower has short- and long-staminal whorls; and the long-styled flowers feature mid- and short-staminal whorls (Darwin 1877). In similar fashion to the characteristics of polymorphism outlined for distylous plants, in tristylous species these include the common pollen polymorphisms (colour, size, shape, and exine sculpturing), as well as anther levels, style levels, and stigma polymorphisms (Darwin 1877; Barrett 1992; Dulberger 1993; Hermann et al. 1999).

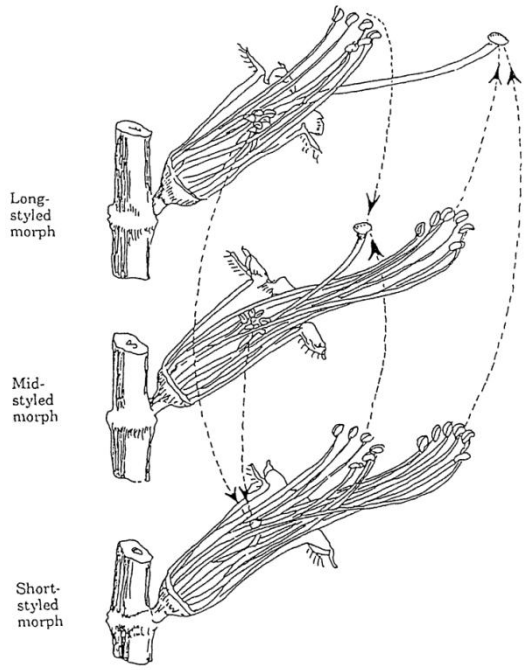


Figure 1.2. The three floral morphs of *Lythrum salicaria* drawn with much of the calyx and corolla removed, illustrating tristyly in this species. Arrows indicate "legitimate" pollen flow (taken from Darwin 1877).

Pollen size (as well as other pollen polymorphisms) is correlated with anther levels as it was described in dimorphic species. The long stamens (from short- and mid-styled flowers) produce the largest pollen grains, but the least amount of pollen; the intermediate stamens (from the long- and short-styled flowers) produce intermediate-sized grains in intermediate quantities; and finally the short stamens (from the mid- and long-styled flowers) produce the smallest grains but in the largest quantities (Darwin 1877; Ganders 1979; Bahadur et al. 1984). This situation appears to be typical in many tristylous species in the Lythraceae, Oxalidaceae, and the Pontederiaceae. However, it is not always clear; *Lythrum junceum* has a weaker pollen polymorphism, because this species showed a considerable overlap in small- and mid-stamen pollen size (Ganders 1979). Yet in Dulberger's (1970) study on tristyly in *L. junceum,* he found clear size trimorphism, as well as starch dimorphism. The starch dimorphism showed positive on a starch test for the larger long-stamen pollen, whereas all other grains were negative.

Pollen of *Lythrum salicaria* was determined also to be strongly trimorphic for pollen size, wherein long-stamens produce the largest pollen grains and mid- and short-stamens produce smaller grains (Darwin 1877; Waites and Ågren 2004). Other polymorphisms associated with the tristylous condition of *L. salicaria* include a pollen colour dimorphism. In *L. salicaria*, green pollen is produced in the long stamens having purple anthers, and yellow pollen is formed in both the mid- and short-stamen levels with greenish anthers (Darwin 1877; East 1927). *L. salicaria* also possesses polymorphic stigmas in association with the three forms of its flowers. The stigma of the long-styled floral morph of *L. salicaria* is larger than that of the mid- and short-styled morphs (Darwin 1877; Mal and Hermann 2000). More precisely, the stigma of the long-style morph was greater in diameter, but also possessed longer and larger papillae, than the mid- or short-styled morphs. Although the diameter of the short-styled stigma to that of the mid-

style was smaller, the difference in size was not statistically significant (Schill et al. 1985; Hermann et al. 1999).

The proportion of long-, mid-, and short-styled plants in natural populations varies as well. Short-styled morphs have the highest probability of being lost in a population, while long-styled morphs have the lowest probability of being lost. Ninety percent of *L. salicaria* populations in the southern Harkskärsfjärden archipelago in central Sweden were trimorphic, whereas only 69% of the populations in the more northern Skeppsvik archipelago possessed all three floral morphs (Ågren and Ericson 1996). In the northern European populations of *L. salicaria,* the mid-styled morph represented only 21% of plants in trimorphic populations (Ågren and Ericson 1996), and a similar though less marked deficiency in the mid-styled morph was found in northern Ontario populations (Eckert and Barrett 1992), indicating variation in morph proportions may be affected by geographic location. Mulcahy and Caporello (1970) noted that in a wild population of *L. salicaria* in Massachusetts, U.S.A., 22% of the plants were of the long-styled morph, 43% were intermediate, and 35% were short-styled plants. However, near Zurich, Switzerland, Schoch-Bodmer (1936) showed that the long-styled morph was most abundant (36.4%), then the mid-styled (33.0%) followed by the short-styled (30.6%) morph.

The movement of pollinators between plants of *L. salicaria* has been investigated extensively. Levin et al. (1971) found that bee and butterfly movements between plants were not random. Bees tended to continue moving in the same direction or with 45° deviations most of the time, while deviations of 135° and 180° were much less frequent. Levin et al. (1971) suggested that this directionality may play a part in reducing the chance of re-visiting previously foraged flowers. Levin and Kerster (1969) showed that the density of *L. salicaria* plants dictates how far pollinators will fly to the next plant, and therefore plays a role in bee-mediated pollen

dispersal. Bees also tend to forage at a similar height when flying between plants of *L. salicaria* (Levin and Kerster 1973). In a more detailed study in Europe, Waites and Ågren (2004) studied differences in pollinator visitation, pollen deposition, and pollen limitation in *L. salicaria*. They found that pollinator visitation rate was correlated with plant population size. The rate of plant visitation increased, and the number of flowers visited per plant decreased, in relation to an overall increase in population size. However, they noted that although this overall trend was observed, in most cases it only approached statistical significance. Thus, Waites and Ågren (2004) postulated that small sparse populations of *L. salicaria* may be less attractive to pollinators, while larger, dense stands will attract more pollinators. However, pollinators will visit fewer flowers per plant in the more dense stands. The results of Waites and Ågren's (2004) study on pollen limitation suggest that no statistically significant differences in pollen limitation occur between floral morphs in populations of *L. salicaria*. However, quantity and composition of pollen did vary depending on population size. The absolute quantities and relative amounts of compatible pollen received increased with population size, whereas the total number of pollen grains received (compatible and incompatible) per flower showed no correlation with population size. However, the number of compatible pollen grains received per flower increased significantly with population size. This outcome occurred in each of two consecutive years. When correlating pollen deposition and seed set in *L. salicaria,* no evidence relating to deposition of incompatible pollen negatively affecting seed set was found (Waites and Ågren 2004), in contrast to the earlier study by Nicholls (1987), where seed set was significantly reduced by the prior deposition of illegitimate pollen. Seed yield per flower was only correlated significantly with the number of compatible pollen grains received per flower.

Heterostyly is usually accompanied by a sporophytically controlled, diallelic incompatibility system that serves to prevent both selfing, and intramorph fertilization (Barrett 1992). Darwin used the terms "illegitimate" to refer to crosses between stigmas and anthers of different levels, and "legitimate" to refer to crosses between anthers and stigmas of the same level (Figure 1.2) (Darwin 1877). Working mainly with *Lythrum salicaria*, Darwin performed illegitimate and legitimate crosses and recorded the seed set. In subsequent generations derived from the seed, he measured ability to reproduce. The results indicated that plants derived through illegitimate crosses were inferior in their fertility, and in some cases were infertile (Darwin 1877). These results present strong evidence that *L. salicaria* is among those species of tristylous plants that does incorporate this incompatibility system.

Lythrum salicaria represents an entomophilous species, its flowers being visited, in both Eurasian and North American habitats, primarily by honey bees and bumble bees (Apidae: *Apis mellifera, Bombus* spp., respectively), and leafcutter bees (Megachilidae: *Megachilie inermis*) (Levin and Kerster 1969; Mal et al. 1992; Diehl et al. 1997; Waites and Ågren 2004). Several other insect groups that visit *L. salicaria* include syrphid flies (Syrphidae: *Helophilus latifrons, H. fasciatus, Sphaerophoria contigua*), and several Lepidoptera species (Nymphalidae: *Nymphalis antiopa, Cercyonis pegala*; Pieridae: *Pieris rapae, Colias philodice*) (Diehl et al. 1997; Henne et al. 2002; Waites and Ågren 2004). However, any insect with a proboscis length greater than 6 mm may potentially effect pollination in this species (Levin 1970). In North America, assortative pollination of *Lythrum* species may occur. Levin (1970) showed that flower visitors in North America prefer to associate with *L. salicaria* over *L. alatum*, indicating that potential pollinators may be associating with this introduced plant species over the native one.

Finally, Mal et al. (1992) described the sexual reproduction of *L. salicaria* as overwhelmingly important in terms of spread of the species. Seed yield in a single healthy purple loosestrife plant can exceed 2.7 million seeds. A single stem of *L. salicaria* is capable of producing approximately 900 capsules (Shamsi and Whitehead 1974). Basal seeds can be dispersed while distal shoots are still flowering (Mal et al. 1992). The seeds of *L. salicaria* are small and light and readily dispersed by wind and water. Seed germination in *L. salicaria* is highly inhibited below 20°C. Seeds of *L. salicaria* can germinate in a variety of soil types and over a wide range of soil pH, from calcareous to very acidic soils (Shamsi and Whitehead 1974; Mal et al. 1992). These attributes, accompanied by high seed viability (i.e., up to 80% viable after 3 years), present favourable characteristics by which dispersal of the species can take place (Mal et al. 1992).

1.3 Research objectives

By gaining new knowledge about the parameters contributing to the successful spread of *Lythrum salicaria*, it may be possible to learn more about possible control methods. Accordingly, the intent of this study was to expand our current knowledge with regard to the pollination biology of *Lythrum salicaria* in central Saskatchewan. In a related study, the single morph of an ornamental cultivar ('Morden Gleam') of purple loosestrife was utilized to advance our understanding of nectary structure in *Lythrum* spp. Accordingly, this study has three main objectives:

i) to determine floral characteristics, i.e., style and stamen lengths; pollen production and viability; ovule production; and nectary location and morphology, as well as

nectar secretion dynamics in the three floral morphs of *L. salicaria*, to ascertain their role in sexual reproduction (Chapter 2).

ii) to examine the pollination biology of *L. salicaria* in natural stands in central Saskatchewan. Self-pollination within this species was re-examined, along with cross-pollination, using hand-crosses. A survey of insect visitors to flowers, as well as a determination of the pollination efficiency of various insect taxa using single visits to previously unvisited (virgin) flowers, were included (Chapter 3).

iii) to determine the development, anatomy, and ultrastructure of the floral nectary in 'Morden Gleam', an ornamentally available cultivar of purple loosestrife (Chapter 4).

2. FLORAL MORPHOLOGY AND NECTAR SECRETION IN *LYTHRUM SALICARIA*

2.1 Introduction

Several studies have investigated floral structure and floral organ morphometrics in *Lythrum salicaria*. The general floral morphology in *L. salicaria* consists of six alternating whorls of floral appendages, three sterile whorls (epicalyx, calyx, corolla), and three whorls of sexual organs (two whorls of stamens and the gynoecium) (Cheung and Sattler 1967; Mal et al. 1992) as discussed in section 1.2.4.

Numerous studies on floral morphometrics between the three morphs of *L. salicaria* exist. In two different regions (Tiverton, Rhode Island and Dresden, Maine) of the northeastern U.S.A., significant differences in style length typically occurred between style morphs, but not between populations (O'Neil and Schmitt 1993). Short stamens were found not to vary significantly in length between populations or floral morph, and the same result was found for long stamens. However, intermediate stamens were found to vary significantly in length, both between floral morphs and populations. Petal lengths were not found to vary significantly either between floral morphs, or populations in those localities (O'Neil and Schmitt 1993). This study also examined perianth, stamen, and pistil mass (dry mass) between populations and floral morph. The perianth was found to vary significantly between populations, but not between morphs; stamen mass was found to vary significantly between both morph and population; and the pistil dry mass varied significantly between morphs, but not between populations (O'Neil and Schmitt 1993). A study from Sweden which investigated morphometric differences in corolla width, petal length, and the length of the calyx in relation to latitude, found that all morphometric parameters examined varied significantly among populations within regions, but

not between regions (Olsson and Ågren 2002). The study found that variation in corolla length and petal width showed no pattern with regard to latitude, but, there was a slight trend to longer sepals at higher latitudes (Olsson and Ågren 2002). A study by Mal (1998) in southern Ontario examined floral organ differences (pistil overall, and ovary and style separately; and long, intermediate, and short stamens) between the three morphs (three genets each) of *L. salicaria* at three stages of development: early, pre-anthesis, and post-anthesis. Mal (1998) found that all organs differed significantly in length at all three stages and between all three floral morphs.

Previous studies of floral nectar production in *L. salicaria* exist from Europe. In studies conducted at the Cambridge Botanical Gardens, Comba et al. (1999) and Corbet et al. (2001) provide some data on nectar secretion rates. Comba et al. (1999) found the peak secretion rate of flowers to be approximately 90 µg of nectar sugar per flower h^{-1}, and Corbet et al. (2001) found a similar peak rate at 80 µg per flower h^{-1}. Comba et al. (1999) indicated that there was a temporal shift in nectar secretion rates, with secretion being much reduced in morning and evening, the peak secretion rates occurring around midday. Preliminary studies showed that rates of nectar secretion between the plants of three floral morphs of *L. salicaria* were not different (Comba et al. 1999). Paper chromatographic analysis indicated that the floral nectar of *L. salicaria* is sucrose dominant, but with both fructose and glucose present. Maltose and an unknown sugar were also detected in the nectar (Percival 1961)

This chapter examines the variation in floral characteristics among the three floral morphs of *L. salicaria*. In particular, style and stamen length, pollen and ovule production, and nectar secretion dynamics were examined at three field sites in central Saskatchewan, which differed in plant density and proportions of the three floral morphs.

2.2 Materials and Methods

2.2.1 Field sites

Flowering spikes of *L. salicaria* were sampled at the same three field sites in the summers of 2006 and 2007. At the field sites, Dutch Growers, Idylwyld, and Lakeshore, flowering began in late July and continued through to early September.

2.2.1.1 Dutch Growers Nursery

The Dutch Growers Nursery (BVR) site (Figures 2.1A, B) was located approximately 6 km south of Beaver Creek Conservation Area and about 20 km south of Saskatoon (N. 51°55'41.1"; W. 106°44'34.1"; elev. 1607 ft.) in a small seasonal creek which was full of water in the spring and usually dried up by late July. This site was the largest used in this project, both by area and by number of plants present. The western edge of the site was dominated by willow (*Salix* spp.) saplings and an alfalfa (*Medicago sativa*) field west of that. East of the site was the Dutch Growers tree nursery. The site proper was almost completely dominated by *L. salicaria,* though some grasses persisted in areas.

2.2.1.2 Idylwyld Bridge

The Idylwyld Bridge (IDY) site (Figures 2.1C, D) was located along the southern shore of the South Saskatchewan River approximately 100 m downstream of Idylwyld bridge, directly adjacent to Rotary Park in Saskatoon (N. 52°07'14.2"; W. 106°40'05.2"; elev. 1576 ft.). IDY was the smallest site used in this study, although its relative abundance of plants and proximity to the university made it a convenient site to collect material such as flowers and seeds to bring back to the lab for analysis. The site was a sandy bank with lots of grasses and small willow

trees. The south side of the site had a steep bank with lots of Canada thistle (*Cirsium arvense*) and invasive grasses like smooth brome (*Bromus inermis*) and crested wheatgrass (*Agropyron cristatum*). Some small elm trees also grew on the southern bank. The northern side of the site abutted the river. *L. salicaria* grew interspersed with small willows, and tall grasses, and dominated the areas that it had invaded. The phytophagous biological control agent, *Galerucella calmariensis* (Coleoptera: Chrysomelidae), was released at this site by the Saskatchewan Purple Loosestrife Eradication Project in the early 2000's, but none of the beetles remained at this site in either of the two years this project was carried out.

2.2.1.3 Lakeshore Garden Centre

Lakeshore (LKS) (Figures 2.1E, F) was located in a farm field directly across 11th Street from Lakeshore Garden Centre (N. 52°06'49.6"; W. 106°45'27.9"; elev. 1660 ft.). The site was a slough with water in the centre all year long. *L. salicaria* grew along the eastern edge of the slough, and was generally submerged until early August when the shoreline retreated to the centre of the slough. This site had a lot of individual plants, but they were spread out along the eastern shoreline, and were relatively sparse. *L. salicaria* grew amongst trembling aspen (*Populus tremuloides*), willow and cat tails (*Typha* spp.). Canada thistle and smooth brome grass dominated the eastern bank of the slough, although white and yellow sweet clover (*Melilotus alba; M. officinalis*) and alfalfa were also very common among smooth brome grass adjacent to the field. The biological control agent, *Galerucella calmariensis*, was released at this site at the same time as it had been released at IDY. However, the beetles have persisted at LKS in abundance, and caused considerable feeding damage to *L. salicaria;* consequently, this site was used less frequently than the other two sites.

Figure 2.1. Field sites utilized during each of the 2006 and 2007 field seasons. (A) Beaver Creek (BVR) field site with many spikes of *Lythrum salicaria* in the foreground. (B) Experimental spikes of *L. salicaria* with white mesh bags shown on some spikes at BVR. (C) Idylwyld Bridge (IDY) site, where the *L. salicaria* is located adjacent to the river seen in the background. (D) Experimental spikes at IDY with mesh bag in place. (E) Lakeshore Garden Centre (LKS) site, where the *L. salicaria* grows along the shoreline of this slough. (F) Close up of an experimental spike at LKS enclosed within mesh bag.


A
B
C
D
E
F

2.2.2 Plant density per site

Plant density at the three field sites was determined at the end of the 2007 field season. At all sites, stems were counted at ground level; therefore, every counted stem does not represent one plant, but one spike. Sampling was carried out at BVR using a 2m x 2m quadrat at six random locations within the site. Because of the relatively compact size of the IDY site, the entire perimeter of the site was measured and all stems counted within the site boundaries. Due to the long and narrow site dimensions of LKS, sub-sampling was carried out using a quadrat measuring 1m x 4m. As in BVR, six random locations within the site were sampled.

2.2.3 Proportions of the three floral morphs per site

Proportions of the three floral morphs were quantified at all three sites during both the 2006 and 2007 field seasons. Censuses were conducted twice at each site, approximately three weeks apart, during August. An exception occurred at LKS in 2006, where only one census was recorded.

The proportions of floral morphs determined at LKS and IDY during both seasons represented every flowering specimen present within these sites on the census dates. A single count was given to each discrete 'clump' of stems; this approach was taken in an attempt to approximate individual specimens rather than every single spike on site. Due to the large number of specimens present at BVR (Figure 2.1A), the site was subsampled as follows. Spikes closest to my left foot on every second step during random walkthroughs of the site, were examined for floral morph. No attempt was made to discern individual specimens at BVR as it was assumed that spikes two steps distant from each other represented different plants, and the high plant density at BVR made it difficult to discern individual 'clumps' of stems.

2.2.4 Scanning electron microscopy

Scanning electron microscopy (SEM) was utilized to examine the floral nectary of *L. salicaria* to compare the surface features (including the total number of stomata) of the nectary between the three floral morphs.

2.2.4.1 Tissue dissection and fixation

Nectaries (n = 12 per floral morph) examined by SEM were taken from flowers sampled at IDY on each day of the nectar collection experiments (section 2.2.9). Once nectar was collected from the flowers, the nectary was dissected from the flower base using a dissecting stereomicroscope on site, in preparation of fixation. Nectary tissues were fixed in 2% glutaraldehyde in 25 mM sodium phosphate (equal quantities of mono- and dibasic forms) buffer for at least one hour. Tissues were rinsed three times in buffer before post-fixing in 1% OsO_4 for 2 hours. Tissues were again rinsed three times in buffer before dehydration in a graded acetone series, culminating at 100% acetone (overnight). Following a series of exchanges with liquid CO_2, tissues were critical-point dried (Polaron Instruments). Dried specimens were then stored in sealed, labelled vials until mounting on SEM stubs.

2.2.4.2 Tissue processing and examination

In preparation for examination, dried samples were removed from vials and placed, nectary surface facing up, on aluminum stubs using double-sided tape. Once mounted, samples were gold coated using an Edwards S150B Sputter Coater. Coated specimens were examined in a Philips SEM 505 at 30 kV and micrographs taken using Polaroid 665 positive/negative film.

Later, SEM negatives were scanned using an Epson 3200 Photo scanner. Image editing (contrast, brightness, and cropping) was carried out using Adobe Photoshop® CS2.

2.2.5 Floral organ morphometrics

Using flowers collected from the LKS field site reproductive organ sizes were compared between the three morphs of *L. salicaria*.

2.2.5.1 Determination of style length

Ten open flowers of each morph were collected from the LKS site and brought back to the lab in sealed Eppendorf® tubes to prevent desiccation. All flowers were collected from spatially distant plants to ensure flowers were not taken from the same individual. Once at the lab, the gynoecium was dissected from each flower. Using a standard ruler, calibrated in millimetres, the style was measured from the point of attachment of the ovary to the tip of the stigma, while viewed with a dissecting stereomicroscope at 6.1x magnification.

2.2.5.2 Determination of filament length

Staminal filaments were harvested from the same specimens collected for the style length data (2.2.5.1). Two stamens from each flower (one each from both staminal whorls) were chosen at random and measured from the point of filament attachment at the hypanthium tube, to the point of filament attachment at the anther base, using a stereomicroscope (6.1x magnification).

2.2.5.3 Statistical methods

The mean of each floral organ measurement within morph-types was determined along with standard deviation, and standard error. Differences in style length between the three floral morphs were analysed using One-Way ANOVA (analysis of variance) to determine if length differences were significant. Direct comparisons of like staminal levels between two floral morphs were analysed using t-tests to determine if there was any significant difference between two like staminal whorls among two different floral morphs. Comparisons of length between the three different staminal levels were analysed using a One-Way ANOVA for significance ($\alpha = 0.05$) with Microsoft Excel 2007.

2.2.6 Pollen characteristics

Several pollen characteristics were examined. Total pollen per anther and per flower was quantified for all three staminal levels in all three floral morphs. Pollen viability was also determined for all three anther levels.

2.2.6.1 Pollen quantity per flower

Floral material for counts of pollen grains per anther at the various staminal levels was collected at IDY and brought back to the lab in closed Eppendorf® tubes (1.5 mL) to prevent desiccation and subsequent anther dehiscence. Flowers were chosen based on being newly open and having indehiscent anthers to ensure that all pollen was still accounted for. In total, all anthers per flower from three flowers per morph (each flower coming from a different plant) were examined. In the lab, all anthers within a flower were harvested, and pollen grains were collected by dissecting each anther in a drop of water on a glass microscope slide. Anthers were

transferred through multiple water droplets on the slide until no more pollen was liberated. A cover slip was applied to the preparation and sealed with clear nail polish to prevent drying out of the slides. The labelled slides were stored in slide boxes until counted.

Pollen counting was done using an Olympus compound light microscope at 100x magnification. Absolute values were determined per anther by counting within the field of view and moving the slide carefully in a linear pattern over the entire cover slip to limit miscounting. Recording of pollen counts was done in such a way as to be able to quantify total pollen per anther, and per flower by summing all pollen counted within a flower.

2.2.6.2 Pollen viability

Pollen viability was determined for all stamens within six flowers from each of the LKS and IDY field sites. Viability was determined between July 5-12, 2006 at LKS and between July 18-20, 2006 at IDY. Pollen viability was determined using the Fluorochromatic Reaction test (FCR) as described by Shivanna and Rangaswamy (1992). A solution of fluorescein diacetate (FDA) in acetone (2 mg/ml) was added to about 2 ml of 0.5 M sucrose solution until the resulting mixture displayed "persistent turbidity". Freshly harvested pollen grains from mature but indehiscent anthers were placed in a drop of FDA solution on a glass microscope slide. The preparation was then placed in an incubation chamber at ~90% RH for 5-10 minutes. When removed from the incubator, a cover slip was placed on the slide and then immediately viewed with a Zeiss fluorescence microscope. Pollen grains were considered viable if they fluoresced green and non-viable if the grains did not glow.

2.2.7 Ovule quantity per flower

Six mature, unopened buds of all three floral morphs of *L. salicaria* were collected at LKS and brought to the lab. The entire gynoecium was removed from these 18 flowers and preserved in 90% ethanol. All ovules per ovary were removed in a Petri dish in 90% ethanol using two fine syringe tips as probes, and then counted under an Olympus dissecting microscope. Descriptive statistics (mean and standard error) were determined for all floral morphs. The three floral morphs were compared to determine if differences in ovule numbers existed using One-Way ANOVA ($\alpha = 0.05\%$).

2.2.8 Pollen-ovule ratio

The pollen-ovule ratio for *L. salicaria* was determined by dividing the average number of pollen grains per floral morph by the average number of ovules per morph. Pollen-ovule ratios were compared with a table of values used to estimate breeding systems based on pollen-ovule ratios (Cruden 1977).

2.2.9 Floral nectar production per flower

Nectar collection experiments were carried out over single days at IDY. The experiment was run twice, once on July 27, 2006, and again on August 17, 2006. On the day prior to these experiments, multiple plants of each morph were cleared of open flowers using forceps and bagged with fine mesh cloth bags to exclude nectar foraging on the following day. Plants of a single morph were numbered, in order to prevent the sampling of flowers from the same specimen during a sampling period. Sampling took place every three hours from 09:00h to 21:00h. Any open flowers not sampled for nectar were re-bagged after each sampling period to

prevent access by insects. After each 3 h interval, total nectar from 5-6 flowers (depending on the supply of newly-open flowers) of all three morphs was collected. Drummond micropipettes (Microcaps®) of 0.2 or 1.0 µL capacity were used to collect the nectar from the surface of the floral nectary. The total volume of nectar collected per flower was determined by measuring the proportion of the micropipette column filled with fluid, using a ruler under a dissecting microscope in the field. Nectar sugar concentration (by weight, or % NCW) of the nectar was determined using a Bellingham and Stanley Pocket Refractometer. Refractometer readings then were corrected according to temperature by using the correction table in the refractometer manual. The corrected % NCW readings were converted to µg sugar per µL nectar (NCV) using the equation: $NCV = NCW^2 (59.6 \times 10^{-6}) + NCW (9.224 \times 10^{-3}) + 7.08 \times 10^{-3}$, which gives an error of less than 1% for nectar sugar concentration readings between 10% and 80% (Burquez and Corbet, 1991). A secretion rate of quantity of nectar sugar produced per flower per hour was determined for all floral morphs by taking the peak nectar sugar quantity value at the 3:00 pm sampling interval and subtracting the nectar sugar quantity value from the first sampling interval (9:00 am) and dividing by the time elapsed (6 hours). Air temperature was recorded at the field site every half hour throughout the day while the experiments were in progress, using a mercury thermometer. Similarly, light readings on July 27th were taken every half hour with a General Electric Triple Range 214 light meter, calibrated in foot-candles. Foot candle readings were converted to lux using the conversion: 1 foot-candle = 10.7639104 lux. Light readings on August 17th were measured every half hour throughout the day with a Li-Cor Incorporated LI-185 light meter calibrated in microeinsteins per m^2 per sec.

2.3 Results

2.3.1 Plant density per site

Plant density for IDY was determined as the total number of stems of *L. salicaria* divided by the site's total area. The density of *L. salicaria* at IDY was 5.54 stems per m^2. However, the number of stems per m^2 assessed by the subsampling of quadrats at LKS was 18.8 ± 2.7 (mean ± s.e.), and at BVR, 83.9 ± 11.0 stems per m^2 (Figure 2.2). Due to the plant density at IDY being a total number rather than a sample, it could not be statistically compared to the other two sites. However, a t-test between BVR and LKS sites showed plant density of *L. salicaria* was significantly higher at BVR (P = 0.002).

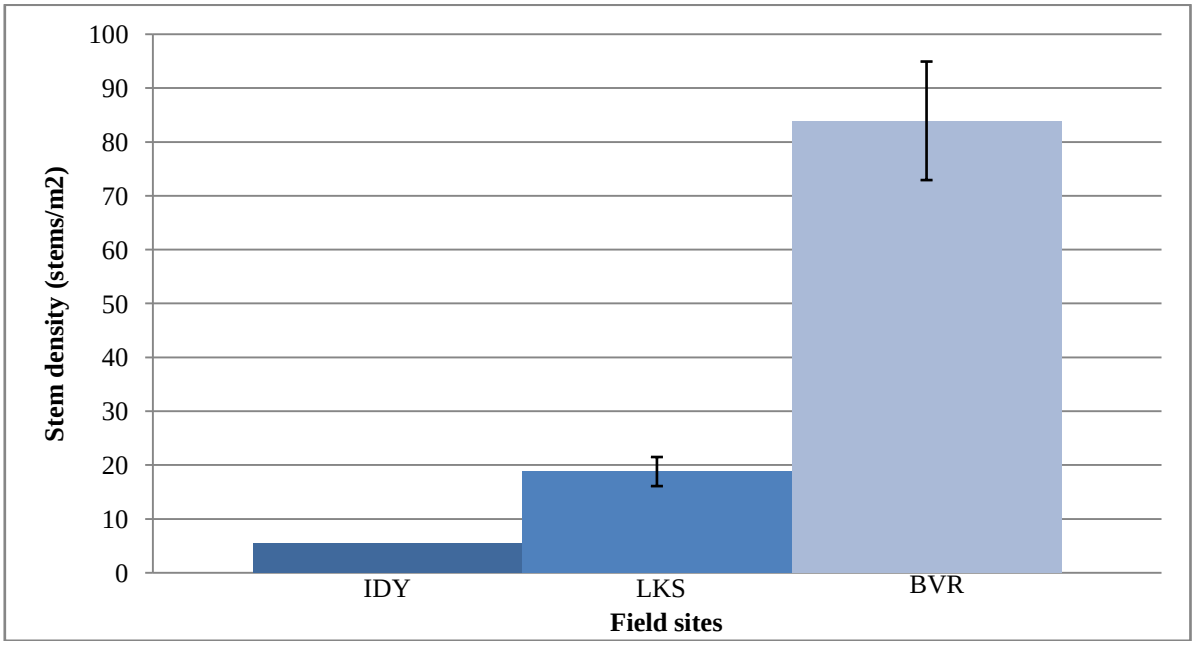


Figure 2.2. Stem density of *L. salicaria* at the three field sites in September 2007. The values for LKS and BVR are reported as mean ± s.e. for six quadrats sampled per site.

2.3.2 Proportions of the three floral morphs per site

Availability of the three field sites in both 2006 and 2007 permitted a comparison of the proportions of the three floral morphs present at each site between the two years. At BVR in 2006, plants of the long (LM) and intermediate (IM) morphs were abundant, with relatively few of the short morph (SM) plants being present (Table 2.1). At LKS in 2006, a somewhat even proportion of all three floral morphs occurred. The IDY site in 2006 had an abundance of SM plants, followed by IM and LM (Table 2.1).

The 2007 census of BVR showed that the majority of flowering spikes at the site were LM and IM, with SM representing 3% or less of all plants in both years (Tables 2.1, 2.2). At IDY in 2007, SM was again abundant during the early August census, but was reduced in late August (Table 2.2). The LKS site had similar proportions of the three floral morphs present in 2007 (Table 2.2), as in 2006 (Table 2.1). Whereas the total number of flowering plants at IDY remained relatively consistent between the two seasons, there was a marked increase in the number of flowering plants at LKS from 2006 to 2007 (Tables 2.1, 2.2), consistent with an expansion of *L. salicaria* at this site.

Table 2.1. Proportion (%) of the three floral morphs (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph) of *Lythrum salicaria* present at each of the three field sites in 2006.
* Number of plants represents a sub-sample of plants present on site.

Site	Date	LM (%)	IM (%)	SM (%)	No. plants
BVR	August 16	48.8	50.7	0.5	201*
	August 29	51.0	45.9	3.1	196*
IDY	August 17	18.7	24.0	57.3	150
	August 29	18.4	32.0	49.5	103
LKS	August 29	32.5	38.6	28.9	197

Table 2.2. Proportion (%) of the three floral morphs (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph) of *Lythrum salicaria* present at each of the three field sites in 2007.
* Number of plants represents a sub-sample of plants present on site.

Site	Date	LM (%)	IM (%)	SM (%)	No. plants
BVR	August 13	46.8	50.2	2.9	203*
	August 29	45.9	52.3	1.8	220*
IDY	August 10	17.8	29.6	52.6	152
	August 30	25.9	34.2	39.8	108
LKS	August 10	38.0	33.5	28.5	313
	August 30	42.5	32.0	25.5	337

2.3.3 Floral morphometrics

2.3.3.1 Style length

Style lengths among the three floral morphs were significantly different (Table 2.3; One-Way ANOVA, $P < 0.001$), with the styles of long-morph (LM) flowers being 1.7 and 5.6 times longer than styles of the intermediate (IM) and short (SM) morphs, respectively.

2.3.3.2 Filament length

No significant differences (t-tests in all three comparisons had $P \geq 0.26$) were found in filament length between staminal whorls of one flower morph, to those of the same staminal level in another floral morph (i.e., LM/IS compared with SM/IS; LM/SS compared with IM/SS; and IM/LS compared with SM/LS) (Table 2.3). One-Way ANOVA analysis of the three staminal levels to each other showed that their differences in length were highly significant ($P < 0.001$). Mal (1998), working with wild *L. salicaria* in Essex County, near Windsor, Ontario, detected similar (often somewhat shorter) dimensions of styles and filaments to those recorded from LKS (Table 2.3). The representation of the three floral morphs indicated by Darwin (1877) (Figure 1.2) accurately depicts what was found for the three floral morphs of *L. salicaria* in Saskatchewan.

Table 2.3. Lengths (mean ± s.e.) of styles and staminal filaments (LS = long stamen; IS = intermediate stamen; SS = short stamen) of the three floral morphs of *Lythrum salicaria* from the LKS field site. Columns in bold type represent corresponding data from Mal (1998). NA= not applicable

	Long Morph		Intermediate Morph		Short Morph	
	Length (mm)	**Data from Mal (1998)**	Length (mm)	**Data from Mal (1998)**	Length (mm)	**Data from Mal (1998)**
Style	9.5 ± 0.2	**9.31 ± 0.01**	5.6 ± 0.1	**5.15 ± 0.02**	1.7 ± 0.1	**1.32 ± 0.01**
LS Filament	NA	**NA**	10.8 ± 0.5	**9.05 ± 0.10**	10.6 ± 0.6	**9.88 ± 0.13**
IS Filament	6.8 ± 0.1	**6.22 ± 0.12**	NA	**NA**	6.4 ± 0.4	**6.61 ± 0.06**
SS Filament	3.4 ± 0.1	**3.20 ± 0.06**	3.1 ± 0.2	**2.89 ± 0.08**	NA	**NA**

2.3.4 Pollen characteristics

2.3.4.1 Pollen quantity per flower

Analysis of the number of pollen grains per anther showed that even within an anther level from the same floral morph, there can be statistically significant differences in average pollen quantity per anther (Table 2.4). Pollen quantities per anther level between floral morphs (i.e., SM/LS compared with IM/LS) were significantly different in all cases (t- tests, $P \leq 0.02$) (Table 2.5). Analysis between staminal levels within a floral morph (i.e., LS and IS in SM flowers) showed that the number of pollen grains between staminal levels was always significantly different (t-tests, $P \leq 0.01$) (Table 2.5). In all instances, LS possessed fewer pollen grains than IS, which typically possessed fewer pollen grains than SS; however, quantities of pollen per IS and SS anthers did overlap (Table 2.4).

2.3.4.2 Pollen viability

As determined by the FCR test (section 2.2.6.2), pollen viability in all anther levels in *L. salicaria* averaged greater than 90% (Table 2.6). T-tests between anther levels in the same floral morph (such as comparing LS with IS in SM flowers) indicated that differences in viability between anther levels within a flower were not significant (Table 2.7). However, the difference

in pollen viability between IS and LS in SM flowers at LKS did approach significance (P = 0.067). The comparisons between like anther levels in different floral morphs (Table 2.8) also showed no significant difference in pollen viability. Thus, pollen viability was likely not affected by which staminal level it was produced in, either within a floral morph, or between floral morphs.

Table 2.4. Number of pollen grains per anther according to the three stamen lengths (LS = long stamen; IS = intermediate stamen; SS = short stamen) in the various floral morphs (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph) of *Lythrum salicaria*. Pollen grains were counted from all anthers per flower, for three replicates per floral morph.

Flower morph	Staminal level	No. anthers	Total pollen grains	Pollen grains per anther (mean ± s.e.)	One-Way ANOVA for total pollen grains per anther (P-value)	Pollen grains per anther overall (grand mean ± s.e.)
SM1	LS	5	6737	1347.3^{A} ± 53.8		
SM2	LS	6	9391	1565.2^{A} ± 79.1	0.11	1487.1 ± 44.2
SM3	LS	6	9152	1525.3^{A} ± 68.8		
IM1	LS	5	10922	2184.4^{A} ± 101.4		
IM2	LS	6	12750	2125.0^{A} ± 56.3	0.045	2057.1 ± 54.6
IM3	LS	6	11299	1883.2^{B} ± 85.9		
SM1	IS	5	11855	2371.0^{A} ± 65.6		
SM2	IS	6	13952	2325.3^{A} ± 265.2	0.71	2406.8 ± 96.1
SM3	IS	6	15108	2518.0^{A} ± 81.2		
LM1	IS	6	22741	3790.2^{A} ± 317.2		
LM2	IS	5	15835	3167.0^{A} ± 146.2	0.13	3592.2 ± 135.9
LM3	IS	6	22492	3748.7^{A} ± 98.2		
IM1	SS	5	20686	4137.2^{A} ± 116.8		
IM2	SS	6	19199	3199.8^{B} ± 191.4	0.006	3554.4 ± 134.3
IM3	SS	6	20539	3423.2^{B} ± 180.5		
LM1	SS	6	22556	3759.3^{A} ± 78.3		
LM2	SS	5	14248	2849.6^{B} ± 130.7	<<0.001	3154.9 ± 123.1
LM3	SS	6	16829	2804.8^{B} ± 82.0		

Table 2.5. Statistical comparison of Table 2.4's data on quantity of pollen between different staminal levels (LS = long stamen; IS = intermediate stamen; SS = short stamen) within a floral morph (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph), and between like anther levels among different floral morphs (t-tests, $\alpha = 0.05$).

Floral morph	Staminal level	Probability (P)
LM	IS SS	0.01
IM	LS SS	<<0.001
SM	LS IS	<<0.001
LM IM	SS	0.02
LM SM	IS	<<0.001
IM SM	LS	<<0.001

Table 2.6. Average pollen viability as determined by the FCR technique for the various staminal levels (LS = long stamen; IS = intermediate stamen; SS = short stamen) for the three floral morphs (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph) of *L. salicaria* from LKS and IDY field sites.

Field site	Floral morph	Staminal level	No. stamens	Average no. pollen grains analyzed per stamen (range)	Average viability of pollen (%) (range)
LKS	SM	IS	6	304.2 (300-311)	94.2 ± 1.2 (89.51-98.02)
		LS	5	305.4 (300-313)	97.2 ± 0.5 (96.10-99.01)
	IM	SS	5	312.4 (307-323)	94.7 ± 1.6 (91.02-98.71)
		LS	6	334.2 (300-440)	98.4 ± 0.5 (96.67-99.71)
	LM	SS	5	323.2 (307-354)	97.2 ± 1.0 (93.97-99.69)
		IS	6	312.8 (304-326)	93.7 ± 1.7 (85.89-97.37)
IDY	SM	IS	6	307.5 (303-313)	97.8 ± 0.5 (95.71-99.35)
		LS	5	316.6 (301-356)	97.5 ± 0.8 (94.72-99.33)
	IM	SS	5	307.2 (306-308)	95.2 ± 3.1 (83.01-98.38)
		LS	5	316.6 (301-356)	98.3 ± 0.5 (97.07-99.68)
	LM	SS	6	326.2 (307-404)	97.6 ± 0.6 (95.56-99.50)
		IS	6	307.3 (302-321)	96.5 ± 0.9 (93.20-99.01)

Table 2.7. Statistical analysis of Table 2.6's data on pollen viability between different staminal levels within the same floral morph (LM = long-style morph; IM= intermediate-style morph; SM = short-style morph) at the LKS and IDY field sites.

Site	Floral morph	Staminal levels within flower morph
LKS	SM	t = -2.23, P = 0.067
	IM	t = -2.28, P = 0.084
	LM	t = 1.76, P = 0.117
IDY	SM	t = 0.31, P = 0.764
	IM	t = -1.00, P = 0.375
	LM	t = 1.10, P = 0.305

P > 0.05 = Not significant

Table 2.8. Statistical analysis of Table 2.6's data on pollen viability between stamens of the same staminal level (LS = long stamen; IS = intermediate stamen; SS = short stamen) among different floral morphs at the LKS and IDY field sites.

Site	Like staminal level between two floral morphs	
LKS	SS	t = -1.34, P = 0.229
	IS	t = 0.21, P = 0.842
	LS	t = -1.65, P = 0.137
IDY	SS	t = -0.77, P = 0.487
	IS	t = 1.32, P = 0.224
	LS	t = -0.83, P = 0.436

P > 0.05 = Not significant

2.3.5 Number of ovules per flower

The average numbers of ovules per floral morph in *L. salicaria* are shown in Table 2.9. Each floral morph averaged over 100 ovules per flower, and differences were not statistically significant (One-Way ANOVA, P > 0.30).

Table 2.9. Number of ovules (mean ± s.e.) per floral morph (LM = long-style morph; IM= intermediate-style morph; SM = short-style morph) in *Lythrum salicaria* from IDY.

Floral morph	No. flowers	Ovules per flower
SM	6	115.2 ± 5.6
IM	6	101.5 ± 2.1
LM	6	105.0 ± 8.9

2.3.6 Pollen-ovule ratio

Pollen-ovule ratios for the three floral morphs are indicated in Table 2.10. The pollen-ovule ratio for the long-style morph (LM) is 1.2 and 1.9 times greater than that for the intermediate-style (IM) and short-style (SM) morphs, respectively. For all morphs, the pollen-ovule ratios lie between the averages for facultative autogamy (168.5 ± 22.1) and facultative xenogamy (796.6 ± 87.7) as determined for many plant species by Cruden (1977), who suggested that pollen-ovule ratios are a conservative indicator of a plant species' breeding system. However, hand pollination work carried out in the field during both field seasons indicates that *L. salicaria,* for all practical purposes, approaches true xenogamy (obligate outcrossing) (see section 3.2.2).

Table 2.10. Pollen-ovule ratios for the three floral morphs (LM = long-style morph; IM= intermediate-style morph; SM = short-style morph) of *Lythrum salicaria* in central Saskatchewan.

Floral morph	No. flowers	Total pollen per flower (mean ± s.e.)	No. flowers	No. ovules per flower (mean ± s.e.)	Pollen/ovule ratio
SM	3	22065 ± 1756.6	6	115.2 ± 5.6	191.5
IM	3	31798 ± 100.4	6	101.5 ± 2.1	313.3
LM	3	38234 ± 4425.4	6	105.0 ± 8.9	364.1

2.3.7 Floral nectar production

2.3.7.1 Morphology of the floral nectary

The floral nectary of *L. salicaria* is located in a ring which forms a slight depression or valley around the gynoecium (Figure 2.3A). Nectar is exuded from the floral nectary through evenly spaced modified stomata located along the surface (Figures 2.3A, B). Stomata are generally found singularly along the surface, but can be found in adjacent pairs on occasion (Figure 2.3C). The number of nectary stomata present per nectary varied among the three floral

morphs, ranging from 17.6 ± 0.89 to 19.2 ± 0.55 (Figure 2.4). However these differences were not statistically significant (One-Way ANOVA, P > 0.23).

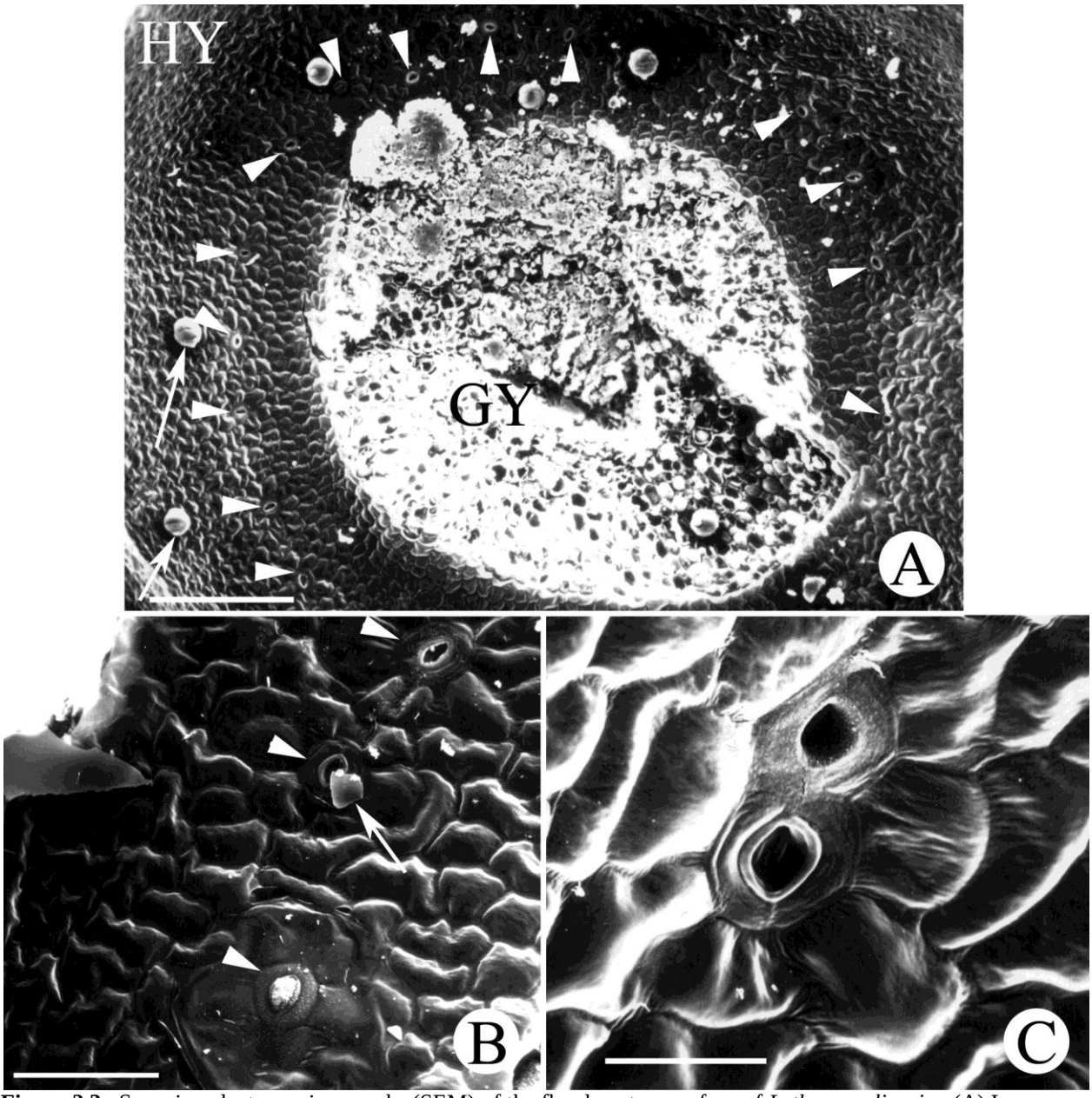


Figure 2.3. Scanning electron micrographs (SEM) of the floral nectary surface of *Lythrum salicaria*. (A) Low magnification of the floral nectary of intermediate-style morph. GY = gynoecium; HY = hypanthium. Arrowheads indicate evenly spaced nectary stomata; pollen grains (arrows). Scale bar = 200 µm. (B) Higher magnification of nectary stomata from a long-style morph (arrowheads); the lower of the three stomata is occluded by an unknown material. Debris (arrow). Scale bar = 50 µm. (C) High magnification of two nectary stomata in an alignment, from a long-style morph. Scale bar = 25 µm.

In SM and LM flowers, paired nectary stomata accounted for 0.87% and 1.4% of all stomata, respectively. There were no paired stomata detected in 12 IM flowers examined; however, twinned stomata were observed on IM nectaries not utilised for the total counts of stomata.

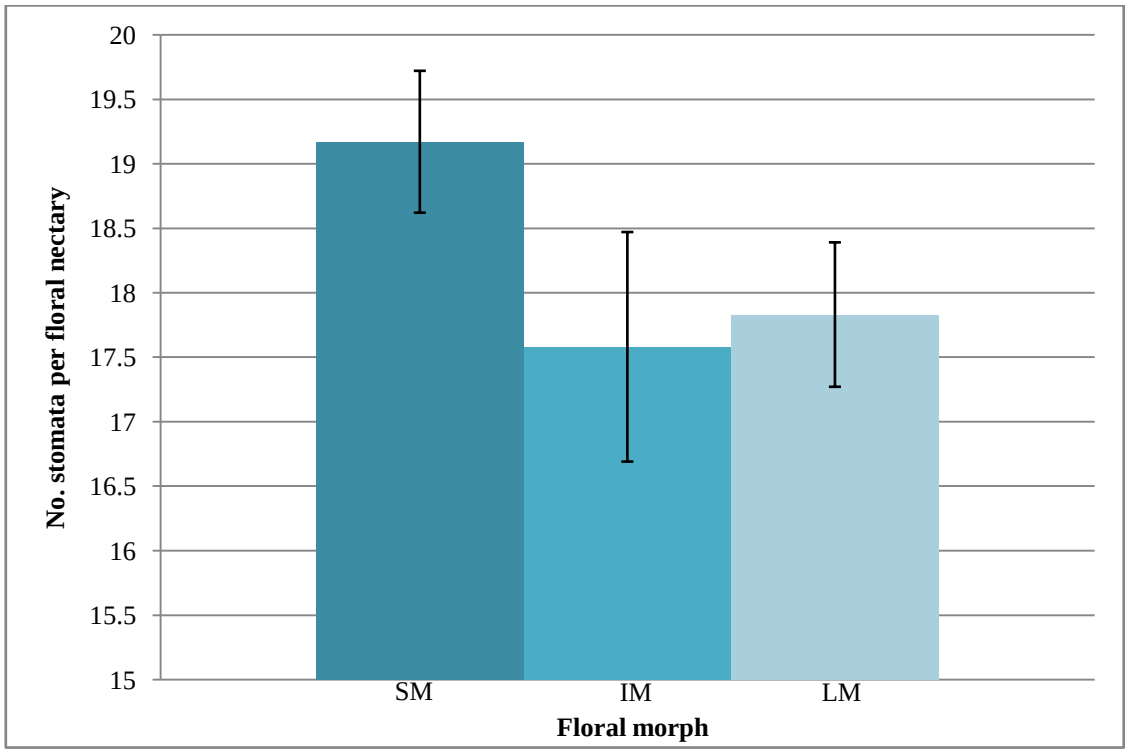


Figure 2.4. Number of stomata (mean ± s.e.) per floral nectary among the three floral morphs (LM = long-style morph; IM= intermediate-style morph; SM = short-style morph). Twelve floral nectaries from IDY were examined per morph.

2.3.7.2 Nectar secretion dynamics

During the inaugural nectar-collection trial on July 27, 2006, no nectar was available from any floral morph at 6:00am. Accordingly, nectar collection began at 9:00am on both sampling dates, and ended at 9:00pm as darkness set in. Both sampling days were sunny and warm, although a few cloudy periods occurred on August 17 (Figure 2.6) compared to July 27 (Figure 2.5).

Nectar sugar concentration typically increased throughout the day, from 9:00am to 9:00pm (Table 2.11). This increase was gradual and steady on July 27, but was more dramatic in the early sampling periods of August 17. Nectar sugar concentrations on July 27 exceeded those of August 17 at almost every sampling interval (Table 2.11).

Table 2.11. Average (mean ± s.e.) nectar sugar concentration (%) per sampling interval for 5-6 flowers sampled for each of the three floral morphs (LM = long-style morph; IM= intermediate-style morph; SM = short-style morph) at IDY. NA = nectar unavailable for collection.

Floral morph	Sampling interval	No. flowers yielding refractometer reading	July 27, 2006 Nectar sugar concentration	No. flowers yielding refractometer reading	August 17, 2006 Nectar sugar concentration
LM	9:00 AM	3	47.4 ± 4.86	3	27.9 ± 6.43
	12:00 PM	5	57.2 ± 2.97	6	51.2 ± 3.14
	3:00 PM	6	61.1 ± 1.74	6	57.7 ± 1.82
	6:00 PM	6	68.7 ± 0.79	6	61.5 ± 1.56
	9:00 PM	5	68.6 ± 1.77	6	63.3 ± 1.40
IM	9:00 AM	1	46.1	2	22.9 ± 3.00
	12:00 PM	5	56.8 ± 2.36	6	51.6 ± 3.39
	3:00 PM	6	62.3 ± 0.79	6	53.9 ± 2.19
	6:00 PM	6	69.2 ± 0.84	6	60.9 ± 2.18
	9:00 PM	5	70.2 ± 1.12	6	57.9 ± 2.97
SM	9:00 AM	4	53.3 ± 1.38	0	NA
	12:00 PM	5	52.4 ± 3.15	6	52.2 ± 2.53
	3:00 PM	6	56.4 ± 3.19	6	56.7 ± 2.01
	6:00 PM	6	65.3 ± 1.14	6	57.9 ± 3.69
	9:00 PM	5	67.0 ± 1.02	6	62.6 ± 3.88

Table 2.12. Estimated rates of nectar sugar (µg) secreted by each of the three floral morphs (LM = long-style morph; IM= intermediate-style morph; SM = short-style morph) on the two sampling dates at IDY in 2006.

Date	Floral morph	Average nectar sugar quantity at 9:00am	Average nectar sugar quantity at 3:00pm	Difference in nectar sugar secreted over 6 h	Rate of nectar sugar secreted per hour
July 27	LM	191.8	594.2	402.4	67
	IM	83.8	446.8	363.0	61
	SM	96.6	556.9	460.3	78
August 17	LM	35.7	505.0	469.3	78
	SM	16.7	494.6	477.9	80
	IM	0	500.2	500.2	83

Mean nectar sugar quantities and mean nectar volume per flower for July 27, 2006 and August 17, 2006 were determined for the three floral morphs (Figures 2.5, 2.6). The daily pattern of secretion was similar between all three morphs, with peak average nectar volumes and sugar quantities per flower at 3:00 pm regardless of morph and day. The only exception occurred for IM flowers on August 17, where highest average nectar sugar quantity per flower occurred at 6:00 pm (Figure 2.6). T-tests of peak nectar sugar quantities among like floral morphs between days indicated that no significant difference in nectar sugar quantity existed ($P \geq 0.380$). The estimated rates of nectar sugar secretion for July 27 ranged from 61-78 µg per flower per h, and from 78-83 µg per flower per h on August 17 (Table 2.12). The relative consistency in nectar sugar concentration as the sampling progressed throughout the day (Table 2.11) helps to explain the close similarity between the plots of nectar volume and nectar sugar quantity (Figures 2.5, 2.6).

2.4 Discussion and Conclusions

Plant density at the three field sites used in this study varied significantly, ranging from 5.5 (IDY) to 84 (BVR) stems per m^2. Various factors may have influenced this density disparity. The BVR site, which had the highest density, was located literally in the bottom of a seasonal creek. This location may provide optimal conditions for the growth and spread of *L. salicaria* at that site. The amount of time that BVR has been infested with *L. salicaria* may also have played a role, but the period that plants have been present at any of the three sites used is unknown. The LKS site also possessed a significant source of water, and its density was intermediate. IDY had the lowest density among the three sites and was situated in such a way that the plants

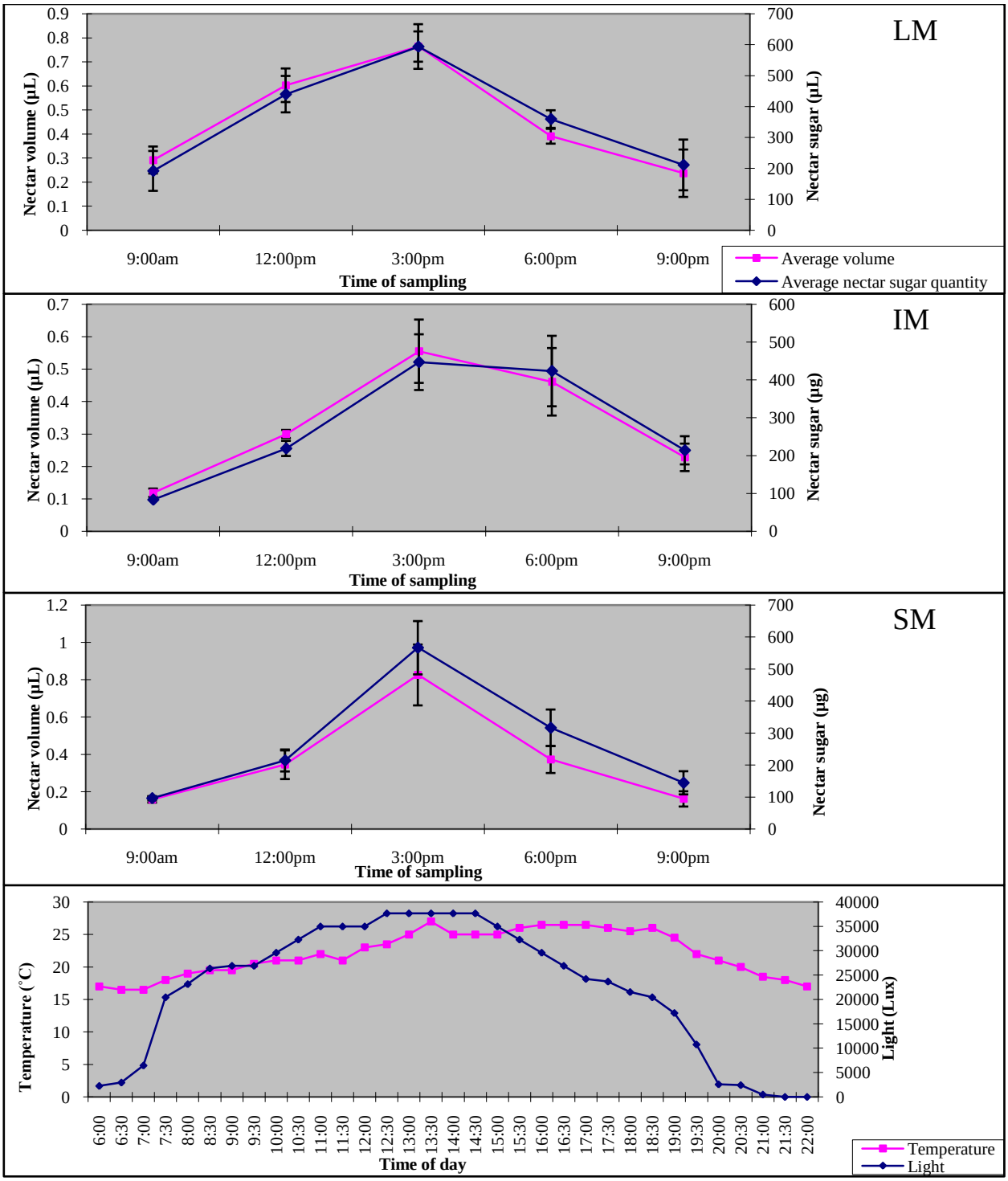


Figure 2.5. Average volume and nectar sugar quantity per flower for long-style (LM), intermediate-style (IM), and short-style (SM) morphs of *Lythrum salicaria*, and temperature and light intensity throughout the day at IDY (July 27, 2006).

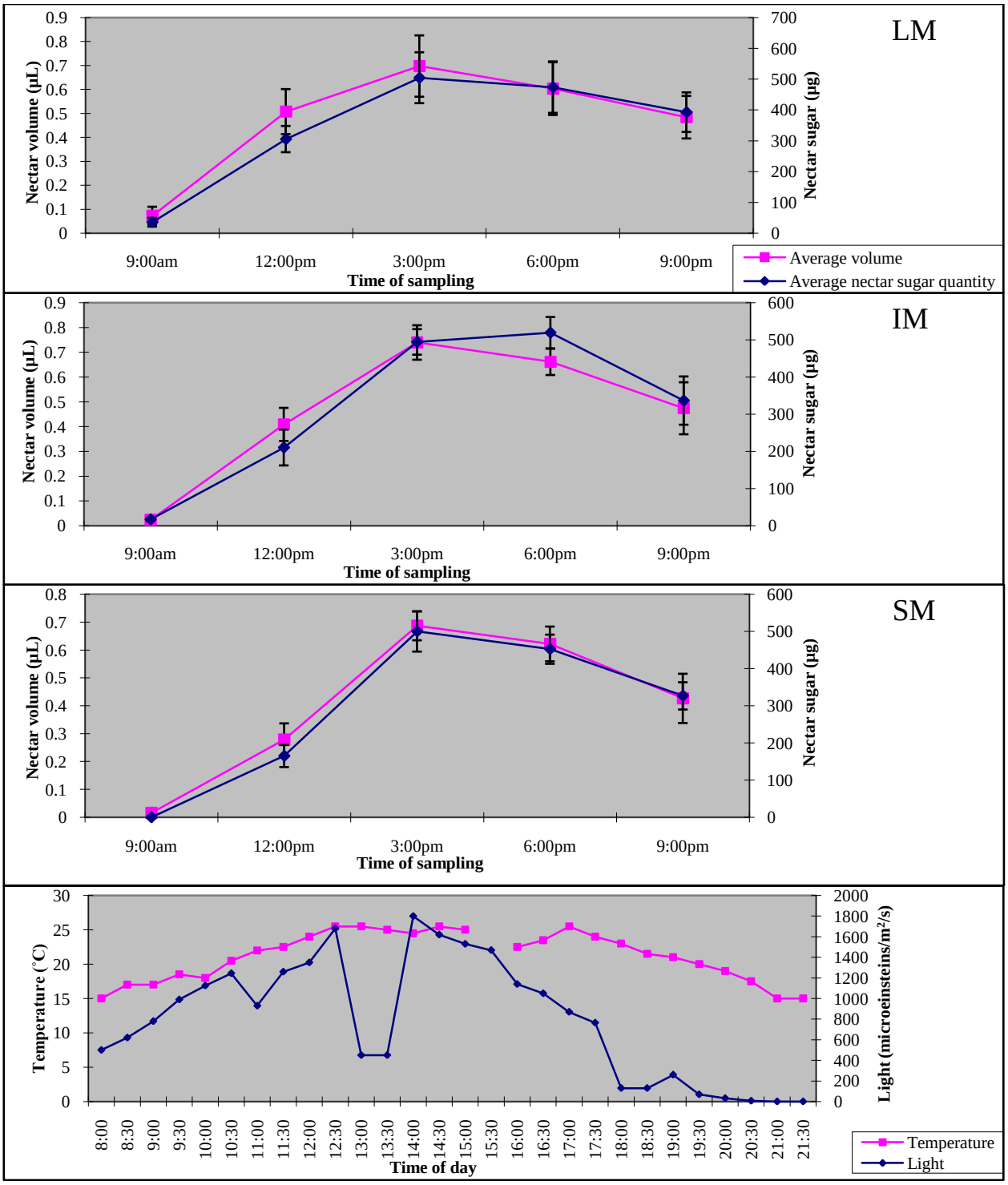


Figure 2.6. Average volume and nectar sugar quantity per flower for long-style (LM), intermediate-style (IM), and short-style (SM) morphs of *Lythrum salicaria*, and temperature and light intensity throughout the day at IDY (August 17, 2006).

themselves were never submerged. These values do correspond (though somewhat higher for BVR) to densities of *L. salicaria* at two sites (1-17, and 4-54 stems per m^2) in Connecticut, U.S.A., which also showed variation, both within site, and between the two sites (Farnsworth and Ellis, 2001). Mal et al. (1997) reported that in 74 populations of *L. salicaria* in Ontario and Quebec, the mean population density was 17 plants per m^2, with a maximum recorded density of 86 plants per m^2. Certain dense patches of *L. salicaria* on Turkey Island in the Detroit River could reach densities of around 110 plants per m^2 (Mal et al. 1997). It is difficult to say how "plants per m^2" corresponds to "stems per m^2", because it is very difficult to discern individual plants in the field and Mal et al. (1996) likely counted stems as well. If so, these data from various locations in North America are similar to those densities reported here for central Saskatchewan. The data from Turkey Island indicate that in certain discrete patches, the density of *L. salicaria* can exceed that determined for any of the three field sites used.

The proportions of short-, intermediate-, and long-style morphs of *L. salicaria* vary in wild populations (Eckert and Barrett 1992; Ågren and Ericson 1996) (see section 1.2.4). With the exception of the BVR site, the IDY and LKS field sites had these three floral morphs in abundance; however, they were not always of equal proportions. The BVR site had all three morphs as well, but SM plants were very scarce. Eckert and Barrett (1992) examined 102 populations of *L. salicaria* throughout Ontario and found that small populations (3-50 ramets) were less likely to be trimorphic than larger populations. Populations consisting of 500 or more ramets were found to be uniformly trimorphic, meaning at least one specimen of each morph was present at such sites. In the case of the BVR site, it had the largest population, but the most conspicuous deficiency of any floral morph (SM is limiting) between any of our field sites. Although this apparent lack of SM in BVR seems to go against the findings of Eckert and Barrett

(1992), it must be noted that although there was very little SM present at BVR, it was represented at the site, and this site was, therefore, trimorphic. Eckert and Barrett (1992) also investigated the frequency with which one morph was lost over another and found that out of the 20 dimorphic populations of *L. salicaria* in Ontario, 75% of them had lost the SM plants, 20% lost the IM plants, and only 5% lacked LM plants. In two Swedish archipelagos, Ågren and Ericson (1996) found that in the Northern Skeppsvik archipelago, 11 dimorphic populations were found within a total of 35 populations examined, and of those 11, nine lacked IM plants, one lacked SM, and the other one lacked LM plants. In the Harkskärsfjärden archipelago in Central Sweden, 28 out of 31 populations were trimorphic. Of the three dimorphic populations, two lacked SM, and one lacked IM specimens (Ågren and Ericson 1996). In the case of central Saskatchewan populations, all three (BVR, IDY, LKS) were trimorphic, however the BVR site had very few SM plants. This result fits well with the data from Ontario and central Sweden, which would suggest that the SM is the most likely to be lost within a discrete population of *L. salicaria*. What caused the low proportion of SM in BVR is difficult to ascertain. It is possible that many factors of environment and genetic diversity within the population may play a role. Because BVR was located in a seasonal creek bed, it could be speculated that in prolonged drought periods where water is scarce, conditions may be advantageous for LM and IM plants to flourish, or conversely could be disadvantageous to SM plants.

The reproductive floral organ morphometrics performed in Saskatchewan compared well with the results Mal (1998) found for populations of *L. salicaria* in Ontario. Mal (1998) determined that the number of cells does not increase in the short style during development, but does increase in the intermediate and long styles. This developmental difference in styles of different lengths may, in part, be responsible for the significantly different lengths of styles

between morphs. The number of epidermal cells in the outer staminal filaments of a floral morph was also found to increase, whereas the number of epidermal cells of the inner staminal filaments was found either not to change or to change only slightly, which conforms well with the observation of longer outer stamens within a floral morph (Mal 1998).

Average numbers of pollen grains produced per anther at the different staminal levels varied in *L. salicaria*, as well as for all like staminal levels in different floral morphs. Interestingly, differences in pollen quantities between staminal levels in trimorphic *Lythrum junceum*, had been noted previously by Ganders (1979), citing Ornduff (1975). It was found that LS produced 6666 pollen grains per flower in IM flowers and 6088 in SM flowers; IS produced 13599 grains per flower in LM flowers and 12999 in SM flowers; SS produced only 11643 pollen grains per flower in LM flowers, but 17687 pollen grains in IM flowers. There are a few similarities between *L. junceum* with the present data for *L. salicaria*, in that SS of IM flowers consistently produced more pollen than SS of LM flowers. Furthermore, SM flowers always had smaller quantities of pollen per anther than LS of IM and IS of LM flowers.

On the other hand, average ovule numbers per flower between the three floral morphs in *L. salicaria* did not differ significantly. Therefore, with these constant numbers of ovules per flower, the pollen-ovule ratio differed between the three floral morphs. However, all three ratios fall within the range of facultative autogamy (Cruden, 1977). This result seems to contradict what one would expect of *L. salicaria* due to its tristylous self-incompatibility system (Barrett, 1992), which makes it closer to being an obligate outcrosser (i.e. xenogamous).

Pollen viability was consistently high in wild plants of *L. salicaria* at the two sites investigated in central Saskatchewan. There was no statistical significance to differences in

pollen viability between anther levels within a floral morph, or between like staminal levels between two floral morphs.

No difference in nectar secretion rates were found for *L. salicaria* at the IDY field site on either day the experiments occurred. *L. salicaria* in England was also found, in a less intensive study than undertaken here, to have no significant difference in nectar secretion rates between floral morphs (Comba et al., 1999). The secretion rate of *L. salicaria* at Cambridge Botanical Gardens was found to be around 80 µg nectar sugar per flower per h (Corbet et al. 2001). This observation correlates well with the findings at IDY, where estimated secretion rates for all floral morphs ranged between 61 and 83 µg nectar sugar per flower per h. Therefore, it would appear that floral nectar is produced by *L. salicaria*, invasive to central Saskatchewan, in a fashion similar to plants at least in parts of its native Europe.

3. POLLINATION BIOLOGY OF *LYTHRUM SALICARIA* IN CENTRAL SASKATCHEWAN

3.1 Introduction

Lythrum salicaria possesses the rare self-incompatibility syndrome, tristyly, meaning that successful pollination is only achieved when 'legitimate' pollen is transferred to a receptive stigma of up to two correct floral morphs (see section 1.2.4). Although direct experimentation is lacking, cross pollination of *Lythrum salicaria* in the wild is thought to be performed by various insect visitors observed on the flowers (Mal et al. 1992; Diehl et al. 1997; Waites and Ågren 2004), thereby suggesting that this species is entomophilous.

The most common insect visitors to flowers of *L. salicaria* in North America, and its native Eurasia, are bees of the family Apidae (Hymenoptera); specifically bumble bees (*Bombus* spp.) and the European honey bee (*Apis mellifera*). Moreover, leafcutter bees of the family Megachilidae (Hymenoptera) also have frequently been reported to visit flowers of *L. salicaria* (Mal et al. 1992; Diehl et al. 1997; Waites and Ågren 2004). Other insect groups reported to visit flowers of *L. salicaria* are the hover flies (Diptera: Syrphidae) and butterflies (Lepidoptera) (Diehl et al. 1997; Henne et al. 2002; Waites and Ågren 2004) (see section 1.2.4).

Non-pollinating insect activity such as herbivory and other related interactions with plants of *L. salicaria* are also well known. Insects from many orders including the Coleoptera, Hemiptera (including the Homoptera), Thysanoptera, Lepidoptera (generally as larvae), Diptera, and Hymenoptera have been recorded in association with various parts of purple loosestrife plants (Diehl et al. 1997; Henne et al. 2002). Several beetles (Coleoptera: Curculionidae, Chrysomelidae), including *Galerucella calmariensis* and *G. pusilla*, have been studied as possible biological control agents for import to North America from Europe. Some native

phytophagous coleopterans are also attracted to purple loosestrife (Blossey and Schroeder 1995; Blossey et al. 2000; Hunt-Joshi and Blossey 2005).

The objective of this portion of the project was to investigate the functional reproductive biology of *L. salicaria* in central Saskatchewan, specifically by conducting experimental pollinations of the three floral morphs by hand, and ultimately to determine and quantitatively rank various flower visiting insects as pollinators.

3.2 Materials and Methods

3.2.1 Survey of insect diversity

Censuses of insect visitors to *L. salicaria* were carried out at both IDY (two days) and BVR (three days) sites in 2007. The transect used at IDY was 10 m long and at BVR the transect was divided into three lengths of 10 m each. Insects foraging on flowers of *L. salicaria* were recorded while walking the transect. Each 10 m transect (or section) was walked for one minute at three times throughout the day; once between 9:00 and 11:00 am, once between 12:00 and 1:10 pm, and again between 3:00 and 4:00 pm. Thus, observations were made along 30 m and 90 m of transect at IDY and BVR, respectively, on each census date. Insect diversity was not measured at LKS due to the difficulty of creating a walkable transect.

3.2.2 Controlled pollination experiments by hand

3.2.2.1 Self-pollination

Self-pollination experiments were conducted by hand at IDY in 2006 and at both IDY and LKS during 2007, to determine the potential ability of *L. salicaria* to self-pollinate in wild

stands in Saskatchewan. However, at the LKS field site the coleopteran biological control agent, *Galerucella calmariensis* (Chrysomelidae), resided in the flowers and on the leaves of *L. salicaria*. Considerable feeding damage was observed, both on the foliage and within the flowers, including many experimental flowers which had to be discarded due to damaged or missing styles, presumably caused by the feeding activities of this species.

Self-pollination trials included a receptive stigma of SM being brushed with pollen from IS and LS anthers of its own flowers; each IM stigma crossed with pollen from SS and LS anthers of its own flowers; and each stigma of LM crossed with pollen from SS and IS anthers of its own flowers, as described below.

In preparation for hand-pollinating experiments, several spikes of all three floral morphs were cleared of open flowers using forceps. Then each cleared spike was carefully enclosed in mesh bags sewn from fine netting (Century Textiles and Supplies, Saskatoon, SK) with mesh openings approximately triangular in shape and having dimensions of about 0.25 mm (base) x 0.65 mm (height). Each mesh bag was open on both ends and was slid down the spike over mature buds using draw-strings to close the bag securely around the stem, to prevent visitor access to flowers which opened later within the bag. The following day, bagged spikes were carefully unveiled to allow access to the newly open flowers. Forceps were used to remove a stamen and gently touch the stigma of the same flower that yielded the stamen. This procedure was repeated separately on different flowers for both staminal levels present within each floral morph. All flowers on a bagged spike were given the same treatment, marked, and then the plant was labelled with the treatment in order to prevent confusion between different treatments and plants. Hand-pollinated flowers were then carefully re-bagged and any pollen tubes from germinated grains were allowed to elongate for 24 hours. The following day, hand-pollinated

flowers were collected using forceps and placed into labelled vials containing 3:1 ethanol: glacial acetic acid to fix the styles for at least 24 hours. After fixation, styles with the stigma and upper portion of the ovary intact were dissected from flowers and rinsed three times in 70% ethanol. Styles were then placed in 0.1% aniline blue, a UV-fluorescent stain for the polysaccharide callose (β-1, 3 glucan) present in pollen tubes as callose plugs (Martin 1959).

Testing for successful pollination and potential fertilization was accomplished using fluorescence microscopy. Accordingly, after a period ranging from a few days to several months, stained styles were removed from vials and placed in a drop of aniline blue stain on glass microscope slides. Styles were gently squashed under a cover glass before sealing with clear nail polish to retard desiccation of the slides. Slides were viewed between 63x and 400x magnification under ultraviolet light using a Zeiss epifluorescense microscope. Pollen tubes, if present, could be distinguished by the presence of callose in the tube walls (Figures 3.1A, B) or as callose plugs (Figure 3.1F). Pollen tubes detected at the ovary end of the style were quantified. For those experiments, it was assumed that if a pollen tube entered the ovary, there was the potential for fertilization of an ovule.

3.2.2.2 Illegitimate crosses

The experimental procedure for illegitimate crosses was performed similarly to the selfing experiments above; however, instead of placing pollen from a stamen within the same flower onto the stigma, "illegitimate pollen" (i.e., from a stamen not matching the style's length) from another plant was brushed upon the stigma of a bagged, virgin flower. Illegitimate crosses made were: LS pollen on SM and IM stigmas; IS pollen on SM and LM stigmas; and SS pollen on IM and LM stigmas. Collection and processing of styles, and the eventual visualisation and

quantification of pollen tubes, were also undertaken using fluorescence microscopy (section 3.2.2.1.).

3.2.2.3 Legitimate crosses

"Legitimate crosses" were performed between plants by hand using combinations of stamens and gynoecia of the same length. In other words, SS pollen was placed upon SM stigmas, IS pollen on IM stigmas, and LS pollen on LM stigmas. Otherwise, the procedure utilised for these legitimate crosses was the same as for self- and illegitimate crosses (sections 3.2.2.1 and 3.2.2.2). Visualisation and quantification of pollen tubes at the style bases of legitimately crossed flowers were accomplished using the fluorescence microscopy technique described in section 3.2.2.1.

3.2.3 Pollination efficiency as determined by single insect visits

3.2.3.1 Experiments involving single insect visits to virgin flowers

Experiments allowing single insect visits (SIVs) to virgin flowers (Davis 1992a) were conducted from July 25 - September 1, 2006, and from July 17 - September 5, 2007, and performed primarily at the IDY and BVR field sites. The LKS site was also used for some SIV trials during both field seasons, however because of the risk of data loss due to insect herbivory (see section 3.2.2.1), LKS use was restricted. Entire sections of a flowering spike were enclosed using fine mesh cloth bags (see section 3.2.2.1) to prevent visitor access. Before being closed in the mesh bags, spikes were cleared of open flowers by forceps. This practice ensured that any flowers later found open within the bag had opened after spikes were enclosed, thereby providing a source of non-visited (virgin) flowers for experimentation. The following day (i.e.,

9:00 am – 4:00 pm), bags were removed from spikes and virgin flowers watched closely until a visitor approached. The duration of the solitary visit was recorded and an attempt was also made to capture the departing visitor with an insect net. The visited flower was marked on a petal with black ink, then immediately and carefully re-bagged to prevent further visitor access. The SIV flowers were left bagged for approximately 24 hours and then collected and placed into fixative in labeled vials, and their styles processed for pollen-tube quantification, as explained in section 3.2.2.1.

Collected insect specimens were pinned and labeled at the lab, placed into like groups and, where possible, determined to species using Curry (1984); Vockeroth (1992); Michener et al. (1994); and the Discover Life website (2007). Tentatively identified specimens were sent to taxonomic experts for confirmation. With this taxonomic verification in hand, descriptive statistics (mean and standard error of the mean) were determined for the identified insect taxa participating in SIVs for all field sites, and also for pooled field sites after first checking for significant differences between years and sites. For each year separately, percentages of insect taxa involved with SIV experiments at all sites combined were determined by dividing the total number of insects per taxon by the total number of visitors.

3.2.3.2 Controls

Various treatments (controls) were conducted to quantify levels of pollination inadvertently performed by the investigator, rather than insect visitors. By carefully unbagging and immediately re-bagging virgin flowers of *L. salicaria*, thereby preventing an insect visit, it was possible to attribute pollination levels caused by non-insect factors. Unvisited flowers from the 2006 field season were marked as controls on non-visited spikes to distinguish them from

spikes receiving SIVs, and individual control flowers then processed in the same manner as SIVs. In 2007, the technique for acquiring controls was changed slightly, as follows. Controls were taken from unvisited, open flowers near the same region on a spike that had received a SIV to a virgin flower nearby. Before the flowering spike was re-bagged following an SIV, unvisited flowers were identified using a different colour mark on the petals to distinguish these control flowers from that receiving a SIV. The collection and processing of control flowers in 2007 was the same as for SIV flowers (see section 3.2.2.1).

Furthermore, normal (i.e., never bagged) flowers were also sampled randomly during both field seasons from BVR and IDY during 2006 and from BVR and LKS in 2007. However, flowers with a darkened stigma typical of aging were not collected. These open flowers of imprecise age and unknown visitation history were collected from the field sites in order to investigate natural pollination levels in *L. salicaria*. These randomly collected flowers were processed in the same manner as SIV flowers and the standard control flowers (see section 3.2.2.1).

3.2.3.3 Statistical analysis

Prior to statistical analysis, SIV data underwent data transformation using a variant of the square root transformation ($\sqrt{y+\frac{1}{2}}$) recommended for use with data sets containing several small numbers (under 10-15) and zeros (Steel and Torrie 1980). After data transformation, analysis of variance (One-Way ANOVA; $\alpha = 0.05$) was performed to determine statistical significance between three or more different sets of SIV data. Two-Tailed t tests were used when comparing between two data sets. Regression analyses were performed between visit

duration and number of pollen tubes following single insect visits. Statistics were performed using Microsoft Excel 2007.

3.3 Results

3.3.1 Diversity of insect visitors

Three taxonomic orders of insects were observed visiting flowers of *L. salicaria* over the course of the censuses made at IDY and BVR. Various bee taxa from three families constituted the majority (629 of 661 visitors; 95.2%) of visitors overall, with three other taxa (one vespid wasp, nine butterflies and 22 hover flies) also represented (Table 3.1). Taxonomic diversity of flower visitors to *L. salicaria* was notably different between the two sites studied. IDY was visited almost exclusively by *Bombus* spp. and *Apis mellifera*, with only two visits by other insect taxa observed on the census dates. Insect diversity at BVR was higher at all times than at IDY, comprising all four taxa observed at IDY plus four more taxa (Table 3.1). The overall larger number of visitors recorded at BVR over IDY may also have been related to the greater, 90 m transect distance at BVR compared to the 30 m transect used at IDY.

3.3.2 Controlled pollination experiments by hand

3.3.2.1 Self-pollination

In all styles but one, self-pollinations by hand yielded no pollen tubes at the ovary end of the style. The exception was a single IM of the eleven flowers of that morph selfed by LS pollen, which had five pollen tubes at the ovary end of the style (Table 3.2). All styles included in the data had copious quantities of germinated pollen grains on the stigma, indicating that all

replicates had successful pollen transfer to the stigma, but the pollen tubes did not grow to the ovary.

Table 3.1. Number of insect visitors per taxonomic group to non-experimental flowers of *Lythrum salicaria*, according to census date and time at BVR and IDY.

			No. of Visitors								
				Hymenoptera							
				Apidae						Lepidoptera	Diptera
Site	Date	Time	Total	*Bombus* spp.	*Apis mellifera*	*Anthophora* spp.	Halictidae	Megachilidae	Vespidae		Syrphidae
IDY	04-Aug-07	9:38	6	5							1
		12:11	9	5	4						
		15:00	5	3	1		1				
	15-Aug-07	10:55	2	2							
		12:52	2	1	1						
		15:05	3	2	1						
BVR	05-Aug-07	9:00	60	41	2		10	6		1	
		12:00	42	29			7	2		1	3
		15:00	33	31					1		1
	14-Aug-07	10:30	31	24	5		2				
		13:09	99	74	11	2	3	5		4	
		15:00	95	84	10	1					
	29-Aug-07	9:10	14	8							6
		12:40	143	76	59			3		3	2
		15:00	117	55	51			2			9

Table 3.2. Results of self- and illegitimate-pollinations performed by hand using flowers of *Lythrum salicaria*, pooled from the BVR, IDY, and LKS field sites. LM = long-style morph; IM = intermediate-style morph; SM = short-style morph; LS = long stamens; IS = intermediate stamens; SS = short stamens.

Recipient floral morph	Source of donor pollen	No. flowers pollinated	No. pollen tubes observed per style (mean ± s.e.)
SM	self IS	10	0 ± 0
SM	self LS	13	0 ± 0
IM	self SS	7	0 ± 0
IM	self LS	11	0.45 ± 0.45
LM	self SS	11	0 ± 0
LM	self IS	11	0 ± 0
SM	LM/IS	8	0 ± 0
SM	IM/LS	7	0 ± 0
SM	SM/IS	6	0 ± 0
SM	SM/LS	3	0 ± 0
IM	LM/SS	7	0 ± 0
IM	SM/LS	5	0.2 ± 0.2
IM	IM/SS	5	0 ± 0
IM	IM/LS	5	0 ± 0
LM	SM/IS	5	0 ± 0
LM	IM/SS	12	0 ± 0
LM	LM/SS	6	0 ± 0
LM	LM/IS	5	0 ± 0

3.3.2.2 Illegitimate crosses

As with self-pollination by hand, illegitimate hand-crosses yielded virtually no pollen tubes at the ovary end of the style. The single exception (IM stigma receiving SM/LS pollen) resulted in a single pollen tube at the base of one style from five styles examined (Table 3.2). Pollen tubes that issued from "illegitimate pollen" were often swollen and rarely possessed callose plugs (Figures 3.1C, E). Thus, illegitimate pollen transfer to the stigma of another specimen rarely resulted in a successful fertilization event.

3.3.2.3 Legitimate crosses

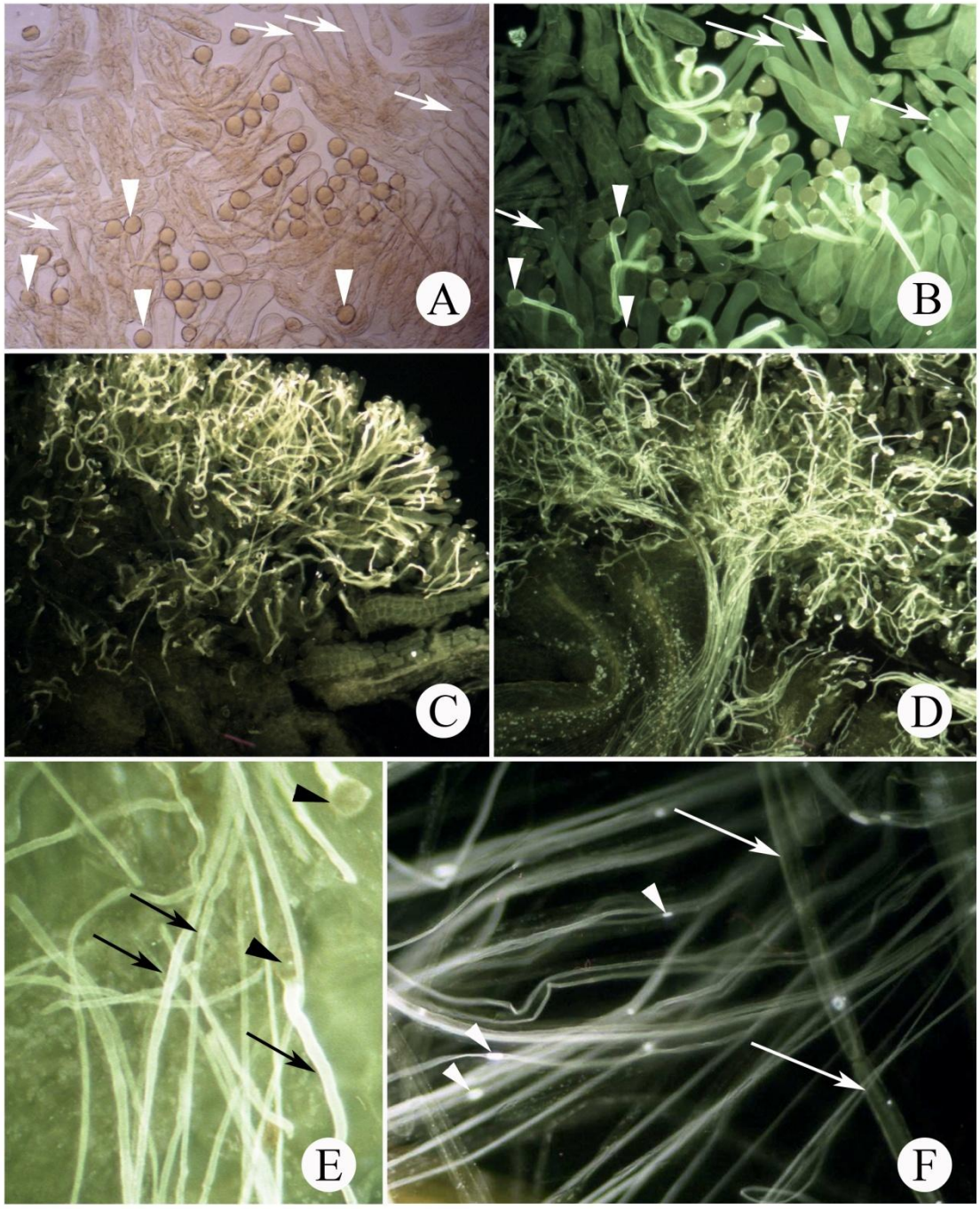
Legitimate pollen transfer to the stigma resulted in pollen tube growth to the ovary, without exception (Figure 3.2). The lowest number of pollen tubes recorded at the ovary end of the style in a legitimate cross was five, the numbers reaching several hundred pollen tubes per style. Pollen tubes arising from crosses with "legitimate pollen" typically were narrow and possessed distinct callose plugs (Figures 3.1D, F). Hand-crossing experiments showed that when legitimate pollen is involved, pollen tubes do reach the ovary, and presumably succeed in fertilizing ovules. These results, along with those from the experiments involving self- and illegitimate pollinations, indicate the strong level of outcrossing that *L. salicaria* requires from stamens of precisely the same length of the gynoecium, in order for the species to achieve sexual reproductive success.

3.3.3 Single insect visits

3.3.3.1 Frequency of insect taxa as floral visitors

During the 2006 field season, a total of 107 single insect visits (SIVs) were recorded when SIVs were combined from the three field sites. The majority (65%) were made by bumblebees (*Bombus* spp.) (Figure 3.5A), followed distantly by honeybees (*Apis mellifera*) (Figures 3.3, 3.5B). Appreciable numbers of visits by both sweat bees (Halictidae) and hoverflies (Syrphidae) (Figure 3.5F) were also registered (Figure 3.3). A single visit each from an unknown fly (Diptera), a pierid butterfly (Figure 3.5C), and a vespid wasp were also recorded during SIV trials in 2006 (Figure 3.3).

Figure 3.1. Fluorescence and light micrographs of pollen tubes on the stigma and in the styles of *Lythrum salicaria*. (A) Light micrograph showing region of intermediate-style morph flower of unknown visitation history with multiple germinated pollen grains (arrowheads) among the stigmatic papillae (arrows). (B) Fluorescence micrograph of the same region as seen in "A" showing the fluorescence of pollen tubes after staining with aniline blue. Germinated pollen grains (arrowheads) with pollen tubes; stigmatic papillae (arrows). (C) Stigmatic region of intermediate-style morph flower of unknown visitation history showing many illegitimate germinated pollen grains with short pollen tubes. (D) Stigmatic region of an intermediate-style morph flower of unknown visitation history showing many legitimate germinated pollen grains with many pollen tubes entering the transmitting tract of the style. (E) Pollen tubes (arrows) and some germinated illegitimate pollen grains (arrowheads) near the point of attachment of stigma to the style. Such pollen tubes frequently lack callose plugs. (F) Fluorescence micrograph of pollen tubes from legitimate pollen grains with callose plugs (arrowheads) in the transmitting tract of a squashed style from an intermediate-style morph. Cells of the transmitting tract (arrows).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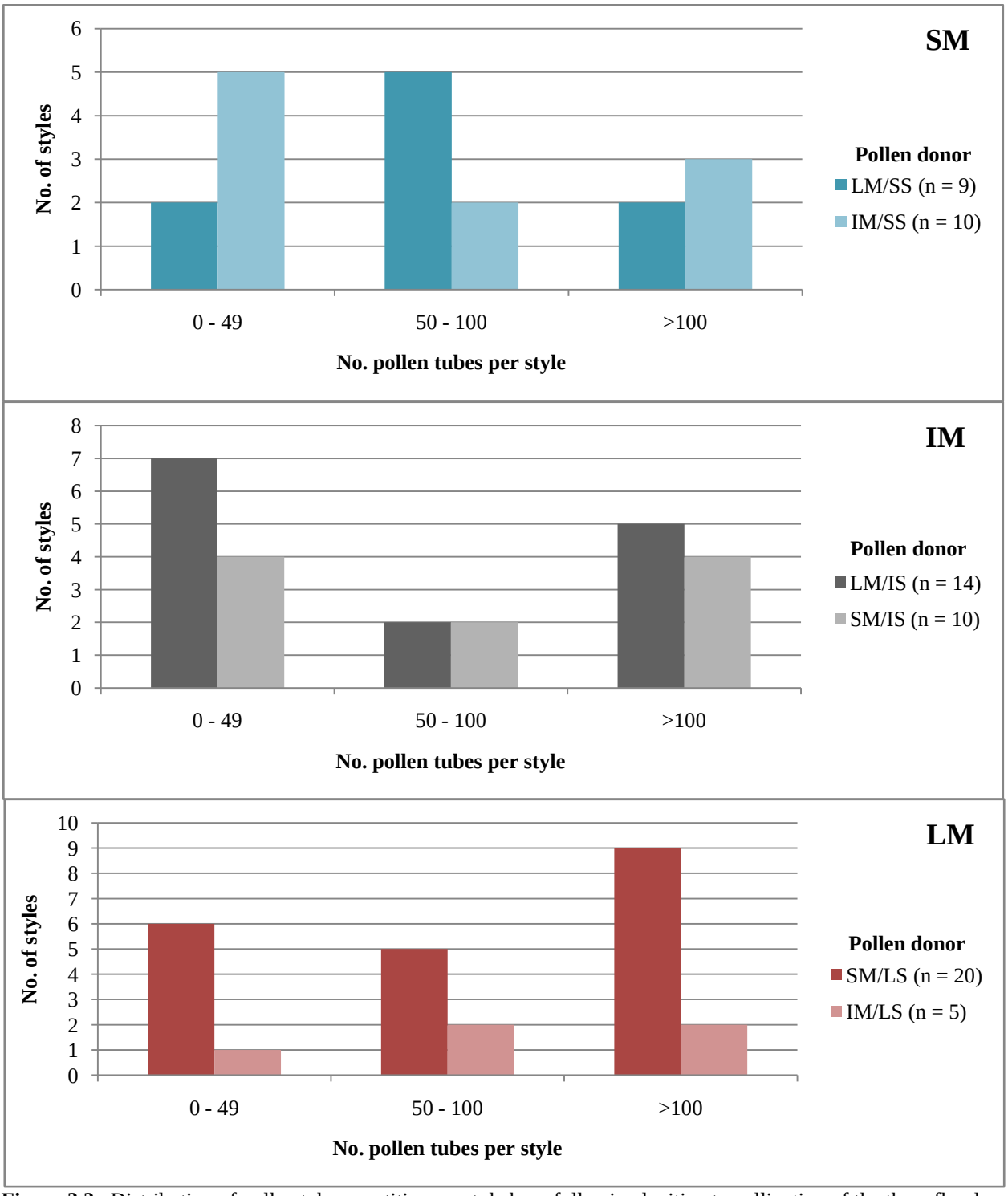


Figure 3.2. Distribution of pollen tube quantities per style base following legitimate pollination of the three floral morphs (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph) of *Lythrum salicaria*, with pollen from the corresponding legitimate anthers (LM/SS = long-style morph/short stamens; IM/SS = intermediate-style morph/short stamens; LM/IS = long-style morph/intermediate stamens; SM/IS = short-style morph/intermediate stamens; SM/LS = short-style morph/long stamens; IM/LS = intermediate-style morph/long stamens).

In 2007, 130 SIVs to virgin flowers were obtained, and insect diversity was generally higher than the year before (Figure 3.3). Although bumblebees still performed the majority (45%) of visits in 2007, their numbers were down in proportion to other taxa. Honeybees (21%) still performed the second highest numbers of SIVs, but were followed closely by sweat bee (16%) and hoverfly (11%) visits (Figure 3.3). In 2007, SIVs were also recorded by leafcutter bees (Megachilidae) (Figure 3.5D), *Anthophora furcata* (Figure 3.5E), vespid wasps, two unknown flies (Diptera), and one dragon fly (Odonata) (Figure 3.3).

3.3.3.2 Randomly collected flowers of unknown visitation history and age

Microscopic examination of the styles of randomly collected 'normal' flowers of unknown history and age collected during both field seasons showed that a gradation in pollination success was present in wild *L. salicaria*. In 2006, interestingly, the majority of flowers (67.8%) collected at random did not have pollen tubes at the base of the style (Figure 3.4). However, successful pollination had occurred for all three floral morphs, with some styles possessing over 150 pollen tubes. In 2007, again a high number of styles lacking pollen tubes (35.6%) were present (Figure 3.4), however only about half as many as in 2006. In contrast to 2006, in 2007 many styles possessed over 100 pollen tubes each, and at least one surveyed flower occurred within each pollen tube range (Figure 3.4).

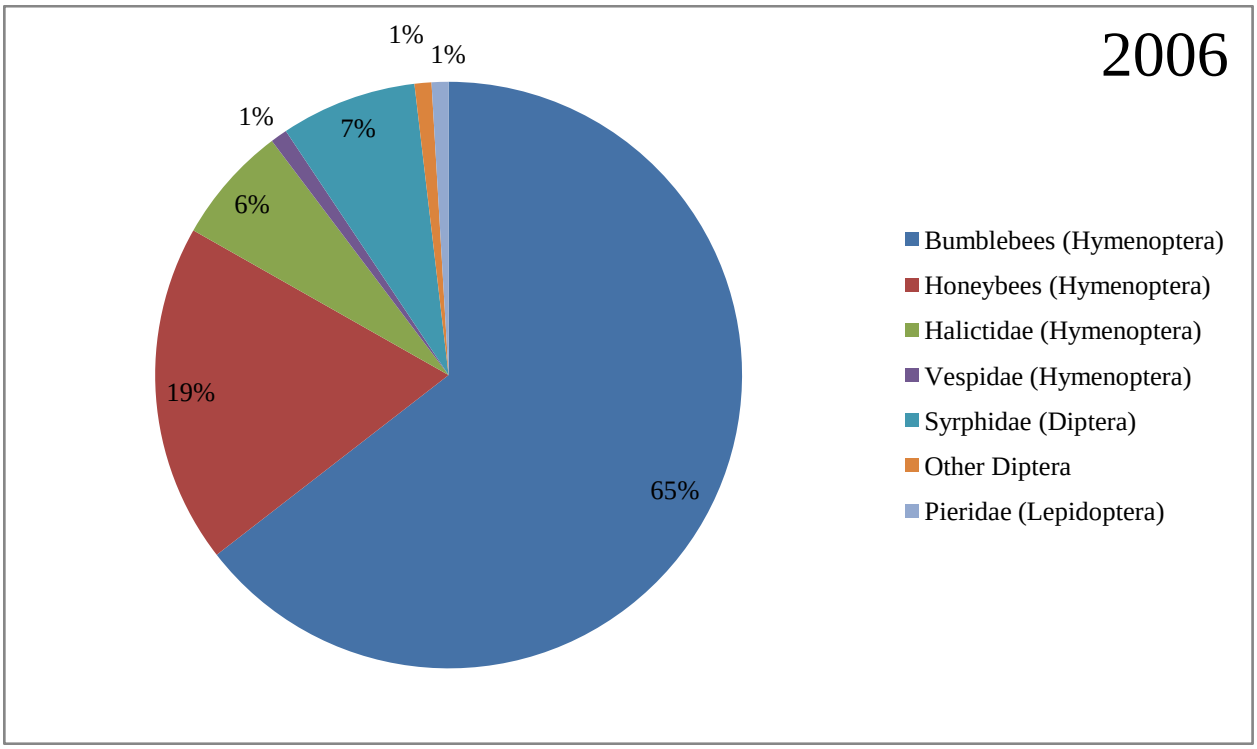
3.3.3.3 Pollination efficiency of single insect visitors

During the 2006 and 2007 field seasons, a total of 237 single insect visits to virgin flowers of *L. salicaria* were recorded. Overall, 114 insects (48.1%) landed on virgin flowers of LM, 84 (35.4%) on IM flowers, and 39 (16.5%) on SM flowers (Table 3.3).

As reflected in the census data of Table 3.1, members of the Apidae were also the most common visitors to virgin flowers of *L. salicaria* (Figure 3.3; Table 3.4). Of the 237 total SIVs recorded in this study, *Bombus* accounted for 129 (54.4%) and *Apis* had 47 (19.8%). Beyond the Apidae, sweat bees (Halictidae) were represented next with 27 SIVs (11.4%), followed by 22 SIVs (9.3%) by hover flies (Syrphidae). Thirteen styles (*Apis mellifera* -3; *Bombus ternarius* -2; *Bombus* spp. -5; Halictidae -1; Syrphidae -2) unfortunately were lost during processing, leaving a total of 224 styles that yielded pollen-tube data (Table 3.4).

Not only did individuals of *Bombus* participate in over half of the SIVs, all seven identified species contributed as pollinators of *L. salicaria*. Of these, *B. vagans* was the most frequent, followed by *B. ternarius* and *B. huntii* (Table 3.4). Within a species, there were no significant differences in pollination effectiveness with regard to floral morph. That is, for example, *B. huntii* pollinated the three floral morphs (LM, IM, SM) of *L. salicaria*, equally (Table 3.4). The same held true when all *Bombus* data were combined. One exception occurred for the unknown *Bombus* spp. visitors, wherein SM flowers possessed significantly more pollen tubes than in the other two morphs (Table 3.4). However, this result may be anomalous due to the extremely small number of SM flower visits (n = 2) by these unknown *Bombus* species, and may not represent a true statistical difference.

Within an individual floral morph (LM, IM, SM), individual *Bombus* species showed no significant difference in pollination effectiveness (i.e., left-most column of Table 3.4).



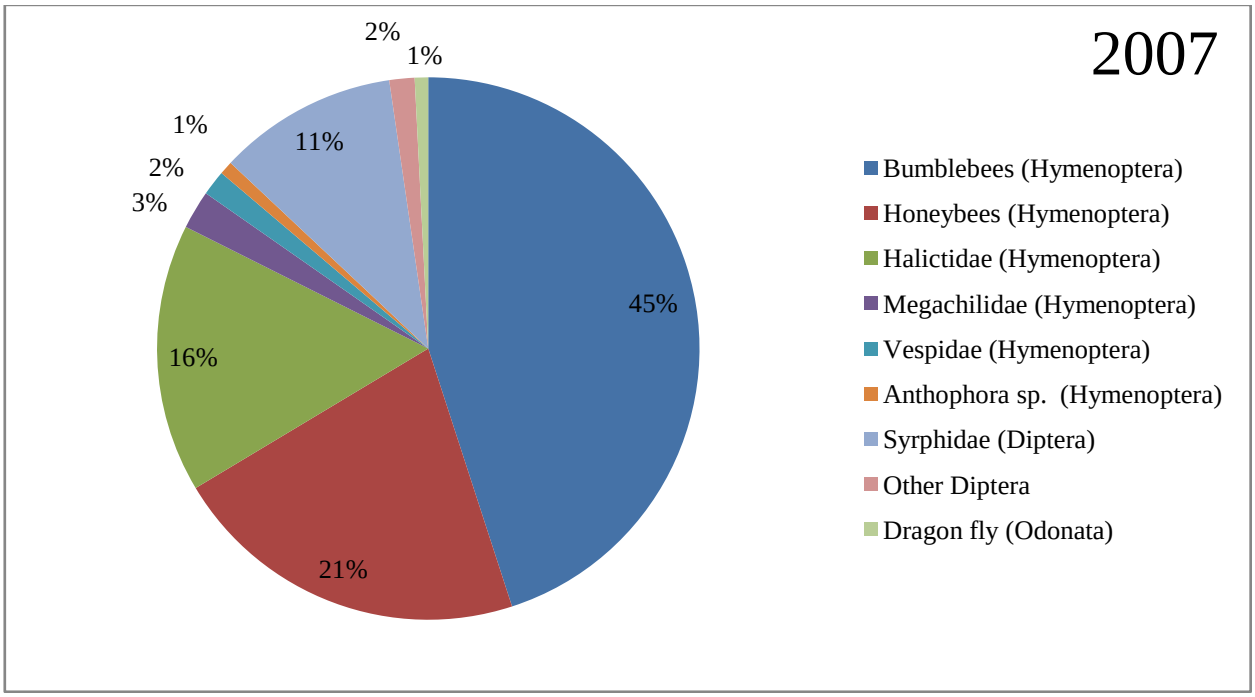


Figure 3.3. Relative proportions of different taxa recorded as single insect visitors to virgin flowers of *Lythrum salicaria* during the 2006 (n = 107) and 2007 (n = 130) field seasons at the BVR, IDY, and LKS sites combined.

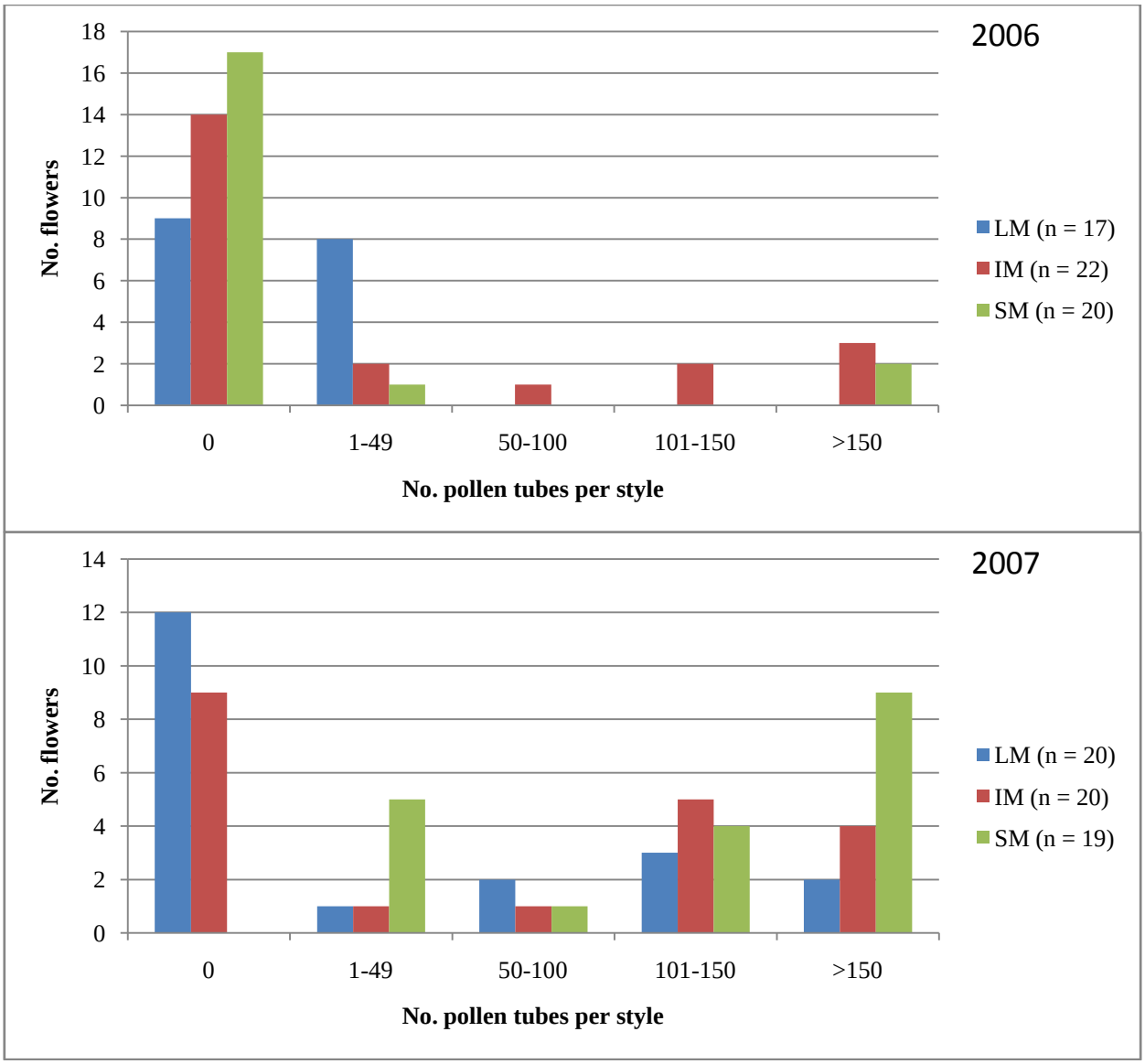


Figure 3.4. Approximate number of pollen tubes per style for randomly collected flowers of *Lythrum salicaria* of unknown visitation history pooled from all three field sites in 2006 and 2007. LM = long-style morph; IM = intermediate-style morph; SM = short-style morph.

Table 3.3. Percentage of the total (n = 237) single insect visits observed for each of the three floral morphs (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph) at the three field sites in 2006 and 2007.

LM		IM		SM	
No. SIVs	%	No. SIVs	%	No. SIVs	%
114	48.101	84	35.443	39	16.456

Bumblebee visits resulted in several of the highest numbers of pollen tubes recorded at the style base. For example, a single visit by *B. huntii* to a SM flower yielded 123 pollen tubes (Table 3.4). Whereas this result is exceptional, other noteworthy SIVs yielded 50 (IM; *B. huntii*), 52 (SM; unknown *Bombus* sp.), 74 (IM; *B. vagans*), and 88 (LM; *B. vagans*) pollen tubes each. On the other hand, 51 of 122 SIVs (41.8%) involving bumblebees resulted in styles without a single pollen tube (i.e., zero), despite the receptive stigma present in those flowers.

Like bumblebees, honeybees (*Apis mellifera*) successfully and equally pollinated the three floral morphs of *L. salicaria* (Table 3.4). Indeed, the highest number of pollen tubes following a SIV was recorded for a honeybee foraging on IM (n = 138 tubes). Other noteworthy honeybee visits yielded 44, 44, 45 (all IM) and 43 (LM) pollen tubes, each (Table 3.4). Still, of 44 visits by honeybees to virgin flowers, 14 (31.8%) failed to yield a single pollen tube at the style base.

The only other apid to have visited virgin flowers in either field season was *Anthophora furcata* which, although visiting only once, yielded 11 pollen tubes at the LM style base (Table 3.4).

Apart from the Apidae, the two other bee families (Halictidae, Megachilidae) that visited newly-unbagged flowers of *L. salicaria* also were successful as pollinators. Although two species were identified, most SIVs by sweat bees involved unknown species of *Lasioglossum*. From the latter group, and when all *Lasioglossum* were combined, a significant difference in pollen tube quantities per floral morph occurred, with SM exceeding LM but not IM (Table 3.4). Two individual visits by *L. laevissimum* furnished 38 (IM) and 53 (SM) pollen tubes per style, and an unknown *Lasioglossum* achieved 35 (SM) pollen tubes (Table 3.4). However, 15 (57.7%) of the 26 total visits by *Lasioglossum* to virgin flowers generated styles lacking pollen tubes.

Figure 3.5. Various insect visitors foraging on *Lythrum salicaria*. (A) Bumblebee (*Bombus terricola*). (B) Honeybee (*Apis mellifera*). (C) Sulphur butterfly (*Pieris* sp.). (D) Leafcutter bee (*Megachile* sp.). (E) Flower bee (*Anthophora furcata*). (F) Hover fly (*Sphaerophoria* sp.).

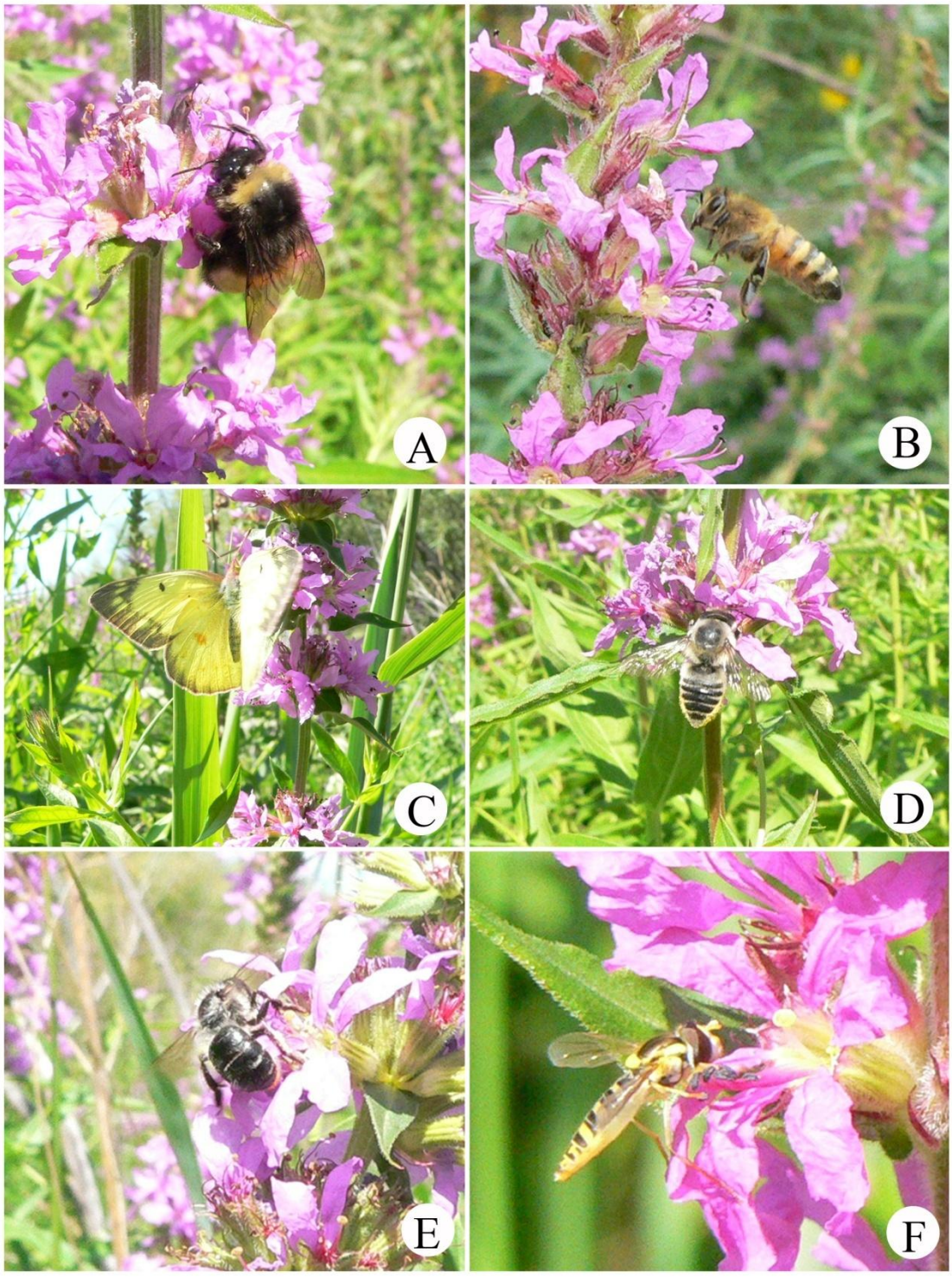

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Table 3.4. Pollination effectiveness of various taxa involved as single insect visitors to virgin flowers (n) of the three floral morphs (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph) of *Lythrum salicaria*. Pollen tube (y) data (mean ± s.e.) are given following a square-root transformation ($\sqrt{y+\frac{1}{2}}$). Actual (i.e., non-transformed) pollen tube counts are shown as a range. Within a row, means denoted by different letters as a superscript are significantly different ($P < 0.05$). Similarly, non-bold superscripted letters in the flower visitor column (LM/IM/SM) denote significantly different quantities of pollen tubes between individual species ($P < 0.05$). Bold superscripted letters in the flower visitor column (LM/IM/SM) denote significant differences between pollen-tube means for the four major visitor groups.

		LM			IM			SM		
Flower visitor	**Taxonomic family**	**n**	**No. pollen tubes (mean ± s.e.)**	**(range)**	**n**	**No. pollen tubes (mean ± s.e.)**	**(range)**	**n**	**No. pollen tubes (mean ± s.e.)**	**(range)**
Anthophora furcata	Apidae	1	3.39	11						
Apis mellifera$^{A/A/A;\ \mathbf{A/A/A}}$	Apidae	23	2.27 ± 0.35^{A}	0-43	14	4.01 ± 0.87^{A}	0-138	7	2.76 ± 0.70^{A}	0-27
Bombus borealis$^{-/-/A}$	Apidae	1	0.71	0				3	1.99 ± 0.78	0-11
Bombus centralis$^{A/-/-}$	Apidae	2	1.63 ± 0.92	9-13	1	0.71	0			
Bombus huntii$^{A/A/A}$	Apidae	8	1.90 ± 0.67^{A}	0-25	4	2.31 ± 1.60^{A}	0-50	5	4.68 ± 1.83^{A}	0-123
Bombus rufocinctus	Apidae	1	4.18	17	1	0.71	0			
Bombus ternarius$^{A/A/A}$	Apidae	10	1.65 ± 0.20^{A}	0-6	15	2.19 ± 0.42^{A}	0-10	2	0.71 ± 0.00^{A}	0-0
Bombus terricola$^{-/-/A}$	Apidae							2	3.37 ± 2.67	0-36
Bombus vagans$^{A/A/A}$	Apidae	28	2.97 ± 0.48^{A}	0-88	13	3.09 ± 0.65^{A}	0-74	2	0.71 ± 0.00^{A}	0-0
Unknown *Bombus* spp.$^{A/A/A}$	Apidae	13	1.04 ± 0.19^{A}	0-6	9	1.69 ± 0.31^{A}	0-10	2	4.68 ± 2.56^{B}	4-52
All *Bombus* combined$^{\mathbf{AB/B/A}}$	Apidae	63	2.17 ± 0.26^{A}	0-88	43	2.30 ± 0.30^{A}	0-74	16	3.02 ± 0.80^{A}	0-123
Lasioglossum laevissimum	Halictidae	3	0.71 ± 0.00^{A}	0-0	3	3.28 ± 1.60^{A}	0-38	1	7.31	53
Lasioglossum zephyrum	Halictidae				1	0.71	0			
Unknown *Lasioglossum* spp.	Halictidae	6	0.88 ± 0.11^{A}	0-1	7	1.16 ± 0.38^{AB}	0-11	5	3.10 ± 1.04^{B}	0-35
All *Lasioglossum* combined$^{\mathbf{BC/B/A}}$	Halictidae	9	0.82 ± 0.08^{A}	0-1	11	1.70 ± 0.54^{AB}	0-38	6	3.80 ± 1.10^{B}	0-53
Megachile latimans	Megachilidae							2	4.52 ± 2.93	2-55
Megachile sp.	Megachilidae	1	0.71	0						
Vespidae					1	0.71	0	2	0.71 ± 0.00	0-0
Eupeodes americanus	Syrphidae	3	0.71 ± 0.00	0-0						
Heringia sp.	Syrphidae	1	0.71	0						
Sphaerophoria contigua	Syrphidae				1	0.71	0			
S. philanthus OR *asymmetrica*	Syrphidae	1	0.71	0	1	0.71	0			
Syritta pipiens	Syrphidae				2	1.29 ± 0.58	0-3			
Syrphus vitripennis	Syrphidae	1	0.71	0						
Toxomerus marginatus	Syrphidae	3	0.71 ± 0.00	0-0	1	2.12	4	1	0.71	0
Unknown Syrphidae		1	0.71	0	2	0.71 ± 0.00^{A}	0-0	2	0.71 ± 0.00^{A}	0-0
All Syrphidae combined$^{\mathbf{C/B/A}}$		10	0.71 ± 0.00^{A}	0-0	7	1.08 ± 0.24^{A}	0-4	3	0.71 ± 0.00^{A}	0-0
Unknown Diptera		1	0.71	0	3	0.71 ± 0.00	0-0			
Pieris rapae	Pieridae				1	0.71	0			
Odonata	Suborder: Anisoptera	1	0.71	0						

Although only three SIVs were registered by leafcutter bees (*Megachile* spp.), both visits to SM resulted in pollen tubes at the style base (once with 55), whereas the LM visit did not (Table 3.4).

Only three visits by yellow jacket wasps (Vespidae) were registered as SIVs over both years, and all lacked pollen tubes at the style base (Table 3.4).

Although a diversity of hover flies (six genera, seven species identified) visited newly-unbagged flowers of *L. salicaria* over both field seasons, the Syrphidae (combined) led to few, or more often, no pollen tubes at the style base (Table 3.4). Indeed, only two visits to IM, by *Syritta pipiens* (3 pollen tubes) and *Toxomerus marginatus* (4), yielded any pollen tubes; 90% of the 20 SIVs resulted in an absence of pollen tubes (Table 3.4).

Of the remaining, infrequent flower-visiting taxa, none of the four unknown flies (Diptera), nor the cabbage-white butterfly (*Pieris rapae*), nor the dragonfly (Odonata), led to any pollen tubes at the style base (Table 3.4).

When comparing the combined pollen-tube data of Table 3.4 for the four taxa which most frequently visited virgin flowers of *L. salicaria*, significant differences were apparent for LM (One-way ANOVA; $P = 0.020$) and IM ($P = 0.012$), but not for SM ($P = 0.441$) flowers. For the latter morph, the relatively lower quantity of SM flowers visited overall, may have been influential. The mean number of pollen tubes per style base for all *Lasioglossum* (Halictidae) was 3.80 ± 1.10 ($n = 6$), not being significantly greater than that for all hover flies (Syrphidae) combined (0.71 ± 0.00, $n = 3$; $P = 0.096$, two-tailed t test).

For IM flowers, on the other hand, SIVs by honeybees (*Apis mellifera*) gave significantly more pollen tubes per style base (4.01 ± 0.87) than all *Bombus* combined (2.30 ± 0.30; $P = 0.020$, two-tailed t test), all *Lasioglossum* (Halictidae) together (1.70 ± 0.54; $P = 0.045$), and all

Syrphidae (1.08 ± 0.24; P = 0.030). However, when compared on an individual species basis rather than to the large number of visits (43) amassed when pooling all *Bombus* together, honeybees were found not to be superior pollinators of IM flowers than any *Bombus* species in particular (Table 3.4).

Finally, for LM flowers, determination of a clearly superior pollinating taxon was complex. Honeybees visiting virgin flowers yielded an average of 2.27 ± 0.35 pollen tubes per style, similar to all *Bombus* together (2.17 ± 0.26), but statistically superior to all *Lasioglossum* (Halictidae) combined (0.82 ± 0.08; P = 0.015, two-tailed t test) and Syrphidae combined (0.71 ± 0.00; P = 0.006). Bumblebees were not superior to Halictidae (P = 0.053), but were significantly better pollinators than the Syrphidae combined (P = 0.027). Halictids and syrphids were equally inferior pollinators of LM flowers (Table 3.4)

In 2006, microscopic examination of styles from bagged (control) flowers sampled from inflorescences separate from those hosting SIVs, indicated a very low level of inadvertent pollination had taken place (Table 3.5). For those controls, there was no significant difference in pollination levels between the three floral morphs (One-Way ANOVA, P = 0.624). Similarly, in 2007, the isolated-inflorescence controls, as well as SIV-associated controls, showed no significant differences in pollination levels between morphs (P = 0.165 and P = 0.402, respectively). When comparing the different control treatments within a morph, LM SIV-associated controls from 2007 had significantly fewer pollen tubes than those control flowers from isolated inflorescences in 2006 (P = 0.042); however, there was no difference between either of the two control treatments in 2007, or between the 2006 and 2007 isolated-inflorescence controls. One-way ANOVA indicated that between treatment analysis of IM controls was almost significant (P = 0.052). In comparison, between SM control treatments there is no

significant difference in the level of inadvertent pollination (One-way ANOVA, P = 0.343) (Table 3.5).

Table 3.5. Numbers of pollen tubes per style base for unvisited flowers (negative controls) of *Lythrum salicaria* collected from all field sites in 2006 and 2007. LM = long-style morph; IM = intermediate-style morph; SM = short style morph.

	LM		**IM**		**SM**	
Type of control	**No. flowers**	**mean ± s.e.**	**No. flowers**	**mean ± s.e.**	**No. flowers**	**mean ± s.e.**
2006; isolated inflorescence	22	0.41 ± 0.19	21	0.24 ± 0.15	18	0.67 ± 0.52
2007; isolated inflorescence	8	0.13 ± 0.13	24	0.00 ± 0.00	5	0.00 ± 0.00
2007; inflorescence associated with SIV	52	0.06 ± 0.04	29	0.00 ± 0.00	32	0.13 ± 0.08

In order to determine whether insect visitors overall had preferentially chosen one floral morph of *L. salicaria* over another at the three field sites, a chi-squared test ($\chi^2 = \sum \frac{(0-E)^2}{E}$, α = 0.05) was used. The observed proportions to each floral morph of the total SIVs recorded are given in Table 3.3, and expected, weighted values were determined based on the resident proportions of the three floral morphs among the three field sites (Table 3.6). It became evident that there was no preference to any particular floral morph shown by the pooled insect visitors ($\chi^2_{2d.f.} = 3.0415, P \simeq 0.228$).

Table 3.6. Average percentage (upper value) of each of the three floral morphs (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph) present at the three field sites in 2006 and 2007 (from Tables 2.1 and 2.2). The lower values (in brackets) represent the weighted percentages of the three floral morphs based on the location of the SIVs performed.

Field site	No. SIVs	Proportion SIVs (No. SIVs/237) per field site	LM	IM	SM
BVR	159	0.6709	48.1% (32.270)	49.8% (33.410)	2.1% (1.409)
IDY	58	0.2447	20.2% (4.943)	30.0% (7.342)	49.8% (12.187)
LKS	20	0.0844	37.7% (3.181)	34.7% (2.928)	27.6% (2.329)
Weighted percentages of floral morphs, according to the number of SIVs acquired per field site (i.e., expected values)			(40.394)	(43.680)	(15.925)

3.3.3.4 Duration of visits to virgin flowers by single insect visitors

Of the 237 total insects that visited newly-unbagged flowers, visit duration was recorded for 214 SIVs as shown in Table 3.7. The average duration of visits to virgin flowers varied between different taxa (Table 3.7). However, within a specific visitor taxon, there was never an instance where visitors would remain statistically longer on one floral morph over another (Table 3.7). Visitors of the Syrphidae tended to spend the longest time on individual flowers per visit, with averages ranging from 5.3 to 62.1 seconds. Similarly, halictid bees remained on the flowers for comparatively long periods, ranging from 35.9 (*Lasioglossum laevissimum* on LM) to 57.8 (unknown *Lasioglossum* spp. on LM) seconds per single visit (Table 3.7). The dragon fly remained stationary for a few minutes before flying off again, as would be expected. On the contrary, bees of the family Apidae tended to stay for relatively shorter times, with averages ranging from 2.3 (*B. borealis* on SM) to 15.7 (honeybees on SM) seconds per single visit.

A comparison of average duration per flower visit to virgin flowers of *L. salicaria* (Table 3.7) among the individual *Bombus* species plus *Apis mellifera*, yielded several significant differences for LM (One-way ANOVA, $P = 0.0002$) and SM ($P = 0.020$) flowers, but not for IM ($P = 0.213$).

On virgin flowers of LM, on average *Apis mellifera* (10.7 sec) remained significantly longer than *Bombus huntii* (4.7 sec, $P = 0.013$, two-tailed t test), *B. ternarius* (5.9 sec, $P = 0.015$), *B. vagans* (5.8 sec, $P = 0.0005$) and the unknown *Bombus* spp. (5.5 sec, $P = 0.003$). Although having only two visits by *B. centralis* to LM flowers brings sample size into question, analysis of the data available from this study showed that *B. centralis* (11.0 sec) foraged significantly slower than *B. huntii* ($P = 0.013$, two-tailed t test) and the unknown *Bombus* spp. ($P = 0.004$), but not *B. ternarius* ($P = 0.060$) and *B. vagans* ($P = 0.059$).

Table 3.7. Average time (seconds) spent on a flower per single visit by various insect visitors to *Lythrum salicaria* at BVR, LKS, and IDY. LM = long-style morph; IM = intermediate-style morph; SM = short-style morph. Statistical tests of significance between floral morphs used One-Way ANOVA, $\alpha = 0.05$. Within a row, means denoted by different letters as a superscript are significantly different ($P < 0.05$). Similarly, non-bold superscripted letters in the flower visitor column (LM/IM/SM) denote significantly different quantities of pollen tubes between individual species ($P < 0.05$). Bold superscripted letters in the flower visitor column (LM/IM/SM) denote significant differences between pollen-tube means for the four major visitor groups.

		LM		**IM**		**SM**	
Flower visitor	**Taxonomic family**	**No. visits**	**mean ± s.e.**	**No. visits**	**mean ± s.e.**	**No. visits**	**mean ± s.e.**
Anthophora furcata	Apidae	1	2.9				
Apis mellifera$^{A/A/A;\ \mathbf{A/B/B}}$	Apidae	23	10.7 ± 1.1^{A}	14	9.9 ± 1.9^{A}	7	15.7 ± 2.8^{A}
Bombus borealis$^{-/-/B}$	Apidae	1	14.3			3	2.3 ± 0.3
Bombus centralis$^{AC/-/-}$	Apidae	2	11.0 ± 2.0	1	2.8		
Bombus huntii$^{B/A/C}$	Apidae	6	4.7 ± 0.8^{A}	4	10.2 ± 5.0^{A}	5	4.6 ± 0.6^{A}
Bombus rufocinctus	Apidae	1	4.3	1	8.4		
Bombus ternarius$^{BC/A/A}$	Apidae	10	5.9 ± 1.0^{A}	11	6.3 ± 1.0^{A}	2	6.2 ± 0.7^{A}
Bombus terricola$^{-/-/AB}$	Apidae					2	7.7 ± 6.3
Bombus vagans$^{BC/A/AB}$	Apidae	28	5.8 ± 0.7^{A}	13	7.5 ± 2.2^{A}	2	6.8 ± 3.2^{A}
Unknown *Bombus* spp.$^{B/A/AB}$	Apidae	12	5.5 ± 0.6^{A}	8	3.6 ± 0.5^{A}	2	4.7 ± 1.3^{A}
All *Bombus* combined$^{\mathbf{A/A/A}}$	Apidae	60	6.0 ± 0.4^{A}	38	6.5 ± 1.0^{A}	16	5.1 ± 0.8^{A}
Lasioglossum laevissimum	Halictidae	3	35.9 ± 14.3^{A}	3	49.8 ± 31.1^{A}	1	67
Lasioglossum zephyrum	Halictidae			1	7.09		
Unknown *Lasioglossum* spp.	Halictidae	6	57.8 ± 18.1^{A}	6	54.1 ± 16.7^{A}	5	47.2 ± 12.6^{A}
All *Lasioglossum* combined$^{\mathbf{B/C/BC}}$	Halictidae	9	50.5 ± 12.9^{A}	10	48.1 ± 13.3^{A}	6	50.5 ± 10.8^{A}
Megachile latimans	Megachilidae					2	4.7 ± 1.4
Megachile sp.	Megachilidae	1	1				
Vespidae				1	8.6	2	2.2 ± 1.2
Eupeodes americanus	Syrphidae	3	5.3 ± 1.2				
Heringia sp.	Syrphidae	1	62				
Sphaerophoria contigua	Syrphidae			1	87.8		
S. philanthus OR *asymmetrica*	Syrphidae	1	47.5	1	10		
Syritta pipiens	Syrphidae			2	75 ± 45		
Syrphus vitripennis	Syrphidae	1	52.2				
Toxomerus marginatus	Syrphidae	3	54.1 ± 25.3	1	63.4	1	96.3
Unknown Syrphidae		1	7.1	2	33.7 ± 31.3	1	27.8
All Syrphidae combined$^{\mathbf{B/C/C}}$		10	31.7 ± 9.9^{A}	7	54.1 ± 16.1^{A}	2	62.1 ± 34.3^{A}
Unknown Diptera		1	68	2	7.4 ± 5.3		
Pieris rapae	Pieridae			1	11		
Odonata	Suborder: Anisoptera	1	224.9				

Upon visitation to newly-unbagged flowers of SM, honeybees again spent longer average visits (15.7 sec) than certain *Bombus* visitors: *B. borealis* (2.3 sec, P = 0.017, two-tailed t test), *B. huntii* (4.6 sec, P = 0.009), and *B. ternarius* (6.2 sec, P = 0.013), but not *B. vagans* (6.8 sec, P = 0.090). Also, within *Bombus*, *B. borealis* foraged significantly faster than *B. huntii* (P = 0.040) and *B. ternarius* (P = 0.012), but not *B. vagans* (P = 0.103).

When the data for duration of floral visit to virgin flowers of *L. salicaria* were combined for each of the four most frequent insect taxa and then compared by a One-Way ANOVA, highly significant differences (P << 0.001) occurred for each floral morph. In LM flowers, average honeybee visits (10.7 sec) lasted as long as the combined *Bombus* (6.0 sec, P = 0.101, two-tailed t test), but significantly shorter than those of combined *Lasioglossum* (50.5 sec, P = 0.003) and all Syrphidae (31.7 sec, P = 0.001). *Bombus* foraged significantly more quickly than all sweat bees together (P << 0.001) and all hover flies (P << 0.001). The latter two taxa visited flowers for lengthy periods that were not significantly different (P = 0.778).

However, for IM flowers, bumblebees together (6.5 sec) foraged more quickly than *Apis mellifera* (9.9 sec, P << 0.001, two-tailed t test), *Lasioglossum* combined (48.1 sec, P << 0.001), and Syrphidae combined (54.1 sec, P << 0.001). Honeybees, on the other hand, spent significantly less time per IM flower than the sweat bees (P << 0.001) and combined hover flies (P = 0.003). The latter two spent long periods on IM flowers, which were not significantly different (P = 0.9).

Finally, for SIVs to SM flowers, when combined, *Bombus* again stayed for significantly shorter periods (5.1 sec) than *Apis mellifera* (15.7 sec, P << 0.001, two-tailed t test), all *Lasioglossum* (50.5 sec, P = 0.009), and Syrphidae combined (62.1 sec, P << 0.001). Again the average duration of each honeybee visit per SM flower was significantly shorter than for the

hoverflies (P = 0.021), but not for the sweat bees (P = 0.585). However, the relatively lengthy visits by sweat bees and hover flies did not differ (P = 0.117).

3.3.3.5 Correlation between flower visit duration and pollination efficiency

For the insect taxa which made frequent visits to newly-unbagged flowers of *L. salicaria* in this study, a relationship between pollination effectiveness (i.e., quantity of pollen tubes per style base) and the duration of floral visit was sought by regression analysis for each floral morph, where possible (Figures 3.6 – 3.12).

As a generalization, this study of SIVs demonstrated that pollination efficiency was not correlated with duration of visit for these most frequent pollinators of *L. salicaria*. For the honeybee (*Apis mellifera*), for instance, no correlation between number of pollen tubes and visit duration to LM flowers (P = 0.953) existed (Figure 3.6). For IM flowers, there was a trend toward a positive correlation (P = 0.059), but no statistically significant relationship existed either for SM flowers (P = 0.470) nor when all three morphs (LM, IM, SM) were considered together (AM; P = 0.100) (Figure 3.6).

For all floral morphs (LM, IM, SM, and even when combined (i.e., AM)) visited by *Bombus huntii*, a positive correlation between pollen-tube quantities per style base and visit duration occurred (Figure 3.7). However, this relationship was only significant for IM flowers ($P << 0.001$), but may be due partially to the relatively few observations available.

For *B. ternarius* (Figure 3.8) and *B. vagans* (Figure 3.9), there were only 2 SM flowers available as SIVs for each species (Table 3.7), and hence regression analyses for that particular morph were not meaningful. However, the SM data were included for the analysis of all morphs (AM) together. For *B. ternarius*, there was no significant correlation between number of pollen

tubes per style and duration of visit for LM, IM or AM (Figure 3.8), although a weak positive correlation occurred for LM (P = 0.094). Likewise, for *B. vagans*, no significant correlation occurred for any floral morph or when all were taken together. It was evident that a large quantity of pollen tubes per style base were attainable even after relatively short visits (e.g., 3 sec), and that for the IM and AM plots, there was a large influence by an outlying, poor-pollinating visit of long duration (Figure 3.9).

When SIV data available for all *Bombus* species were combined, there was no correlation for LM (P = 0.168), IM (P = 0.417) or SM (P = 0.277) flowers separately, and especially not when all morphs were taken together (AM; P = 0.821) (Figure 3.10).

Similarly, no significant correlations existed for the sweat bees (*Lasioglossum* spp.) taken collectively, for any floral morph (LM, P = 0.886; IM, P = 0.736; SM, P = 0.376; AM, P = 0.843) (Figure 3.11).

Finally, as anticipated by their relatively poor performance per SIV (Table 3.4, section 3.3.3.3), no significant correlation was found for the hover flies (Syrphidae) combined, for any floral morph (LM, P = 1.00; IM, P = 0.195), although when all floral morph data were combined (AM), there was a slightly positive trend (P = 0.064) but with a slope of very low magnitude, owing to the large number of non-pollinating visits (Figure 3.12).

Of the 25 plots illustrated in Figures 3.6 – 3.12, 14 regression lines had a positive slope suggesting a general trend for a weak positive correlation between pollination effectiveness and time spent per virgin flower. This trend was most obvious for *B. huntii* (all four regressions had a positive slope) and *A. mellifera* (three of four regressions had a positive slope). However, six of the 25 regression lines were essentially horizontal, indicating the lack of a strong relationship

between pollination effectiveness and visit duration. Finally, five of the 25 regressions resulted in a negative slope, including all three regressions for *B. vagans*.

Accordingly, taken overall, the data for single insect visits to intact, virgin flowers of *L. salicaria* do not support the hypothesis that pollination effectiveness, as measured by pollen-tube quantity at the style base, is correlated with time spent foraging on the flower.

3.4 Discussion and Conclusions

The hand pollination experiments performed throughout the 2006 and 2007 field seasons were used to establish the levels of selfing and of possible illegitimate pollination (i.e., pollen from stamens whose length differed from the style) that could lead to seed set of purple loosestrife in central Saskatchewan. Darwin (1877) noted that illegitimate pollination frequently lead to no seed production whatsoever, however, when seed was produced, resultant plants frequently were irregular, or even sterile. Darwin (1877) also showed that *L. salicaria* did not generate seed when self-fertilised. The findings of Darwin (1877) with regard to self-fertilisation were confirmed by Stout (1922), where in a series of selfing experiments, he frequently found seed set failures. These findings correspond very well with what has been determined here for invasive *L. salicaria* in central Saskatchewan. Although very infrequently (i.e., 2 of 137 flowers), a small number (n = 1, 5) of pollen tubes were recorded at the ovary end of two styles, the vast majority of self- and illegitimately crossed replicates yielded no pollen tubes at the ovary. It was not possible to completely eliminate the potential for smaller insects such as thrips (Thysanoptera) from moving between plants and crawling up the stem into bagged flowers, thus yielding the possibility that pollen tubes detected in the style at the ovary end in self- or illegitimately crossed experimental flowers may have resulted from the unanticipated

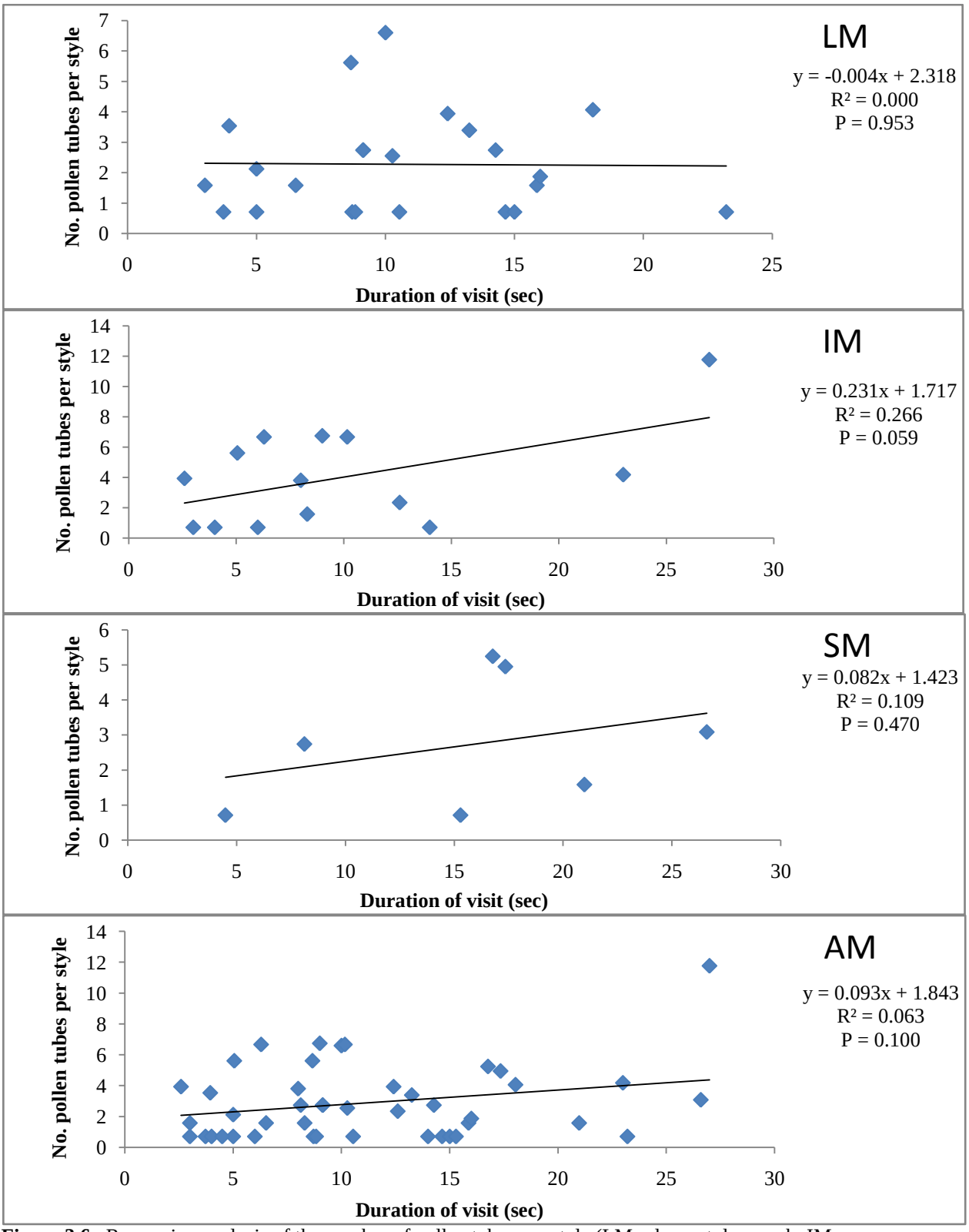

Figure 3.6. Regression analysis of the number of pollen tubes per style (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph; AM = all morphs combined) on duration of visit for *Apis mellifera*. Quantities of pollen tubes per style (y) are shown as transformed values ($\sqrt{y+\frac{1}{2}}$).

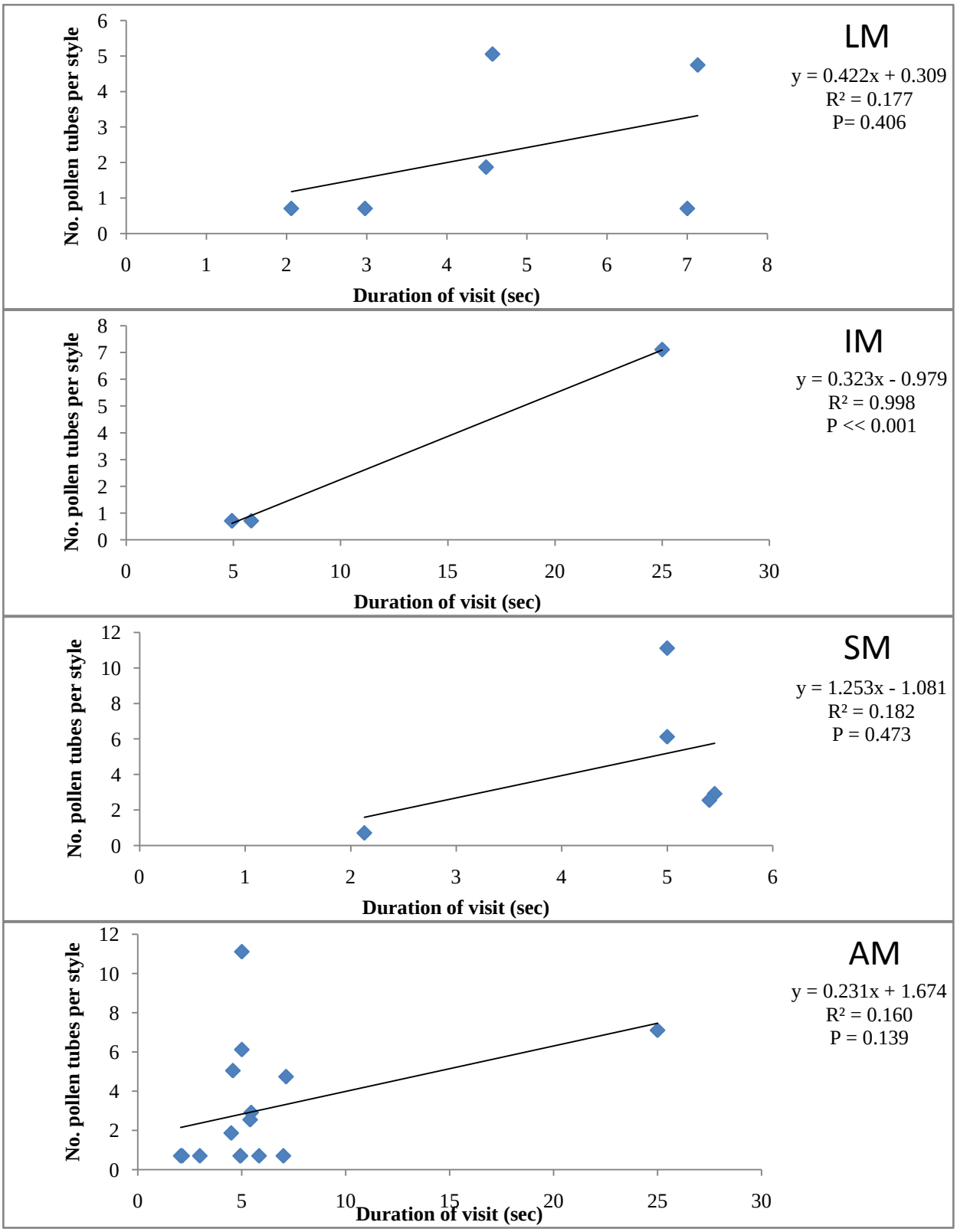

Figure 3.7. Regression analysis of the number of pollen tubes per style (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph; AM = all morphs combined) on duration of visit for *Bombus huntii*. Quantities of pollen tubes per style (y) are shown as transformed values ($\sqrt{y+\frac{1}{2}}$).

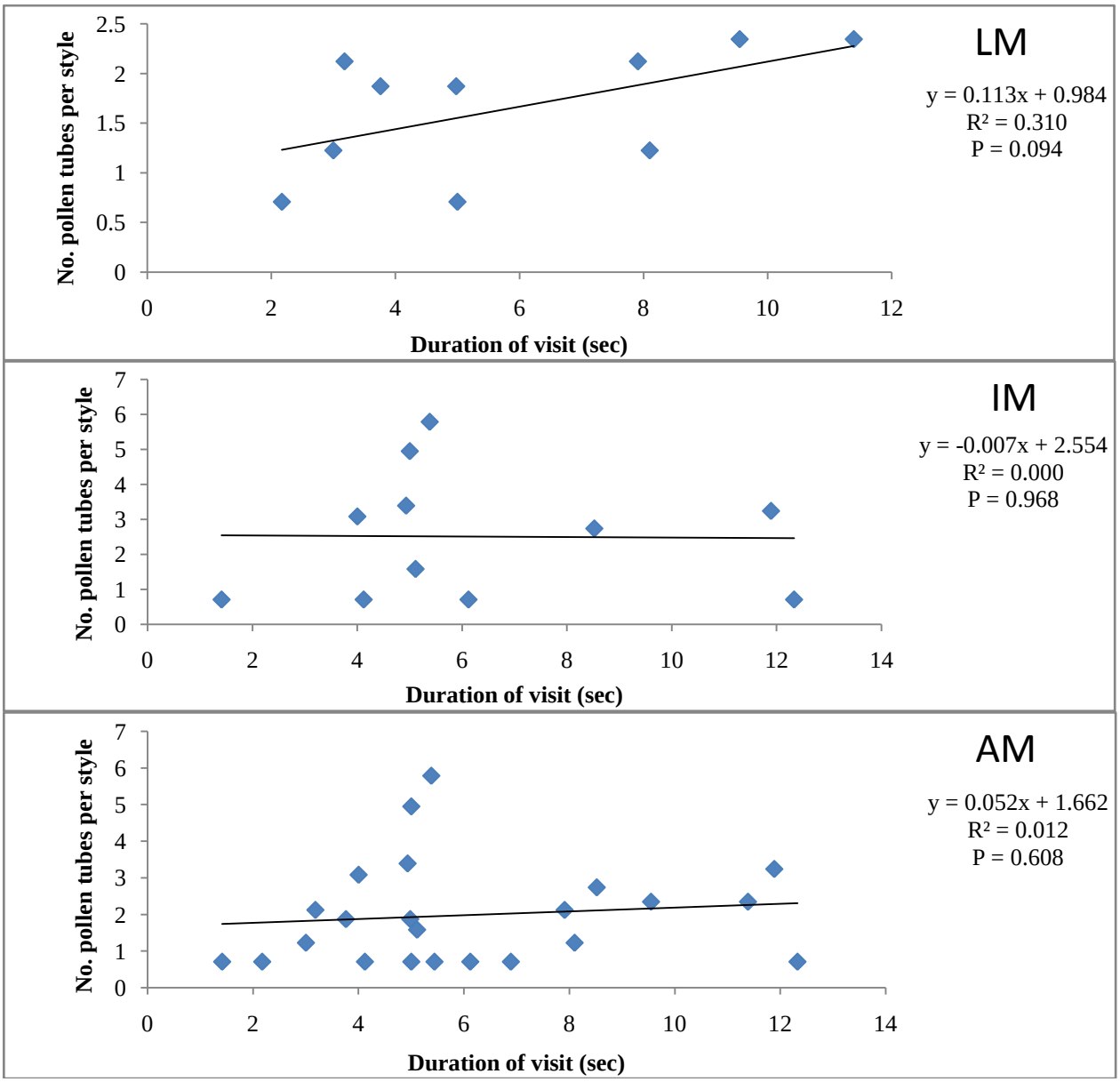

Figure 3.8. Regression analysis of the number of pollen tubes per style (LM = long-style morph; IM = intermediate-style morph; AM = all morphs combined) on duration of visit for *Bombus ternarius*. Quantities of pollen tubes per style (*y*) are shown as transformed values.

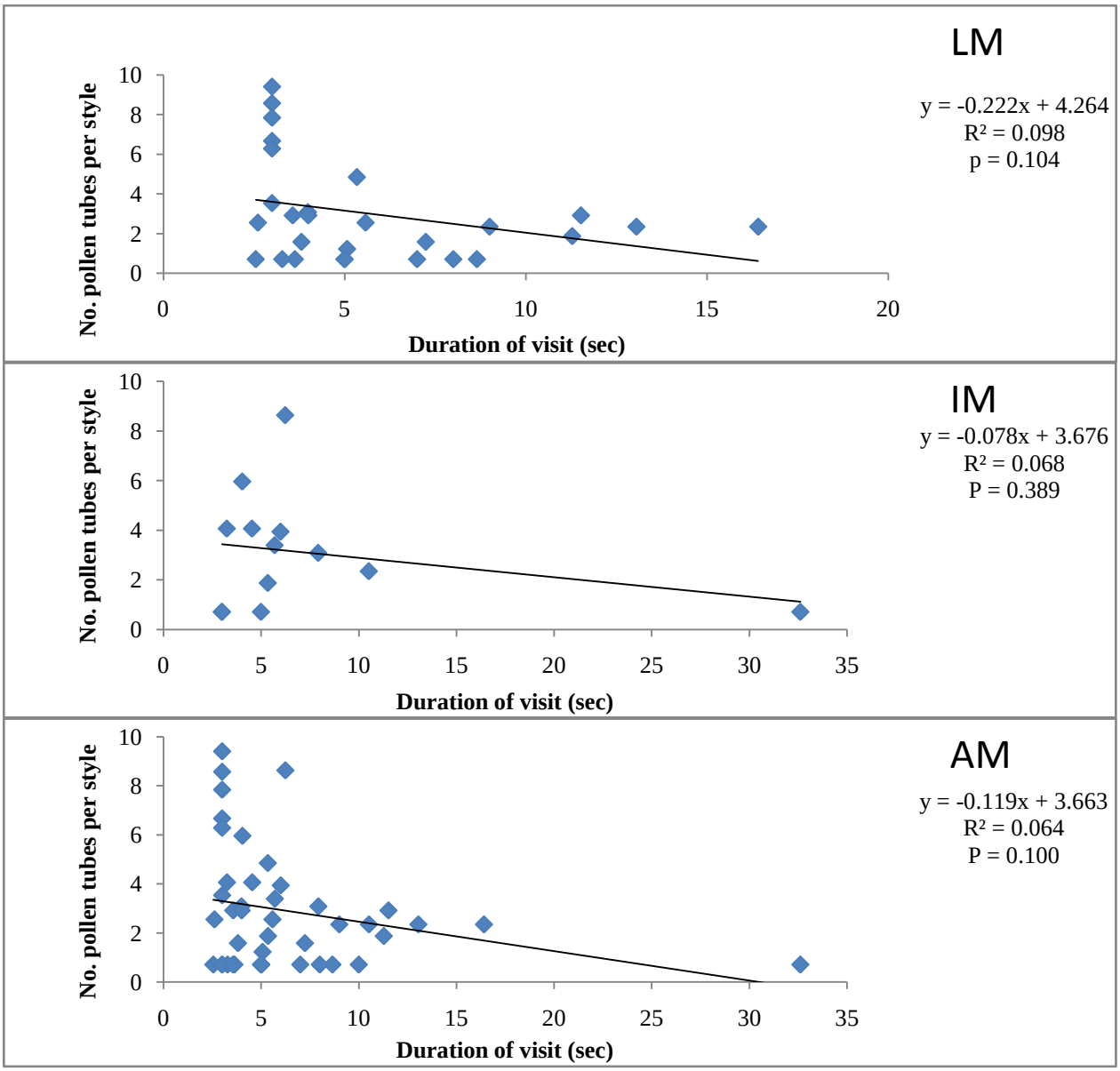

Figure 3.9. Regression analysis of the number of pollen tubes per style (LM = long-style morph; IM = intermediate-style morph; AM = all morphs combined) on duration of visit for *Bombus vagans*. Quantities of pollen tubes per style (y) are shown as transformed values ($\sqrt{y+\frac{1}{2}}$).

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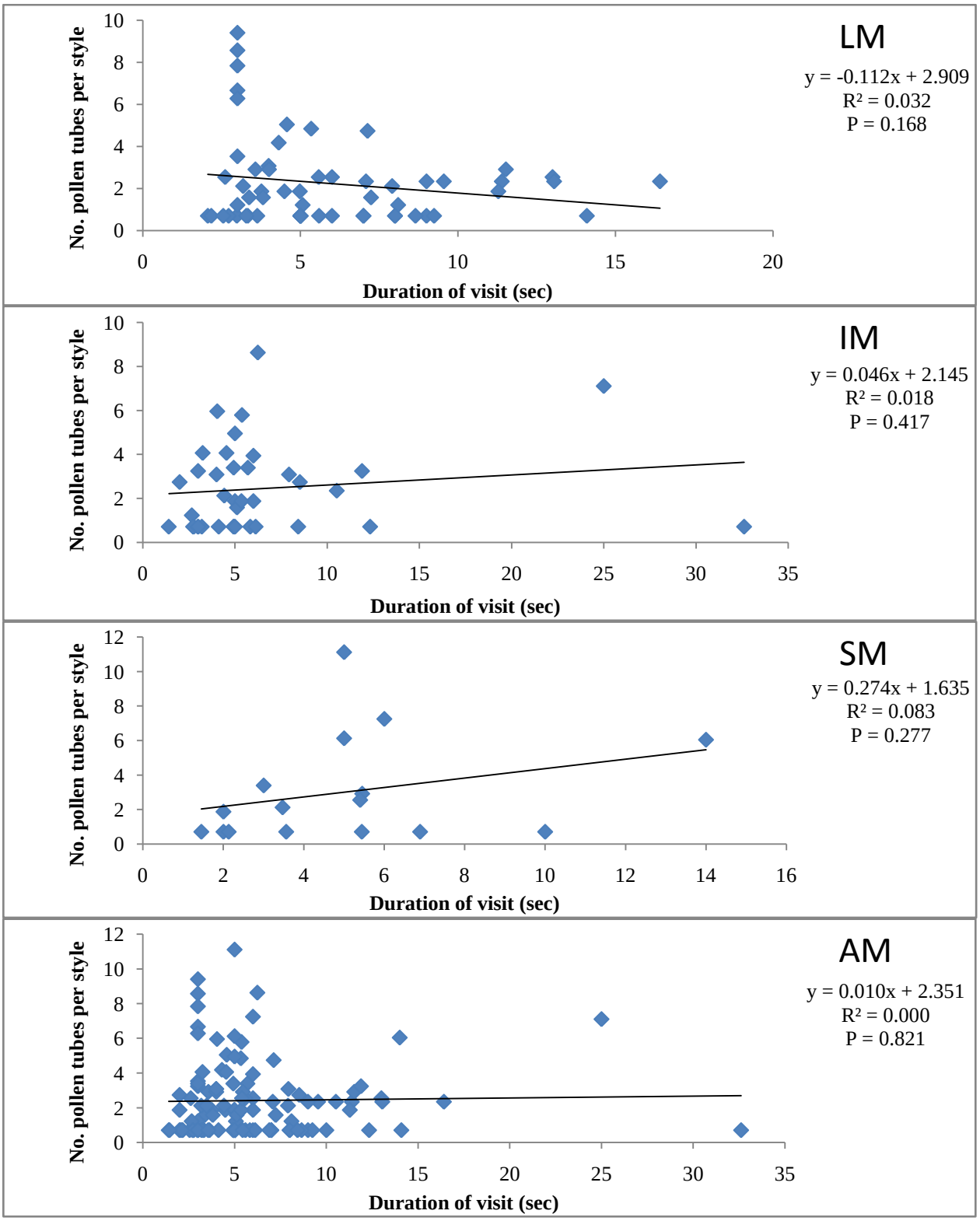

Figure 3.10. Regression analysis of the number of pollen tubes per style (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph; AM = all morphs combined) on duration of visit for *Bombus* spp. combined. Quantities of pollen tubes per style (y) are shown as transformed values ($\sqrt{y+\frac{1}{2}}$).

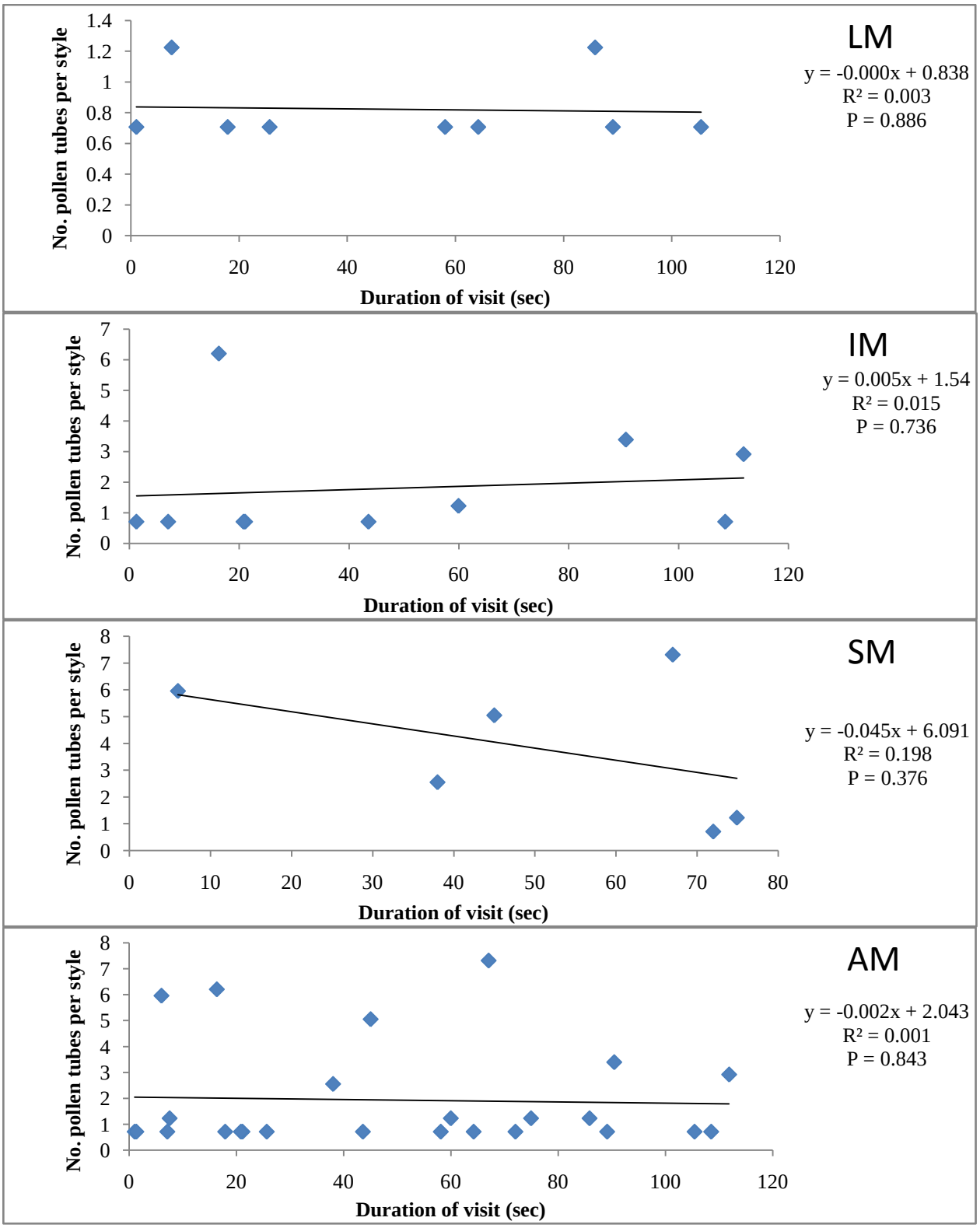

Figure 3.11. Regression analysis of the number of pollen tubes per style (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph; AM = all morphs combined) on duration of visit for all *Lasioglossum* (Halictidae) combined. Quantities of pollen tubes per style (y) are shown as transformed values ($\sqrt{y+\frac{1}{2}}$).

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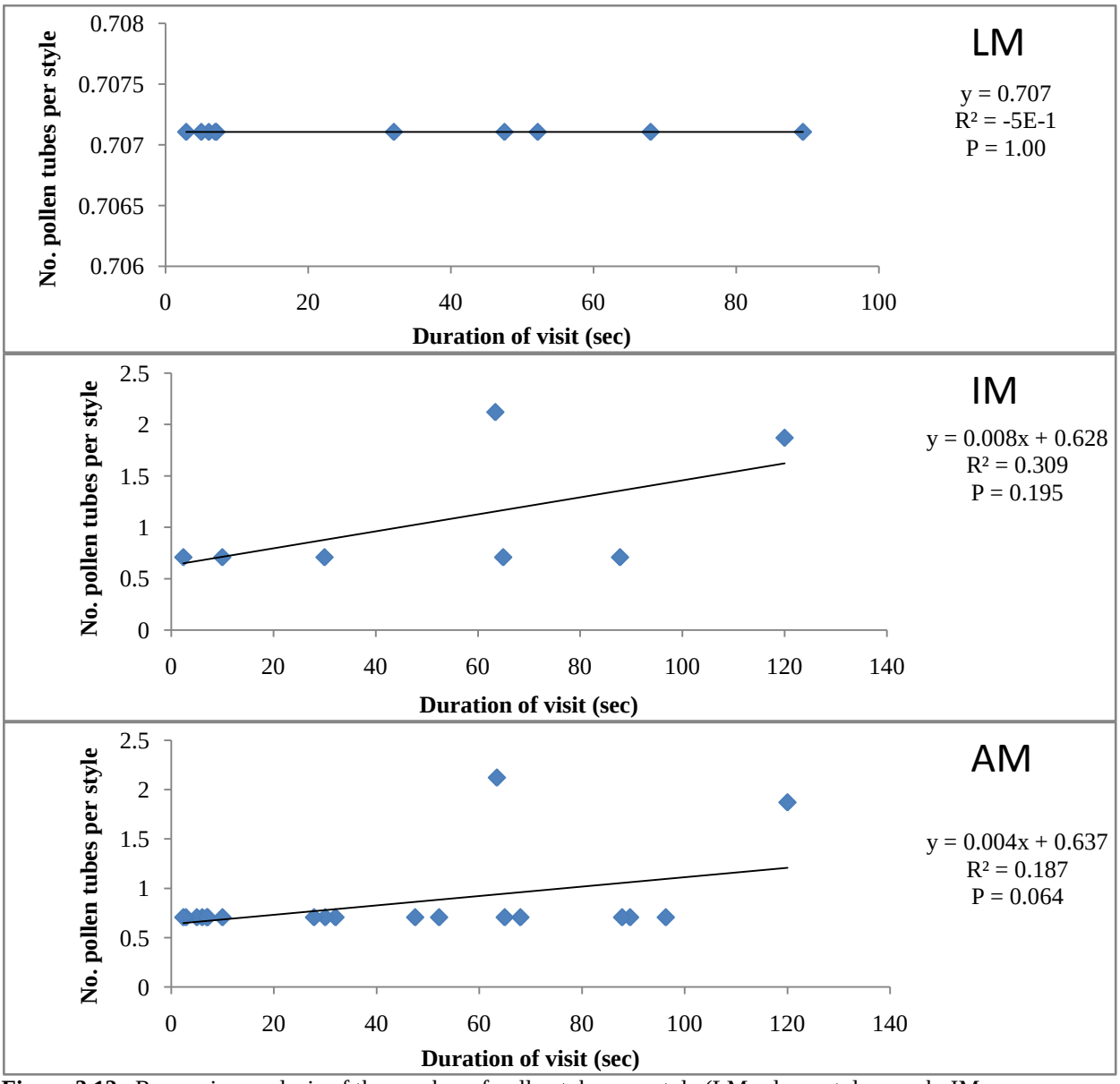

Figure 3.12. Regression analysis of the number of pollen tubes per style (LM = long-style morph; IM = intermediate-style morph; SM = short-style morph; AM = all morphs combined) on duration of visit for all hover flies (Syrphidae) combined. Quantities of pollen tubes per style (y) are shown as transformed values ($\sqrt{y+\frac{1}{2}}$).

introduction of some legitimate pollen. Furthermore, the consistently successful pollination that resulted from hand crosses involving legitimate pollen (i.e., from stamens taken from other plants, in which stamen length matched style height) completes this story in central Saskatchewan, and indicates that *L. salicaria* in this area is strongly xenogamous, as opposed to the facultative autogamy based upon the pollen-ovule ratios discussed in section 2.3.6. Our observations on the sexual reproductive behaviour of *L. salicaria* also accord with the suggestion by Barrett (1992) that the heterostylous condition is accompanied by a diallelic incompatibility system which prevents selfing, as well as intramorph fertilisation (see section 1.2.4).

The present study relied heavily on the microscopic detection and quantification of pollen tubes at the style base, as a measure of a flower visitor's effectiveness as a pollinator. The method had proven reliable for other plant species (Davis 1992a) because pollen tubes are preserved in the style's transmitting tract as a past record of tube elongation which transports non-flagellated sperm cells from the germinated pollen grains on the stigma, to ovules located within the flower's ovary. In this study of *L. salicaria*, it was determined that pollen tubes arising following illegitimate pollination frequently showed aberrant growth patterns when compared with tube growth involving legitimate pollen. Very often, the former did not even grow into the style, terminating growth on the stigma. Those pollen tubes following illegitimate pollination that did grow into the style almost always lacked discrete callose plugs (Figures 3.1C, E), and grew only a very short distance, terminating at the top of the style. Legitimate pollen tubes tended to grow down the style into the transmitting tract in relatively large bundles or aggregations of pollen tubes that appeared morphologically similar to one another (Figure 3.1D, F). Regularly spaced callose plugs were visible throughout the length of pollen tubes arising from legitimate pollination. Pollen tubes that grew down to the base of the style where it

connects the ovary always possessed callose plugs. This disparity of pollen tube growth patterns following illegitimate versus legitimate pollination allowed the assumption that pollen tubes detected at the base of the style arose from the latter cross. Although there are apparently few previous studies describing pollen tube growth characteristics in *L. salicaria*, Scribailo and Barrett (1991) did describe pollen tube characteristics in another tristylous species, *Pontederia sagittata* (Pontederiaceae). With legitimate pollination, pollen tube growth in *P. sagittata* is similar to that of *L. salicaria* in that the tubes also tend to grow down the transmitting tract of the style in discrete groups. However, even in *P. sagittata* tubes emanating from legitimate pollen demonstrated abnormal growth habits, with regard to their failure to grow down the style, plus their swollen nature, absence of callose plugs, and an increase in particulate callose causing the entire pollen tube to fluoresce (Scribailo and Barrett 1991). Abnormal pollen tube characteristics like those described for legitimate pollination, such as tubes terminating growth early in the style of *P. sagittata,* match well with the abnormal characteristics observed for illegitimate pollen tubes in this study on *L. salicaria*. Illegitimate pollen tubes in *P. sagittata*, though terminating growth before the ovary, did possess callose plugs at regular intervals, and did not display more abnormal growth than legitimate pollen tubes (Scribailo and Barrett 1991).

In this study of the pollination biology of *Lythrum salicaria* in central Saskatchewan, bees were, by far, the predominant flower-visiting taxon. Bees represented 95.2% of all flower visitors in the 2007 census data, and 87.5% (2006 – 90%; 2007 – 85%) of all insects that participated as visitors to previously-bagged (virgin) flowers.

The diversity of various floral visitors to wild stands of *L. salicaria* in central Saskatchewan reflects very closely the previous observations made both in North America, and in its native Europe. In Manitoba, Diehl et al. (1997) observed that honey bees, bumble bees,

and leafcutter bees were common visitors to flowers of *L. salicaria*, and that hover flies and butterflies also frequently visited flowers in that province. Honey bees, bumble bees, and leafcutter bees also represent important flower visitors of *L. salicaria* in its native European habitats (Waites and Ågren 2004). Moreover, other bees newly recorded in the present study as visitors to *L. salicaria* include at least two species of sweat bee (*Lasioglossum*; Halictidae) and *Anthophora furcata* (Apidae), plus other insects like yellow-jacket wasps and a dragonfly.

Whereas confirmation of similarities in the flower-visiting insect taxa of invasive *L. salicaria* in Saskatchewan is important, the current study is unlike any previous study of purple loosestrife in that it goes beyond strictly recording observations of flower-visiting insects, to quantitatively discriminate mere flower visitors from pollinators and to rank these pollinators according to their effectiveness in terms of pollination efficiency of specific insect taxa. This study has demonstrated that honey bees and bumble bees are important pollinators of *L. salicaria* in Saskatchewan. Furthermore, Halictidae also seem to be able to effect pollination quite well, especially in IM and SM flowers. The lack of effective pollination to LM flowers by sweat bees may be a function of their tendency to collect pollen alone (i.e., not nectar), and their small body size, which allows them to collect pollen without contacting the exserted stigma. Although relatively few visits (n = 3) from leafcutter bees to virgin flowers were obtained, the visits did indicate that leafcutter bees can effectively pollinate *L. salicaria*. In terms of frequent visitors that do not appear to be effective pollinators to *L. salicaria*, it is important to mention the hover flies (Syrphidae). Compared to the bee species, hover flies possess relatively few setae on their bodies, and these hairs are unbranched (Lukoschus 1957, in Davis 1997a) suggesting that pollen adherence is inferior. Even though floral visits by hover flies were more than occasional (9% of

visits), the majority (90%) of 20 visits yielded no pollen tubes at the ovary. Thus, sweat bees (Halictidae) and hoverflies (Syrphidae) were equally inferior pollinators of LM flowers.

Despite the capability of various insect species to pollinate *L. salicaria* as demonstrated here, this study also showed that each visit by a *bona fide* proven pollinator does not necessarily ensure that pollination will actually occur. For example, 32% of SIVs performed by honeybees (*Apis mellifera*) resulted in a lack of pollen tubes at the style base, and for other frequent-visiting pollinators the proportion of SIVs leading to an absence of pollen tubes was even greater (e.g., *Bombus* combined – 42%; *Lasioglossum* combined – 58%). Thus, many insect visits to virgin flowers of purple loosestrife did not yield quantities of stylar pollen tubes any different from the experimentally bagged (control) flowers, which were non-visited. That is, these control flowers routinely possessed no, or very few, pollen tubes. Furthermore, the investigation of never-bagged, intact flowers of unknown age and visitation history (Figure 3.4) also demonstrated a high proportion (51.7%) of styles lacking pollen tubes, even within flowers that potentially had received multiple visits. Accordingly, it would appear that in natural settings, non-experimental flowers of *L. salicaria* may be missed and/or are never pollinated and never set seed, although these seedless, perfect flowers may still have played an important role as pollen donors. Although apparently unavailable from previous studies of tristylous species, these data for *L. salicaria* are comparable to results obtained from a self-compatible invasive species (*Echium plantagineum*; Boraginaceae) in Australia, wherein 34.7% of SIVs by *Apis mellifera* to flowers with receptive stigmas did not yield pollen tubes, 11% (intact) and 15% (emasculated) flowers available to multiple visitation still lacked pollen tubes at the style base, and 84% of permanently-bagged flowers were deficient of pollen tubes (Davis 1992a).

Problems encountered with beetle herbivory at LKS (a site with 28% SM) and the relative lack of insect foraging activity at the smallest site, IDY (50% SM), led to the majority (67.1%) of SIV experiments being conducted at the largest site with the greatest floral visitation by insects (Table 3.1), BVR (only 2.1% SM). Consequently, these circumstances led to a smaller quantity of SIVs to SM flowers than desired. This relative shortage of SM flowers available denied a rigorous comparison between some of the insect taxa foraging on SM flowers. Yet, numbers of SM flowers for SIVs were sufficient to determine that for the most frequent flower visitors (e.g., *Apis mellifera*; *Bombus*, both for individual species as well as when combined), typically there were no differences in pollination effectiveness between the three floral morphs. Also, a chi-squared analysis of floral morph census data weighted according to site utilisation for SIVs, showed overall that insects participating in SIV trials did not exhibit foraging preferences for one floral morph over others, but rather visited SIV flowers in proportion to which floral morph was available and offered to them. In other words, for example, despite being fewer in number, SM flowers were neither sought after, nor avoided, during the SIV experiments.

No correlation between duration of insect visit and pollination efficiency seems to exist for *L. salicaria* in central Saskatchewan. This discovery agrees with a regression analysis of honeybee pollination of *Echium plantagineum* (Davis 1992a). Length of flower visit as a predictor of an insect's pollination effectiveness appears to be more important for plant species with "platform inflorescences" on which insects can walk, and which require only one or two pollen tubes per tiny floret to satiate all ovules, such as the Apiaceae (e.g., caraway – Langenberger 2000) and the Asteraceae (e.g., *Echinacea* – Wist 2005). The lack of a strong correlation in purple loosestrife between an insect's effectiveness and its duration of visit

indicates that in this species, the frequency of stigmatic contact with compatible pollen does not increase appreciably over time spent on the flower. Instead, because stigmas can be touched upon an insect's arrival to a flower, an event which can happen with regularity even within three seconds of landing (Figures 3.6, 3.9, 3.10), it appears that a better predictor of pollination success of campanulate flowers like *L. salicaria* and *E. plantagineum* is the number of visits that a flower receives during its receptive phase, rather than duration of visits (Davis 1992a).

When comparing visit duration among insect types, it is clear that certain taxa spent differing amounts of time on a flower. Bees of the family Apidae visited individual flowers quickly, generally less than 10 seconds per flower, yet frequently showed high efficiency of pollination. Although they shared an equal rapidity for LM flowers, bumblebees (when taken together) foraged on each IM and SM flower more quickly than honeybees. Halcitid bees spent much longer (mean of 50.5 sec on LM and SM) on individual flowers, and although the halictids do show some proficiency at pollination in *L. salicaria*, they were incapable of pollinating many flowers within the same time period. Syrphid flies also spent long periods (averaging from 31.7 sec on LM to 62.1 sec on SM) on flowers of *L. salicaria*, but nonetheless have been demonstrated here to be poor pollinators of this species in Saskatchewan.

That individual insect species, without exception, spent an equal duration on each of *Lythrum's* three floral morphs (Table 3.6) again is interpreted as strong evidence for a lack of discrimination or preference for an individual morph. Moreover, such indiscriminate insect foraging behaviour corroborated the results (Figures 2.4, 2.5) that the three floral morphs produce equal quantities of nectar sugar and hence appear to be equally attractive for food. It does appear, however, that visits by nectar-foraging insects such as bees were most frequent in

the early afternoon (Table 3.1) when average nectar volume per flower was near its peak (Figures 2.4, 2.5).

These findings also seem in agreement with a bee's interplant flight distance being negatively correlated with plant density in purple loosestrife, evidently without regard to an adjacent plant's floral morph type, but nonetheless with potential significance for the distance pollen is transported on the insect's body (Levin and Kerster 1969). Also potentially relevant is the work of Ågren (1996), who observed a significant positive correlation in Sweden between population size, and seed set in *L. salicaria*. Although difficult to directly compare with my results, it may help to explain the less frequent visitation to flowers at the two less dense and smaller field sites, LKS and IDY, than at BVR.

Finally, this study has demonstrated the highly stochastic nature of pollination itself, in which the pollination success of a single insect species can vary dramatically from zero to over 100 pollen tubes per style per visit. It seems remarkable that on two occasions, SIV trials demonstrated that one solitary visit by a honeybee (138 pollen tubes on IM) and by *Bombus huntii* (123 pollen tubes on SM) could result in pollination sufficient to theoretically fertilize all 100-115 ovules per ovary (Table 2.9) within a single flower of *L. salicaria*.

4. DEVELOPMENT, ANATOMY, AND ULTRASTRUCTURE OF THE FLORAL NECTARY OF THE PURPLE LOOSESTRIFE CULTIVAR 'MORDEN GLEAM' (*LYTHRUM VIRGATUM* x *L. ALATUM*)

4.1 Introduction

The angiosperm order Myrtales consists of up to ten families: the Combretaceae, Lythraceae (including Trapaceae, Sonneratáceae, Punicaceae), Melastomataceae, Memecylaceae, Myrtaceae, Onagraceae and Vochysiaceae (Judd et al. 2008). Of these families, evidently only the Combretaceae, Lythraceae, Melasomataceae, Myrtaceae, Onagraceae, and the Trapaceae have records of floral nectary structure.

In the Combretaceae, the floral nectary has a ribbed surface that lines the lower hypanthium of the tubular flowers of *Combretum fruticosum* and *C. lanceolatum*. The nectary surface possesses scattered stomata (Bernardello et al. 1994) which are distinctly elevated in *C. lanceolatum* (Sazima et al. 2001). The latter species possesses "jelly flowers", because their secretory product is a gelatinous pellet (rather than fluid nectar) consumed by perching birds (Sazima et al. 2001).

Within the Lythraceae, floral nectary stomata have been reported in *Lythrum hyssopifolium* and *L. salicaria* (Bonnier 1879), and also in *Cuphea platycentra* and *Cuphea* sp. (Wolff 1924; Imperatori 1906). In *C. graciliflora*, the floral nectary is formed as a "unilateral retroverse disk bulge" upon which stomata are restricted to the apex and may form clusters where two or three stomata are directly adjacent (Vogel 1998).

In the Melastomataceae, most members lack floral nectaries of any kind. However, some members of this family are able to exude nectar through "rupturing" of the phloem supply to the

staminal filaments (Vogel 1997). Tobe et al. (1989) describe one species of Melastomataceae (*Medinilla magnifica*) which exudes nectar from the tip of each of its petals.

In the Myrtaceae, nectary stomata are known from at least 43 species (Beardsell et al. 1989; Carr and Carr 1987, 1990; Davis 1968, 1969, 1997b; Moncur and Boland 1989; Ronse Decraene and Smets 1991). Detailed structural studies, however, have only been carried out in two species of the Myrtaceae (*Chamelaucium uncinatum* - O'Brien et al. 1996; *Thryptomene calycina* - Beardsell et al. 1989), but some data exists on changes in nectar composition and nectary surface features in three species of *Eucalyptus* (Davis 1997b). In *T. calycina*, droplets of nectar originate in the "cup" formed by the floral tube, these droplets arising in areas of many nectary stomata located around the base of the style of this epigynous flower (Beardsell at al. 1989). A region of 2-3 cell layers of densely packed secretory cells covers 5-10 layers of large parenchyma cells that apparently contain phenols or tannins. The only vascular tissue in the region is associated with the style and stamens; thus, the floral nectary lacks a direct vascular supply (Beardsell et al. 1989).

The floral nectary of *Chamelaucium uncinatum* (Myrtaceae), evidently the only nectar-secreting gland in the Myrtales to have been studied ultrastructurally, is located on the hypanthium plus the entire upper surface of the ovary (O'Brien et al. 1996). The nectary consists of two distinct zones: the secretory zone lying immediately beneath the epidermis, and the subglandular zone located between the secretory zone and the vascular tissue. The epidermal surface is covered with a thick cuticle broken only at sites of stomatal openings. The stomata are uniformly spaced; each stomate is surrounded by a cluster of secretory cells. The epidermal cells contain a very large central vacuole, and are described as lacking organelles. The nectariferous cells of secreting nectaries contain many organelles within granular cytoplasm including

dictyosomes mitochondria and several forms of endoplasmic reticulum. Starch grains in plastids was uncommon. Many vacuoles could be present in these cells, some surrounded by thin membranes only, while others have a thick layer of electron dense material lining the tonoplast. Plasmodesmata are common between many cells, although not all cells appear to possess them. Plastids and other unidentified organelles are also present within the secretory cells of the nectary. Each subglandular and secretory cell contain a single large vacuole which expands with flower age, wherein few organelles remain in the cytoplasm even though cell walls remain intact.

In flowers of the Onagraceae, nectary stomata are known from at least 18 species (listed in Davis 1992b). Nectary stomata in the Trapaceae have been noted in only one species, *Trapa natans* (Caspary 1848). However, for both these families, evidently no detailed work on nectary structure has been undertaken.

Within the Lythraceae and the entire Myrtales, much remains to be investigated in terms of floral nectary structure and ultrastructure in this diverse group of plants. The floral nectary of *Lythrum* appears to have received only passing attention in previous studies. Darwin (1877) reported that nectar in *L. salicaria* is exuded all around the base of the ovary. Fahn (1979) described the nectary in the Lythraceae as a disk surrounding the ovary base, but Kurr (1833) had indicated earlier that the floral nectary of *L. salicaria* was a concave "gutter" located between the calyx tube and the gynoecium. Bonnier (1879) reported stomata on the surface of the floral nectary of *L. hyssopifolium* and *L. salicaria*. Other than a description of the presence of nectary stomata, and some brief references to the location and morphology of the floral nectary, no detailed structural or ultrastructural study on the floral nectaries of any *Lythrum* species is known.

Accordingly, this chapter is devoted to a study of the development, anatomy, and ultrastructure of an "environmentally friendly", non-invasive form of purple loosestrife, namely the 'Morden Gleam' cultivar of *Lythrum virgatum* x *L. alatum*. This transplanted material, kindly donated from her garden by J. Smith, Department of Biology, University of Saskatchewan, became available well before the arrival of the first field season involving *L. salicaria*. Consequently, this cultivar was utilized to study nectary ultrastructure in the Lythraceae, and marks only the second such investigation (following that of *Chamelaucium uncinatum* – Myrtaceae; O'Brien et al. 1996) that exists from the order Myrtales.

4.2 Materials and Methods

4.2.1 Plant material

Plant material used represented multiple cuttings taken from a single stem of the purple loosestrife cultivar 'Morden Gleam' (*Lythrum virgatum* x *L. alatum*) which consists of the intermediate-style floral morph, only. These propagated cuttings developed adventitious roots and were grown, one cutting per 20 cm pot, in soil (Sunshine Mix, Sun Gro Horticulture, Seba Beach, AB, Canada) in a growth chamber set at 14 hours light (20° C): 10 hours dark (16° C). Light intensity was 5650 Lux, from 22 Sylvania 'Cool White' fluorescent tubes.

4.2.2 Scanning electron microscopy

Newly-opened flowers (first day of anthesis) were removed with forceps prior to dissection in the lab. The processing and fixation of tissues, and the procedure for imaging tissues with the SEM, were done using the protocols described in sections 2.2.4.1 and 2.2.4.2.

4.2.3 Light and transmission electron microscopy

4.2.3.1 Tissue selection and fixation

Three stages of flower development were chosen to determine aspects of the development of the floral nectary using both light and transmission electron microscopy (TEM): mature unopened flower buds (pre-secretory stage); first-day anthesis (secretory stage); and spent, 3-4 day old, flowers (post-secretory stage).

Fresh flowers of the three stages were dissected to expose the nectary before fixation in 1.5% glutaraldehyde in 25 mM sodium phosphate buffer for 0.5 hours, then transferred to 3% glutaraldehyde in the same buffer for 2 hours. After rinsing with ice-cold buffer over 1-12 hours, samples were post-fixed in 1% OsO_4 overnight, on ice. After post-fixation, samples were rinsed three times with buffer and distilled water before dehydration in a graded ethanol series, including 100% ethanol overnight. Once in 100% ethanol, samples were stored at 0° C until ready to proceed with tissue embedding.

4.2.3.2 Tissue processing and imaging

Gradual substitution of ethanol with propylene oxide was performed prior to infiltration of the tissues with Epon Araldite 502 resin mixture. Embedding was carried out by polymerizing the resin at 60°C for 1-3 days (Wist and Davis 2006). Semi-thin sections for light microscopy were cut at 0.9 µm on a Reichert-Jung Ultracut E ultramicrotome using glass knives and stained with Toluidine Blue O. All light micrographs were taken using a Zeiss photomicroscope with 400 ISO, 35 mm colour film. Film negatives were scanned using an Epson Perfection 1670 Photo scanner. Image editing (contrast, brightness, and cropping) was performed with Adobe Photoshop® CS2.

Ultrathin sections (50-70 nm) of nectary tissues for TEM were cut on a Reichert-Jung Ultracut E ultramicrotome, using either glass knives, or a JUMDI diamond knife. Ultrathin sections were floated onto 200 or 300 mesh copper grids which had been coated with Formvar. Grids were stained for approximately 30 minutes with a 2% solution of uranyl acetate, and then rinsed with distilled water before staining for 10 minutes in Reynolds lead citrate (Reynolds 1963). Grids were left to dry at least 24 hours before observation in either a Philips EM 410 or a Philips CM 10 transmission electron microscope at 60 kV. TEM negatives were scanned using an Epson 3200 Photo scanner, and edited with Adobe Photoshop® CS2.

4.2.3.3 Stereological analysis

Stereological analysis was performed using 10 subepidermal cells from three nectaries each, of the three floral developmental stages: mature, pre-secretory buds; flowers actively secreting nectar at the first day of anthesis, and older post-secretory flowers. Scanned TEM images were re-sized to 15 000x magnification per micrograph (typically one cell each) and printed on Kodak Premium Photo Paper. A grid of 1.5 cm^2 prepared on a plastic overlay was used to calculate total cell, vacuolar, and nuclear area of each cell profile by counting the number of gridline intersection points for each of these features. Moreover, organelles such as dictyosomes, mitochondria, plastids and their starch grains were also quantified per cell profile. Statistical comparisons of the three stages of nectary development were performed using One-Way ANOVA, $\alpha = 0.05$.

4.3 Results

4.3.1 Surface structure of the floral nectary

The nectary surface of the 'Morden Gleam' cultivar was similar to that seen in *L. salicaria* (see section 2.3.7.1). Like in *L. salicaria* the nectary of this *Lythrum* cultivar was located in a depression formed at the interface between the hypanthium and the gynoecium (Figure 4.1A). Nectary stomata were fairly evenly spaced and generally solitary, although stomata were found in adjacent pairs 4.7% of the time (Figures 4.1D, E). In the floral stages examined here, almost all stomata had fully formed pores (Figures 4.1C-E). However, stomatal stages where the overlying cuticle had barely yet torn (Figure 4.1B) to reveal the pre-formed pore, and where a granular occluding material accumulated at the pore margins (Figure 4.1D left), resembling a similar occurrence in *L. salicaria* (Figure 2.3B bottom), also existed. Guard cells commonly possessed circumferential ridges (Figures 4.1B-E). The average number of stomata per nectary surface in the 'Morden Gleam' cultivar was 21.4 ± 0.47 ($n = 11$), significantly higher than all three floral morphs of *L. salicaria* (t tests, all $P \leq 0.006$) (Figure 2.4). The presence of stomata on the nectary surface of this purple loosestrife cultivar suggests that nectar is secreted through these stomatal pores, and gathers as isolated droplets which eventually coalesce in the gutter formed between the hypanthium and the gynoecium. Direct observations of this postulated process are still required.

4.3.2 Anatomy and ultrastructure of the floral nectary

4.3.2.1 Epidermal features

The three developmental stages of the nectary epidermis were examined by light microscopy. At all three stages, a large vacuole was present (Figures 4.2A-C). Amyloplasts

bearing starch grains were also present in pre-secretory, and actively secreting developmental stages (Figures 4.2A, B); however, starch content appeared to be depleted by the post-secretory stage (Figure 4.2C). By TEM, epidermal cells of the floral nectary examined at various stages of development possessed a large central vacuole (Figure 4.3B), though vacuolar contents, as well as cytoplasmic contents, were comparatively electron dense when compared with the subepidermal cells of the floral nectary (Figures 4.3A, B). Epidermal cells possessed many organelles; amyloplasts with multiple starch grains (Figures 4.2A, B; 4.3A), a single nucleus per cell (Figure 4.3B), plus many mitochondria and dictyosomes were frequently observed.

Guard cells of the stomatal openings were subtended by a substomatal space lined by a cuticle (Figure 4.3C), and a group of subepidermal parenchyma cells which remained densely packed with organelles even at advanced stages of nectary development (Figures 4.3C, D). Guard cells possessed thick periclinal cell walls compared to other epidermal and subepidermal cells of the floral nectary, as well as multiple amyloplasts packed with starch grains even at the post-secretory phase (Figures 4.3C, D).

4.3.2.2 Sub-epidermal features

4.3.2.2.1 Pre-secretory stage

Pre-secretory, subepidermal parenchyma cells of the floral nectary possessed many different organelles. Multiple small vacuoles (or a single highly branched vacuole) were present throughout the cytoplasm of cells of this stage (Figures 4.2A; 4.4A-D). A single nucleus was present with rough endoplasmic reticulum often seen in areas around the nucleus and throughout the cell (Figures 4.4A, D). The presence of large amyloplasts, often with many starch grains per plastid (Figures 4.4A, C, D) was a major feature of subepidermal nectary cells at the pre-

secretory stage of development, as were many mitochondria distributed throughout the cytoplasm (Figures 4.4A-D). Dictyosomes

were frequently encountered in cells at this stage of development (Figures 4.4A, B), however, they were not seen in all cell profiles indicating that though present at this stage, their numbers may have been somewhat smaller than other organelles. Other organelles encountered at this stage of nectary development are plasmalemmasomes (Figure 4.4D), and multilamellar bodies (Figure 4.4C). Intercellular spaces are present in the apoplast around subepidermal cells of this stage of development (Figures 4.4A, C, D).

4.3.2.2.2 Secretory stage

Subepidermal cells of actively secreting floral nectaries possessed all of the same organelles as the pre-secretory nectary cells including a nucleus, rough endoplasmic reticulum, vacuome, mitochondria, amyloplasts with starch grains, dictyosomes, plasmalemmasomes, and multilamellar bodies (Figures 4.5A-D). Some changes did occur in these cells between developmental stages. The vacuome, though still often consisting of multiple vacuoles, or being highly branched, tended to be larger (Figures 4.5A, B), and the amyloplasts though often still possessing many starch grains were smaller and possessed starch grains that were smaller in size (Figures 4.5A-D). Many intercellular spaces persist in the apoplast (Figures 4.5A-C).

4.3.2.2.3 Post-secretory stage

In post-secretory, nectariferous cells, there was a substantial change in the size of the vacuome relative to the amount of cytoplasm visible within a cell profile (Figures 4.6A-C). A large central vacuole was present in these cells which relegated cytoplasmic features to a thin

Figure 4.1. SEMs of the floral nectary and nectary stomata in *Lythrum virgatum* x *L. alatum*. (A) SEM of the floral nectary showing its location between the hypanthium (HY) and the gynoecium (GY) of the flower. Nectary stomata (arrowheads) are visible at relatively even spacing along the surface of the nectary. Pollen grain (arrow). Scale bar = 100 µm. (B) Newly maturing nectary stomate with the cuticle just tearing open. Circumferential ridges (arrow) are visible on the guard cells. Scale bar = 10 µm. (C) Mature nectary stomate with fully open pore (P) surrounded by two guard cells (GC). Circumferential ridges (arrow) on guard cells. Scale bar = 10 µm. (D) Paired nectary stomata arranged more or less parallel to one another. Circumferential ridges (arrow) are clear on one stomate and on the other, the start of deposition of an occluding material (arrowhead) is evident. Scale bar = 10 µm. (E) Paired nectary stomata arranged nearly end to end. Circumferential ridges (arrow) are clearly visible on both stomata. Scale bar = 10 µm.

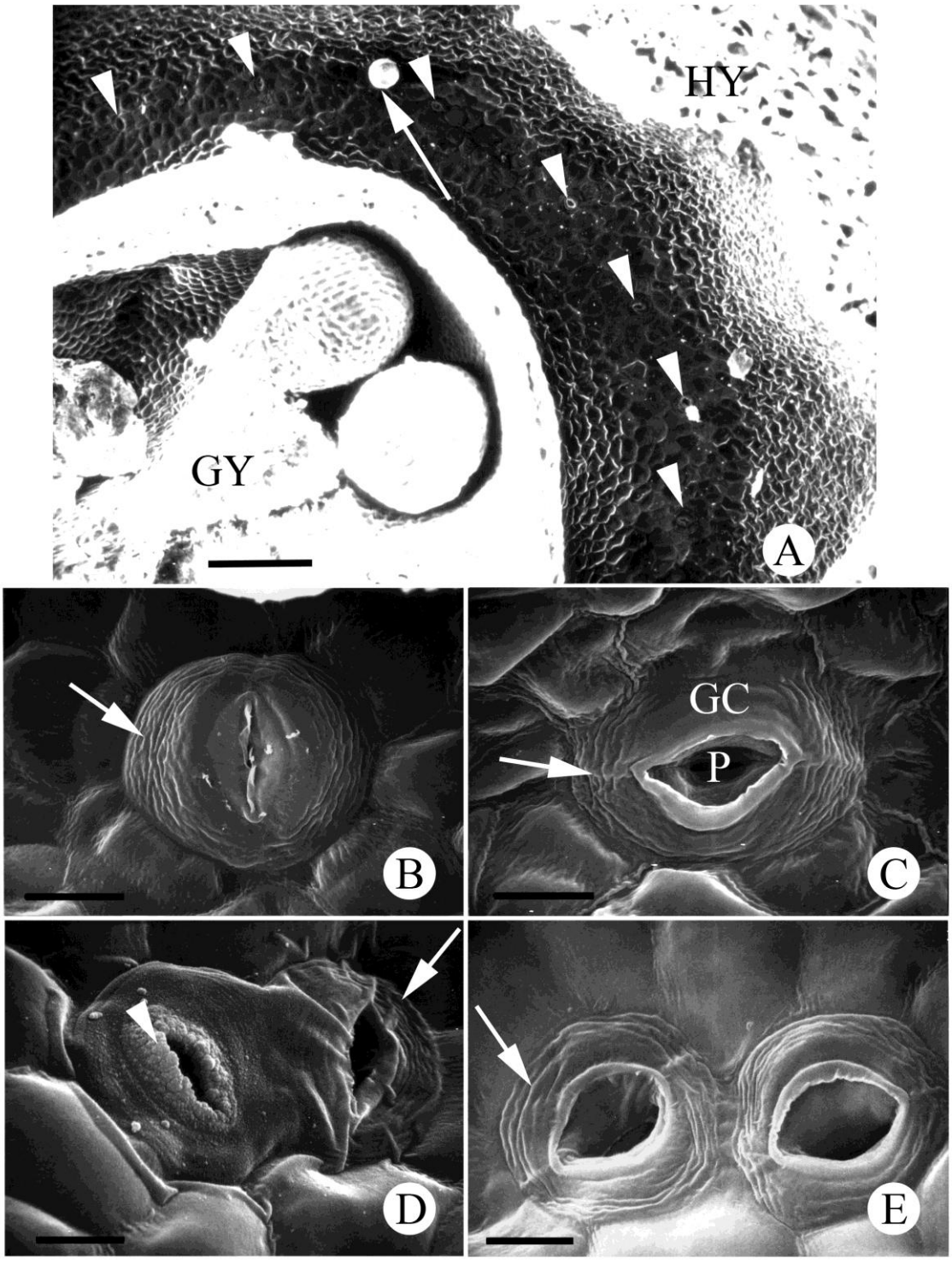
HY
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Figure 4.2. Semi-thin light micrographs of the three stages (pre-secretory, secreting, and post-secretory) of nectary development in *Lythrum virgatum* x *L. alatum*. Sections stained with toluidine blue O. (A) Pre-secretory floral nectary showing the epidermis (E) subtended by several layers of nectary parenchyma cells (NP). Large numbers of starch grains (arrowheads) are present in both epidermal and sub-epidermal cells. Calcium oxalate druses (arrows) are evident in the cell layers immediately under the epidermal layer. Scale bar = 35 µm. (B) Actively secreting floral nectary indicating epidermis (E) and multiple layers of nectary parenchyma cells (NP). Starch grains (arrowheads) and calcium oxalate crystals (arrows) present, though starch content appears reduced. Scale bar = 35 µm. (C) Post-secretory floral nectary with epidermis (E) and several subtending nectary parenchyma (NP) layers. Starch grains (arrowhead) are virtually absent at this stage, however calcium oxalate druses remain (arrows). Scale bar is approximately 35 µm.

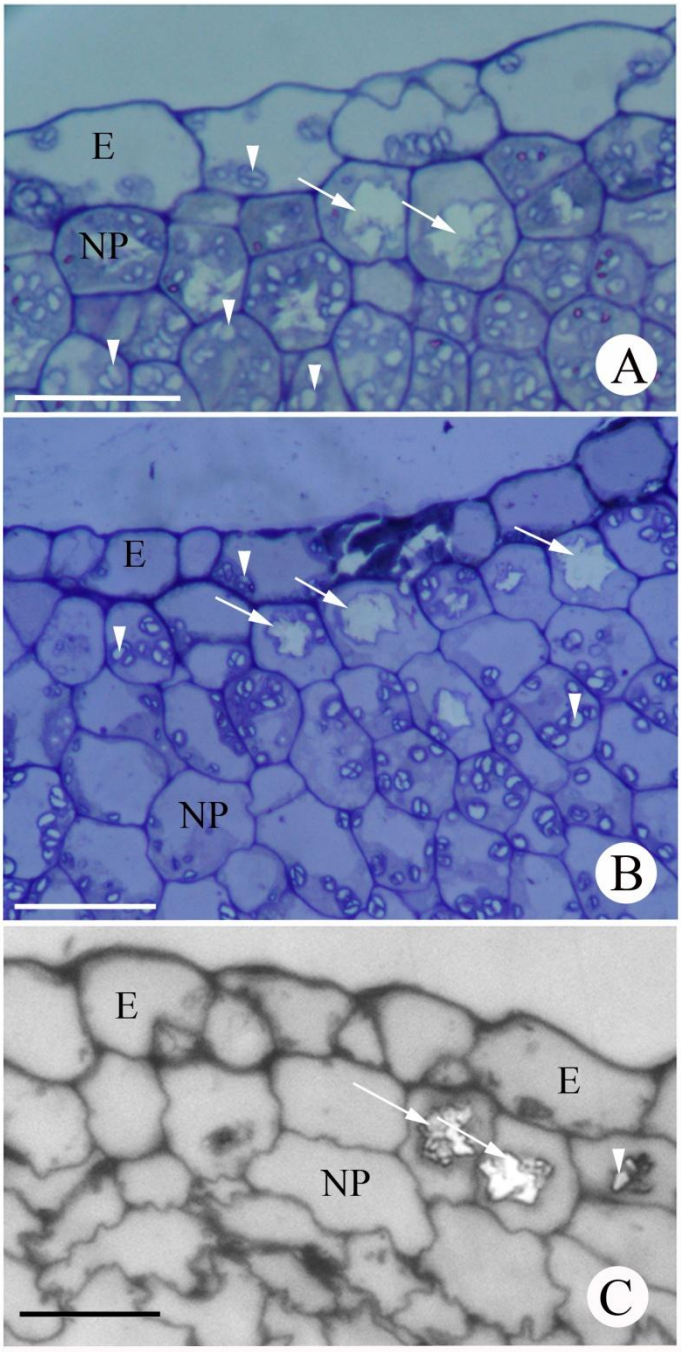
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Figure 4.3. Transmission electron micrographs (TEM) of various epidermal features of the floral nectary of *Lythrum virgatum* x *L. alatum*. (A) Region of a pre-secretory nectary showing contrast in electron density between epidermal cells (above), and the subepidermal parenchyma cells. Within the cytoplasm (CY) of both cell types, are vacuoles (VA) and several plastids (P) containing starch grains. Cuticle (CU) along outer wall of epidermal cells. Scale bar = 5 µm. (B) From a nectar-secreting flower, epidermal cell with electron dense cytoplasm. A large central vacuole (VA) and nucleus (NU) are present. Two plastids with starch grains are also visible on the right side of the cell. Thin uplifted cuticle (CU). Scale bar = 5 µm. (C) Longitudinally-sectioned guard cell with thickened periclinal cell walls (CW) above a substomatal space (SS) lined by thin cuticle, from a post-secretory nectary. Cellular contents of the guard cell are plasmolysed and occupy two distinct areas with organelles present. Highly vacuolated (VA) cells in the epidermis and subepidermal layers are visible. Scale bar = 10 µm. (D) A post-secretory nectary showing a longitudinally-sectioned guard cell with thick inner periclinal cell wall (CW), many starch-bearing plastids and light staining vacuoles, some with granular content. Large vacuoles (VA) with flocculent contents and tonoplasts lined with electron-dense, amorphous deposits occur in two epidermal cells flanking the guard cell. Several nectary parenchyma cells directly below the guard cell maintain a rich organelle content, in contrast to highly-vacuolated cells at bottom left and top right. Scale bar = 10 µm.

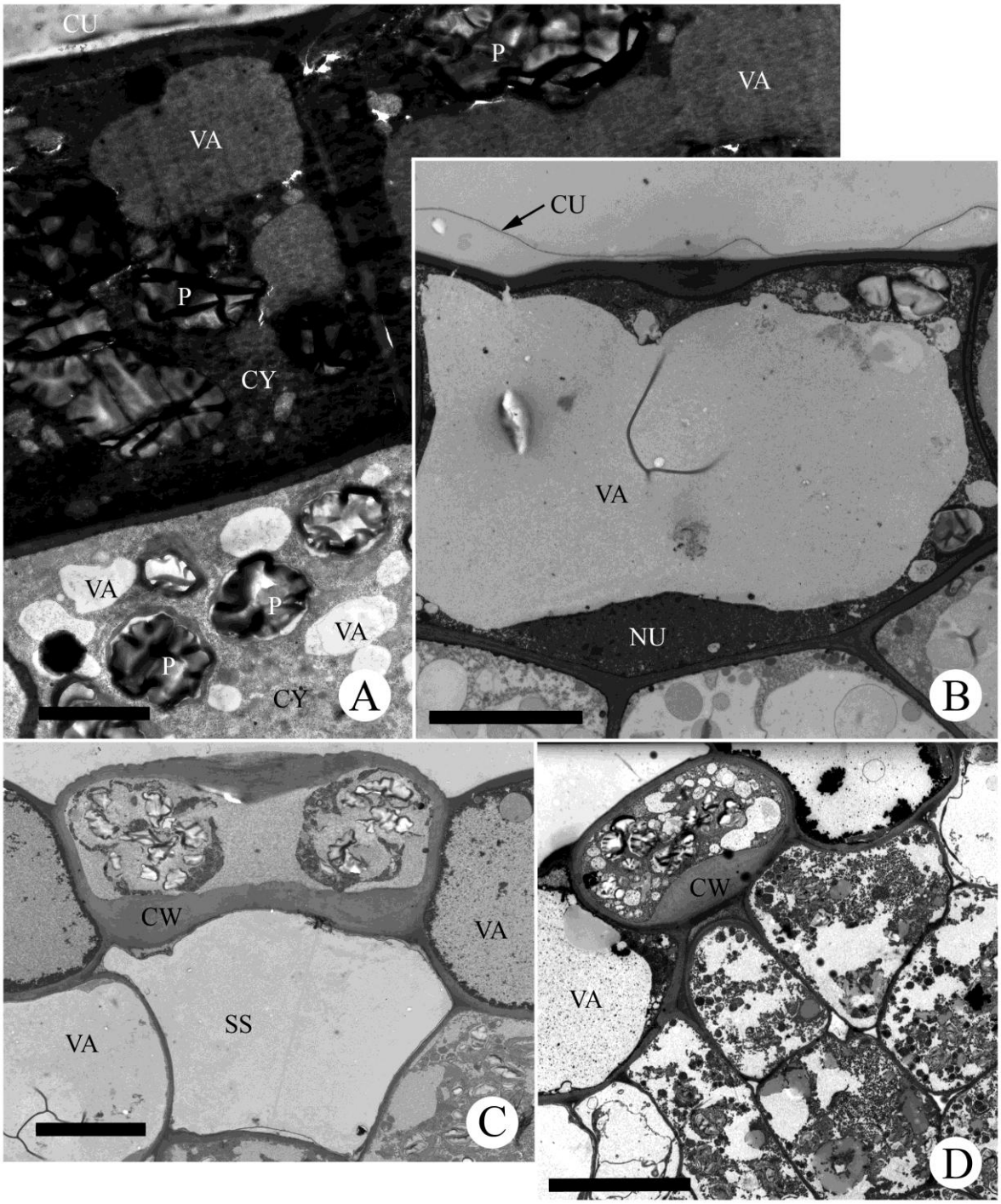
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Figure 4.4. TEMs of parenchyma cells at the pre-secretory stage of nectary development in *Lythrum virgatum* x *L. alatum*. (A) Low magnification TEM of entire nectary parenchyma cell. Multiple small vacuoles (VA) are present in granular cytoplasm that contains a nucleus (NU), several plastids (P) bearing multiple starch grains, several mitochondria (M) and a single dictyosome (DI). Plamodesma (PD). Intercellular spaces (IS) evident at several cell junctions. Scale bar = 5 µm. (B) Parenchyma cell containing a dictyosome (DI), mitochondrion (M) and a small vacuole (VA). Cell walls (CW) surrounding dense deposit of middle lamella. Scale bar = 1 µm. (C) Junction of three cells showing a small intercellular space (IS), plastids (P) possessing starch grains (SG), mitochondria (M), and vacuoles (VA), one of which has a multilamellar body (MB). Scale bar = 1 µm. (D) Three cells situated around an intercellular space (IS); mitochondria (M), vacuoles (VA), a nucleus (NU) and endoplasmic reticulum (ER) can be seen throughout the granular cytoplasm. A plasmalemmasome (PS) is also visible near the right side of the upper cell. Scale bar = 1 µm.

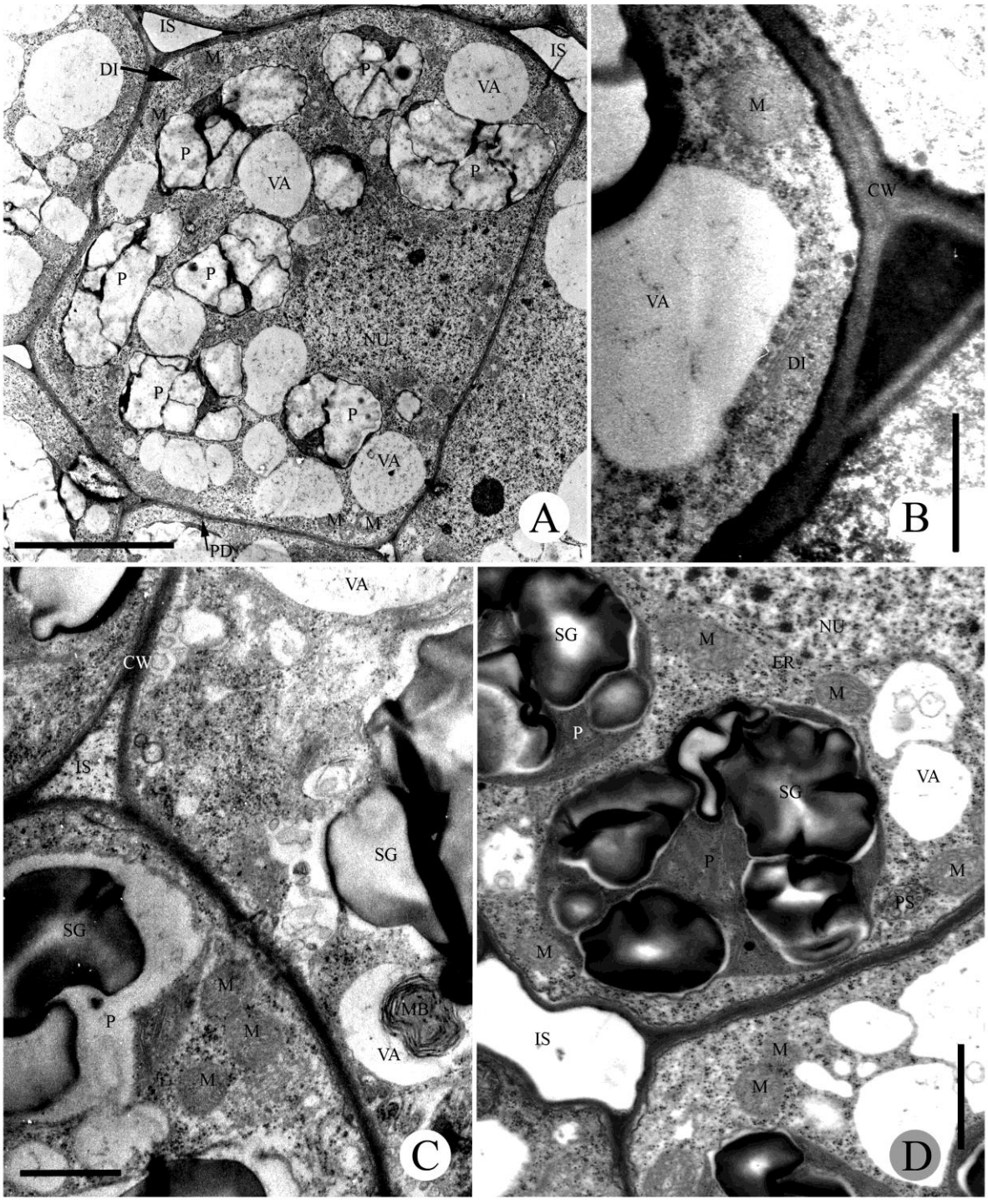
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Figure 4.5. TEMs of parenchyma cells at the secreting stage of nectary development in *Lythrum virgatum* x *L. alatum*. (A) High magnification of cells with thin primary walls (CW) and intercellular spaces (IS). A vacuole (VA), endoplasmic reticulum (ER), several dictyosomes (DI), mitochondria (M), and plastids (P) containing starch grains (SG) are evident. Scale bar = 2 µm. (B) An entire parenchyma cell utilised in the stereological study. Vacuoles (VA), mitochondria (M), a nucleus (NU), and plastids (P) containing starch grains. Intercellular spaces (IS) occur at cell junctions. Scale bar = 5 µm. (C) Adjoining parenchyma cells with large intercellular space (IS) visible. In the lower left cell, a plastid (P) with four starch grains (SG), a plasmalemmasome (PL), as well as several mitochondria (M) can be seen. Various vacuole (VA) profiles are present, one of which contains a multilamellar body (MB). Plasmodesmata (PD) are evident at a glancing section through the cell wall. Scale bar = 2 µm. (D) Several mitochondria (M) can be seen among other organelles such as plastids (P) with starch grains (SG), a vacuole (VA), and a nucleus (NU) with endoplasmic reticulum (ER) located around it. Scale bar = 2 µm.

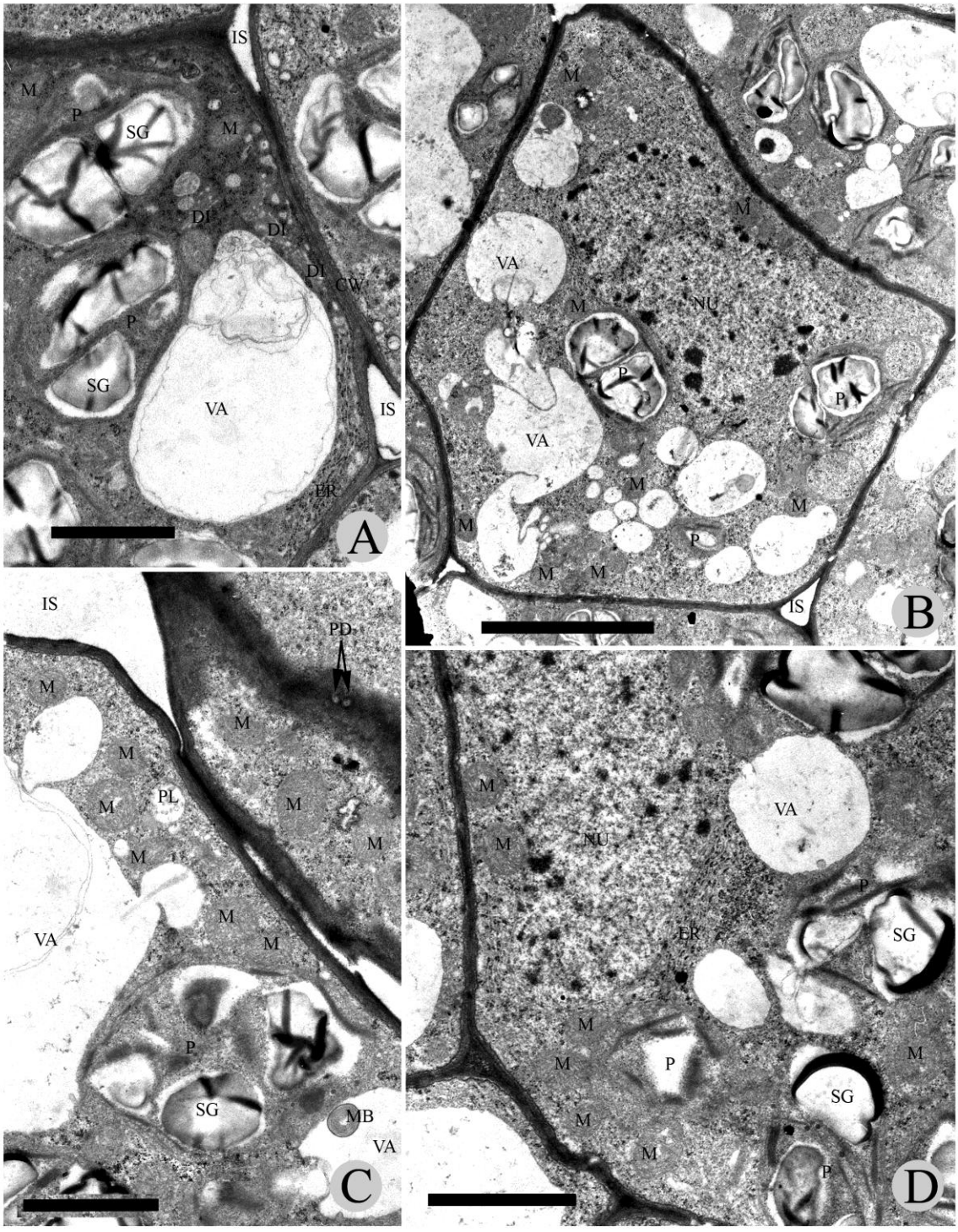
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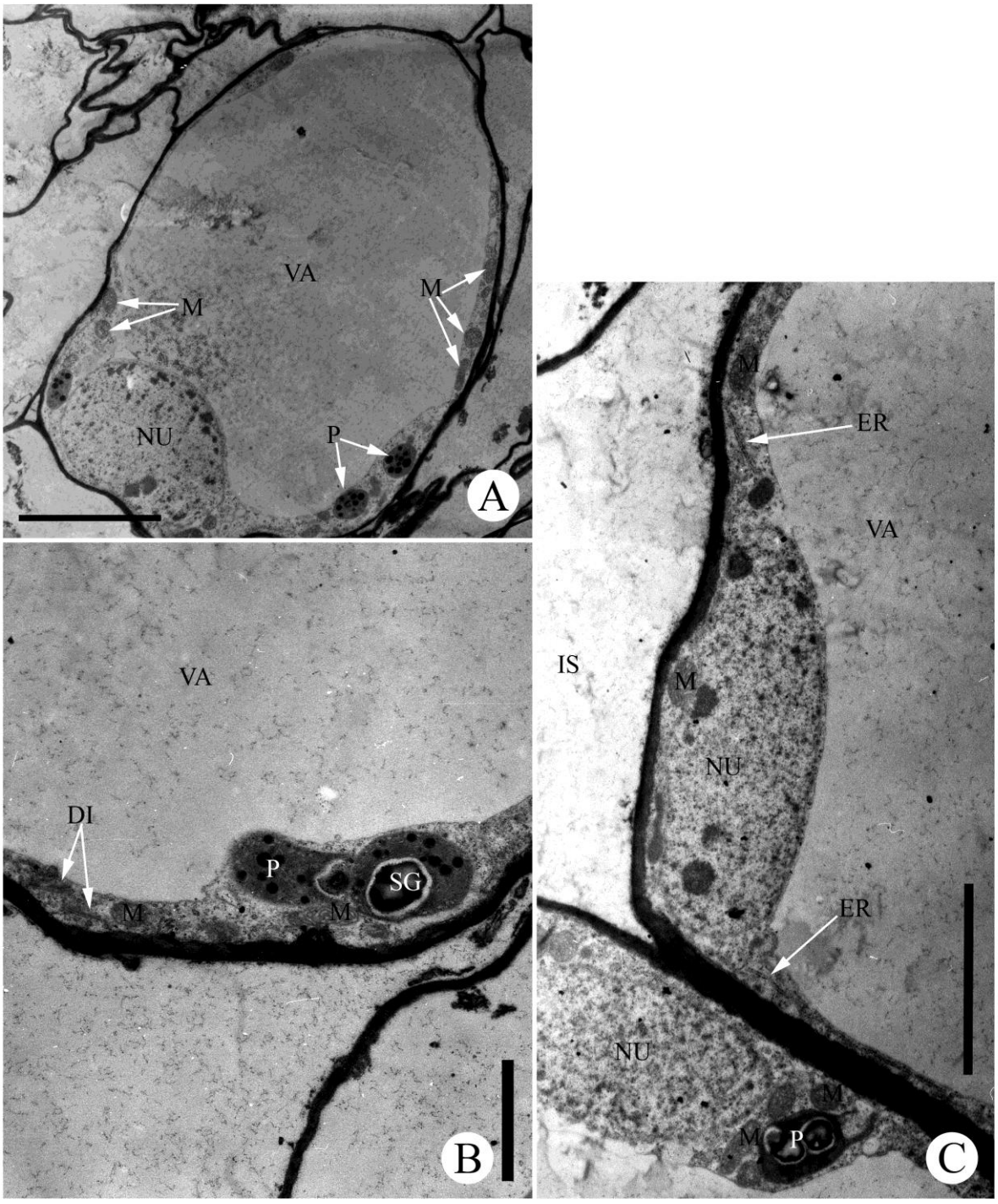
Figure 4.6. TEMs of parenchyma cells of post-secretory nectaries of *Lythrum virgatum* x *L. alatum*. (A) Low magnification micrograph of an entire parenchyma cell with a large central vacuole (VA) relegating cytoplasmic constituents to a thin band at the periphery. Several mitochondria (M) and plastids (P) lacking starch grains can be seen. Nucleus (NU). Several collapsed cells evident at the top left. Scale bar = 10 µm. (B) Parenchyma cell with a large vacuole (VA) containing a flocculent material. Two plastids (P) containing dense plastoglobuli and a small starch grain, mitochondria (M) and dictyosomes (DI) are evident within the thin band of cytoplasm. Two cells at bottom are apparently plasmolysed. Scale bar = 2 µm. (C) Two cells adjacent to a large intercellular space (IS). Both cells possess nuclei (NU) and vacuoles (VA). Endoplasmic reticulum (ER), mitochondria (M), and a plastid (P) with starch grains are evident. Scale bar = 5 µm.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band between the vacuole and the cell walls (Figures 4.6A-C). Mitochondria, dictyosomes, plastids (sometimes with small starch grains), and a nucleus were still present in these cells, as was some rough endoplasmic reticulum (Figures 4.6A-C). Intercellular spaces persist in the apoplast at this advanced stage (Figure 4.6C).

Finally, there was no evidence at any developmental stage to indicate that the nectary of *Lythrum virgatum* x *L. alatum* possessed a direct vascular supply.

4.3.2.3 Stereological analysis

Stereological analysis of various features of the subepidermal parenchyma cells in *Lythrum virgatum* x *L. alatum* demonstrated a number of cellular changes as the floral nectary passed from its pre-secretory through to its post-secretory phase. The total area per cell profile remained unchanged from the pre-secretory to the secretory stage (Table 4.1). However, the total cell area at the post-secretory stage was significantly larger than either the pre-secretory or actively-secreting ones (One-Way ANOVA; $P < 0.001$) (Table 4.1). A significant increase was recorded in the area of the vacuome per cell profile at each progressive stage of nectary development ($P << 0.001$) (Table 4.1). The total area of the nucleus per cell profile also changed significantly as development proceeded. Although there was no significant change in nuclear size from actively-secreting nectary cells to post-secretory cells, nuclear area did decrease in size from pre-secretory to actively-secreting nectary parenchyma cells ($P = 0.001$) (Table 4.1).

In addition to the relative sizes of various cellular components, plastids, mitochondria, and dictyosomes were quantified per unit area of anucleate cytoplasm and starch grains were quantified per plastid per cell profile (Table 4.1). A significant increase in the number of mitochondria per unit area of anucleate cytoplasm occurred between pre-secretory and actively

secreting parenchyma cells (One-Way ANOVA; P = 0.003); however, no significant change in the number of mitochondria was observed between secreting and post-secretory nectaries (Table 4.1). The number of dictyosomes present per unit area of anucleate cytoplasm remained statistically unchanged throughout development of the subepidermal parenchyma cells of the floral nectary (Table 4.1). The number of plastids did not change between the first two stages of development; however, there was a statistically significant increase in the number of plastids per unit area of anucleate cytoplasm present at the post-secretory stage (P << 0.001) (Table 4.1). Finally, the number of starch grains present per plastid decreased significantly at all stages of floral nectary development (P << 0.001) (Table 4.1).

Table 4.1. Characteristics of nectary parenchyma cells in the purple loosestrife cultivar 'Morden Gleam' examined by stereological analysis for three stages of floral nectary development (pre-secretory stage, n = 25; secretory and post-secretory stage, n = 30). Within rows, different superscripted letters denote statistical significance (One-Way ANOVA; $\alpha = 0.05$).

Cellular feature	Developmental stage		
	Pre-secretory	Secretory	Post-secretory
Total area (μm^2) per cell section profile	188.16 ± 8.17^{A}	183.93 ± 9.68^{A}	243.37 ± 12.64^{B}
Vacuolar area (μm^2) per cell section profile	22.16 ± 2.75^{A}	59.60 ± 6.41^{B}	189.77 ± 11.59^{C}
Nuclear area (μm^2) per cell section profile	27.12 ± 2.08^{A}	21.53 ± 1.54^{B}	17.57 ± 1.71^{B}
No. mitochondria*	0.18 ± 0.009^{A}	0.28 ± 0.015^{B}	0.32 ± 0.046^{B}
No. dictyosomes*	0.004 ± 0.001^{A}	0.011 ± 0.004^{A}	0.010 ± 0.005^{A}
No. plastids*	0.059 ± 0.003^{A}	0.058 ± 0.004^{A}	0.126 ± 0.017^{B}
No. starch grains per plastid	2.98 ± 0.18^{C}	1.84 ± 0.17^{B}	0.17 ± 0.11^{A}

* Number of organelles per unit area of anucleate cytoplasm.

4.4 Discussion and Conclusions

Although this study was among the first to examine the structural features of floral nectaries in the Lythraceae, there have been a number of studies that have discussed the presence of nectary stomata in the family, including two species of *Lythrum* (Bonnier 1879) and several species of *Cuphea* (Imperatori 1909; Wolff 1924; Vogel 1998). Stomata on *Lythrum salicaria*

(Bonnier 1879), confirmed in this study as well (see section 2.3.7.1), and now observed on the nectary surface of this *Lythrum* cultivar, suggests that stomata are a common method of floral nectar secretion in the Lythraceae.

Floral nectar secretion in the purple loosestrife cultivar 'Morden Gleam' (*Lythrum virgatum* x *L. alatum*) involves the breakdown of starch stored in subepidermal nectary parenchyma cells before secretion begins. Pre-nectar presumably is exuded to the surface through stomatal pores via a series of apoplastic intercellular canals terminating in substomatal spaces. The lack of vasculature in the floral nectary of this cultivar suggests that starch breakdown products constitute the major source of nectar carbohydrate available, further supported by the significant reduction in cellular starch content as the nectary develops.

There are no previous studies on the fine structure of floral nectaries in the Lythraceae, and only one within the Myrtales. In *Chamelaucium uncinatum* (Myrtaceae), epidermal cells associated with the floral nectary were reported to contain no organelles excepting one large electron translucent vacuole (O'Brien et al. 1996). Although it is unlikely that the epidermal cells contained no organelles in *C. uncinatum,* it does distinguish the floral nectary in this species from that of the *Lythrum* cultivar 'Morden Gleam', in which many cellular organelles were present in epidermal cells throughout development. Nectariferous cells in two-day-old nectaries of *C. uncinatum* possessed multiple small vacuoles, dictyosomes, mitochondria and endoplasmic reticulum (O'Brien et al. 1996), which are features in common with nectariferous cells at all stages of development examined in this study. Where nectary parenchyma cells of *C. uncinatum* do differ from those of *L. virgatum* x *L. alatum* is in the amount of starch present. Although nectary cells of *C. uncinatum* possessed many plastids (O'Brien et al. 1996), starch content of subepidermal parenchyma cells was reported to be very scarce, even at their earliest

developmental stage. On the contrary, *Lythrum* possessed an abundance of starch in early stages. Recognisable organelles were present throughout the development of 'Morden Gleam' nectaries, with all organelles seen at the pre-secretory stage still being present at the post-secretory stage. The vacuome increased dramatically in size at the post-secretory stage, and starch grains disappear or become uncommon. In *C. uncinatum* at 10-16 days post-anthesis, O'Brien et al. (1996) reported that no recognisable organelles remained, and cellular contents were pushed to the edges of the cell by an enlarged central vacuole.

Comparison of nectary development in *Lythrum* with that of the distantly-related species, *Pisum sativum* (Fabaceae), does yield some similarities. In *P. sativum*, Razem and Davis (1999) reported that the total cell area in sub-epidermal nectary cells increased significantly from cells at three days pre-anthesis to cells one day before anthesis. Although this current study did not examine cells of immature flower buds, a significant increase in overall cellular area was recorded from cells of first day anthesis to post-secretory cells. Perhaps a more directly comparable parameter is that of vacuole size. Razem and Davis (1999) reported that there was a significant increase in the size of the vacuome from cells one day prior to anthesis to cells actively secreting nectar. This study also recorded a significant increase in vacuolar area during that developmental period, and went one stage further to note another significant increase in vacuole area during development to post-secretory nectaries. The increase in vacuole size likely corresponds with the consumption of cellular components during the highly metabolic process of nectar secretion. The statistically significant increase in the number of mitochondria present per unit volume of anucleate cytoplasm in *Lythrum* also corresponds with the increase of metabolic activity going from pre-secretory, to actively secreting nectaries (Table 4.1).

The reduction in size of the nucleus from pre-secretory to secreting nectariferous cells is more difficult to explain. The reduction in nuclear area may have been related to the increase in the size of the vacuome; however no significant reduction in nuclear area was recorded from secreting nectary cells to post-secretory cells which corresponds with the most striking increase in vacuolar area. The increase in the number of plastids from secreting to post-secretory cells is difficult to rationalize, especially in light of the distinct decrease in plastid size and the increase in cell size during this same developmental phase. However, if the number of plastids within a given nectary cell are conserved throughout development, the increase in cell size may not have been sufficient to offset the reduction in the area of anucleate cytoplasm caused by the dramatic increase in the size of the vacuole. The increase in the plastid numbers per cell profile at the post-secretory stage may also have been caused by the division of plastids after the secretion phase was complete.

As this study represents only the second ultrastructural analysis of floral nectaries in the order Myrtales, much work remains, in order to build a better general knowledge on nectary development in this diverse group of plants.

5. GENERAL DISCUSSION AND CONCLUSIONS

Purple loosestrife (*Lythrum salicaria*) represents an aggressively invasive threat to wetland habitats of North America. Despite this fact, relatively little is known about certain aspects of it sexual reproductive nature, especially in regard to the ability of the individual insect taxa visiting purple loosestrife in the wild to effect pollination. This study aimed to increase knowledge of the specific effectiveness of various floral visitors as pollinators, as well as to determine specific features of floral morphology and nectar production, both in *L. salicaria* and in a self-sterile cultivar (*L. virgatum* x *L. alatum*), which likely serves as an important floral attractant to potential pollinators.

Due to the tristylous breeding system of *Lythrum salicaria,* aspects of floral morphology and breeding dynamics have been studied fairly rigorously. Work in this field of study conducted in central Saskatchewan has largely verified previous work on this species in other provinces, and other regions of the globe. Tristylous plants differ in their relative lengths of styles and two staminal whorls within the three floral morphs. In Saskatchewan, long-style morph (LM) flowers possessed styles that projected visibly above the other floral organs in the flower. LM flowers possessed intermediate stamens (IS) and short stamens (SS) which also differed in their capacity to produce pollen. SS typically produced more pollen than IS within the LM flowers. Intermediate-style morph (IM) flowers possessed styles that situated the stigma at an intermediate position between long stamens (LS) and SS. As was the case in the LM flowers, SS produced pollen in larger quantities than LS in IM flowers. A short-style morph (SM) flower possessed IS and LS with the stigma located deep within the tube of the hypanthium. Pollen production differentials were typical between the two anther levels in SM flowers as well. This study also determined that pollen production between similar anther levels

in different floral morphs (i.e., IS in both LM and SM flowers) was frequently different. The various floral morphological characteristics determined for this study in Saskatchewan are typical for *L. salicaria* studies elsewhere. Pollen viability for *L. salicaria* in Saskatchewan was very high (all anther levels > 90%).

The number of ovules did not vary between the three floral morphs. The pollen-ovule ratio for all three morphs falls within the range expected for facultative autogamy to facultative xenogamy; however, considerable literature exists regarding reproductive aspects of *L. salicaria*. Tristyly generally represents a very strong self-incompatibility system, and also limits the ability for intramorph fertilisation within the species. Hand-pollination experiments carried out during this study verified the previous literature on this subject. *L. salicaria* in Saskatchewan was strongly outcrossing (obligate xenogamy), requiring pollen from LS to be deposited on LM stigmas (as well as IS on IM and SS on SM) to facilitate reproductive success. Pollination in nature of purple loosestrife is conducted by insects.

There is a relative abundance of literature discussing various floral insect visitors to purple loosestrife, both in its native habitats, and throughout North America. However, the literature does not distinguish the pollination efficiency of the various taxa. This study correlated well with the literature of floral visitors in *Lythrum*, identifying all the major taxa known to visit flowers elsewhere such as honeybees (*Apis mellifera*), bumblebees (*Bombus* spp.), and leafcutter bees (*Megachile* spp.), which have all been confirmed as effective pollinators of purple loosestrife. Two bee taxa not previously mentioned in the literature, *Lasioglossum spp.* and *Anthophora furcata* were also determined to be potential pollinators in Saskatchewan. Many visits from hoverflies (Syrphidae) were also recorded in this study, as in previous studies; however this study has eliminated hoverflies as reliable pollinators of *Lythrum*. Other floral

visitors noted in the earlier literature, also recorded during this study, include butterflies. However, with only a single visit from two field seasons yielding no pollen tubes, it is difficult to evaluate their effectiveness as pollinators, especially in light of the fact that even the best pollinators in Saskatchewan frequently yielded no pollen tubes following a single visit to virgin flowers.

Floral nectar production in *L. salicaria* in central Saskatchewan correlated well with the literature on nectar production in England, and did not differ between the three floral morphs. Floral nectar reached peak secretion values just past mid-day (3:00 pm), and did not differ significantly throughout the day in nectar sugar concentration. Floral nectar likely constituted a very important reward to entice various insect floral visitors to *L. salicaria*.

Literature on the floral nectary structure of *Lythrum* spp., and within the entire Lythraceae is relatively rare, and studies describing the fine structure and development of the floral nectaries in this family are lacking. Hence, this study on nectary ultrastructure and development in *Lythrum virgatum* x *L. alatum* (cultivar 'Morden Gleam') represents the first in this plant family, and only the second within the order Myrtales. Notable features of the floral nectary in 'Morden Gleam' were the apoplastic route of nectar secretion via a series of intercellular channels and the relatively infrequent nectary stomata distributed evenly along the nectary surface, putatively providing the point of nectar escape. The floral nectary of 'Morden Gleam' appeared to lack a direct vascular supply; however, a large quantity of starch was stored prior to secretion, in the nectary parenchyma cells as well as the epidermis, and was reduced to virtually none following the secretory phase of the nectary. This pattern suggests that nectar sugar was derived from the breakdown of this starch. The floral nectary of *Lythrum salicaria* was only examined morphologically; however, the nectary of *L. salicaria* shared many features

with that of ‘Morden Gleam’ including position of the gland within the flower, and the relatively few evenly spaced (typically solitary) nectary stomata, through which nectar secretion takes place.

Due to the past economic and ecological importance of the Lythraceae, both in terms of invasive *Lythrum salicaria* and native *Lythrum* spp. throughout North America, there are abundant future research directions for *L. salicaria* and related species such as the non-*salicaria* hybrid cultivars.

A more detailed look at the nectary morphology of *L. salicaria*, including a stereological analysis using TEM, similar to the one conducted on ‘Morden Gleam’ in this study would be beneficial to our overall knowledge of this aspect of the floral structure in this family. Further detail of the nectary structure in ‘Morden Gleam’ is also still open to examination. An expansion of the stereological study from the subepidermal features to the epidermis would improve the overall understanding of this understudied plant family. The field is still wide open for detailed nectary analysis with the Lythraceae, and a more complete picture to compare to that done on ‘Morden Gleam’ would achieve a broader knowledge of floral nectary structure in this genus, and family.

Nectar component analysis of *Lythrum salicaria* using methods such as high performance liquid chromatography could be performed to determine the types of nectar sugar present to compare with other entomophilous plant species that use floral nectar as a pollinator reward. Nectar component analysis of *L. salicaria* could also be used to determine potential differences in nectar components in invasive North American *Lythrum* contrasted with purple loosestrife from its native range in Eurasia.

In terms of insect visitation to *Lythrum salicaria* in Saskatchewan, it would be advantageous to learn the differences in common visitor species within different ranges throughout Saskatchewan. Pollinator visitation from the south of the province to its northern limits could potentially be significantly different. Analysis of pollinator efficiency at different latitudes in Saskatchewan would improve the knowledge of the reproductive biology of this invasive species. Determination of insect taxa visiting the purple loosestrife cultivar 'Morden Gleam' (*Lythrum virgatum* x *L.* alatum) would be useful to compare visitors of this self-sterile cultivar, to those recorded as visiting invasive *L. salicaria* in central Saskatchewan.

LITERATURE CITED

Ågren J. (1996) Population size, pollinator limitation, and seed set in the self-incompatible herb *Lythrum salicaria*. Ecology 77: 1779-1790.

Ågren, J., and Ericson, L. (1996) Population structure and morph-specific fitness differences in tristylous *Lythrum salicaria*. Evolution 50: 126–139.

Bahadur B., Laxmi S.B., and Swami N.R. (1984) Pollen morphology and heterostyly: A systematic and critical account. Advances in Pollen Spore Research 12:79-126.

Barrett S.C.H. (1992) Evolution and Function of Heterostyly. Springer-Verlag, Berlin Germany.

Beardsell D.V., Williams E.G., and Knox R.B. (1989) The structure and histochemistry of the nectary and anther secretory tissue of the flowers of *Thryptomene calycina* (Lindl.) Stapf. (Myrtaceae). Australian Journal of Botany 37: 65-80.

Bernardello L., Galetto L., and Rodriguez I.G. (1994) Reproductive biology, variability of nectar features and pollination of *Combretum fruticosum* (Combretaceae) in Argentina. Botanical Journal of the Linnean Society 114: 293-308.

Blossey B., Eberts D., Morrison E., and Hunt T.R. (2000) Mass rearing the weevil *Hylobius transversovittatus* (Coleoptera: Curculionidae), biological control agent of *Lythrum salicaria*, on semiartificial diet. Journal of Economic Entomology 93: 1644-1656.

Blossey B., and Schroeder D. (1995) Host specificity of three potential biological weed control agents attacking flowers and seeds of *Lythrum salicaria* (purple loosestrife). Biological Control 5: 47-53.

Bonnier G. (1879) Les nectaires. Etude critique, anatomique et physiologique. Annales Sciences Naturelles Botanique 8: 5-212

Buchmann S.L. (1983) Buzz Pollination in Angiosperms. In Jones C.E., Little R.J. Handbook of Experimental Pollination Biology. Van Nostrand Reinhold Company Inc. New York NY.

Búrquez A., and Corbet SA. (1991) Do flowers reabsorb nectar? Functional Ecology 6: 369–379.

Carr D.J. and Carr S.G.M. (1987) Eucalyptus II- the rubber cuticle, and other studies of the Corymbosae. Phytoglyph Press, Canberra. 372 pp.

Carr S.G.M., and Carr D.J. (1990) Cuticular features of the central Australian bloodwoods *Eucalyptus*, section Corymbosae (Myrtaceae). Botanical Journal of the Linnaean Society 102: 123-156.

Caspary J.X.R. (1848) De nectariis. Dissertation, Elverfeldae (Bonn). 56 pp.

Cheung M., and Sattler R. (1967) Early floral development of *Lythrum salicaria*. Canadian Journal of Botany 45: 1609-1618.

Cody W.J. (1978) The status of *Lythrum alatum* (Lythraceae) in Canada. The Canadian Field Naturalist 92: 74-75.

Comba L., Corbet S.A., Hunt L., and Warren B. (1999) Flowers, nectar, and insect visits: evaluating British plant species for pollinator-friendly gardens. Annals of Botany 83: 369-383.

Corbet S.A., Bee J., Dasmahapatra K., Gale S., Gorringe E., La Ferla B., Moorhouse T., Trevail A., VanBergen Y., and Vorontsova M. (2001) Native or exotic? Double or single? Evaluating plants for pollinator-friendly gardens. Annals of Botany 87: 219-232.

Cruden R. (1977) Pollen-ovule ratios: a conservative indicator of breeding systems in flowering plants. Evolution 31: 32-46.

Curry P.S. (1984) Bumble bees of Saskatchewan (Hymenoptera: Apidae) : a survey of their geographic distribution. Saskatchewan Culture and Recreation, Museum of Natural History. Regina, SK.

Darwin C. (1877) The Different Forms of Flowers on Plants of the Same Species. New York University Press, New York, NY, U.S.A.

Davis A.R. (1992a) Evaluating honey bees as pollinators of virgin flowers of *Echium plantagineum* L. (Boraginaceae) by pollen tube fluorescence. Journal of Apicultural Research 31: 83-95.

Davis A.R. (1992b) Physiological and Structural Aspects of Floral Nectar Secretion. Ph.D. Thesis. Canberra, Australia.

Davis A.R. (1997a) Pollination Efficiency of Insects. In Shivanna K.R., Sawhney V.K. Pollen Biotechnology for Crop Production and Improvement. Cambridge University Press, New York. Pp. 87-120.

Davis A.R. (1997b) Influence of floral visitation on nectar-sugar composition and nectary surface changes in *Eucalyptus*. Apidologie 28: 27-42.

Davis G.L. (1968) Floral morphology and the development of the gametophytes in *Eucalyptus melliodora* A. Cunn. Australian Journal of Botany 16: 19-35.

Davis G.L. (1969) Floral morphology and the development of the gametophytes in *Eucalyptus stellulata* Sieb. Australian Journal of Botany 17: 177-190.

Diehl J.K., Holliday N.J., Lindgren C.J., and Roughley R.E. (1997) Insects associated with purple loosestrife, *Lythrum salicaria* L., in southern Manitoba. Canadian Entomologist 129: 937-948.

Discover Life website (2007) http://pick4.pick.uga.edu/mp/20q?guide=Bumblebees (referenced August, 2007).

Dulberger R. (1970) Tristyly in *Lythrum junceum*. New Phytologist 69: 751-759.

Dulberger R. (1993) Floral Polymorphisms and Their Functional Significance in the Heterostylous Syndrome. In Barrett S.C.H. (1993) Evolution and Function of Heterostyly. Springer-Verlag, Berlin, Germany.

East E.M. (1927) The inheritance of heterostyly in *Lythrum salicaria*. Genetics 12: 393-414.

Eckert C.G., and Barrett S.C.H. (1992) Stochastic loss of style morphs from populations of tristylous *Lythrum salicaria* and *Decodon verticillatus* (Lythraceae). Evolution 46: 1014-1029.

Fahn A. (1979) Secretory Tissues in Plants. Academic Press New York, New York. U.S.A.

Farnsworth E.J., and Ellis D.R. (2001) Is purple loosestrife (*Lythrum salicaria*) an invasive threat to freshwater wetlands? Conflicting evidence from several ecological metrics. Wetlands 21: 199-209.

Ganders F.R. (1979) The biology of heterostyly. New Zealand Journal of Botany 17: 607-635.

Henne D.C., Roughley R.E., and Lindgren C.J. (2002) Additional records of native insects associated with purple loosestrife, *Lythrum salicaria* L., in southern Manitoba. Proceedings of the Entomological Society of Manitoba 58: 10-12.

Hermann B.P., Mal T.K., Williams R.J., and Dollahon N.R. (1999) Quantitative evaluation of stigma polymorphism in a tristylous weed, *Lythrum salicaria* (Lythraceae). American Journal of Botany 86: 1121-1129.

Houghton-Thompson J., Prince H.H., Smith J.J., and Hancock J.F. (2005) Evidence of hybridization between *Lythrum salicaria* (purple loosestrife) and *L. alatum* (winged loosestrife) in North America. Annals of Botany 96: 877-885.

Hunt-Joshi T., and Blossey B. (2005) Interactions of root and leaf herbivores on purple loosestrife (*Lythrum salicaria)*. Oecologia 142: 554-563.

Imperatori L. (1906) Contribuzione allo studio anatomico e funczionaledei nettarii fiorali. Macerata, Unione Cattolica Tipografica. 87 pp.

Judd W.S., Campbell C.S., Kellog E.A., and Stevens P.F. (2008) Plant Systematics: A Phylogenetic Approach. Sinauer Associates, Sunderland, Mass.

Kurr J.G. (1833) Untersuchungen über die Bedeutung der Nektarien in den Blumen. Stuttgart, Germany.

Langenberger M.W. (2000) Studies of Caraway Pollination by Honeybees and Other Insects in Saskatchewan, Canada. M.Sc. Thesis, University of Saskatchewan, Saskatoon, Canada.

Levin D.A. (2003) Ecological speciation: lessons from an invasive species. Systematic Botany 28: 643-650.

Levin D.A. (1970) Assortative pollination in *Lythrum*. American Journal of Botany 57: 1-5.

Levin D.A., and Kerster H.W. (1973) Assortative pollination for stature in *Lythrum salicaria*. Evolution 27: 144-152.

Levin D.A., and Kerster H.W. (1969) The dependence of bee-mediated pollen and gene dispersal upon plant density. Evolution 23: 560-571.

Levin D.A., Kerster H.W., and Niedzler M. (1971) Pollinator flight directionality and its effect on pollen flow. Evolution 25: 113-118.

Mal T.K. (1998) Developmental aspects of tristyly in *Lythrum salicaria*. Canadian Journal of Botany 76: 1214-1226.

Mal T. K., and Hermann B.P. (2000) Quantitative evaluation of pollen polymorphism in a tristylous weed, *Lythrum salicaria* (Lythraceae). Canadian Journal of Botany 78:1086-1094.

Mal T.K., Lovett-Doust J., and Lovett-Doust L. (1997) Time-dependent competitive displacement of *Typha angustifolia* by *Lythrum salicaria*. OIKOS 79: 26-33.

Mal T.K., Lovett-Doust J., Lovett-Doust L., and Mulligan G.A. (1992) The biology of Canadian weeds. 100. *Lythrum salicaria*. Canadian Journal of Plant Science 72: 1305-1330.

Martin F.W. (1959) Staining and observing pollen tubes in the style by means of fluorescence. Stain Technology 34: 125-128.

Michener C.D., McGinley R.J., and Danforth B.N. (1994) The Bee Genera of North and Central America (Hymenoptera: Apoidea). Smithsonian Institution Press, Washington DC.

Moncur M.W., and Boland D.J. (1989) Floral morphology of *Eucalyptus melliodora* A. Cunn. Ex Schau. and comparisons with other eucalypt species. Australian Journal of Botany 37: 125-135.

Morris J.A. (2007) A Molecular Phylogeny of the Lythraceae and Inference of the Evolution of Heterostyly. Doctoral Thesis, Kent State University, U.S.A.

Mulcahy D.L., and Caporello D. (1970) Pollen flow within a tristylous species: *Lythrum salicaria*. American Journal of Botany 57: 1027-1030.

Nicholls M.S. (1987) Pollen flow, self-pollination and gender specialization: Factors affecting seed set in the tristylous species *Lythrum salicaria* (Lythraceae). Plant Systematics and Evolution 156: 151-157.

O'Brien S.P., Loveys B.R., Grant W.J.R. (1996) Ultrastructure and function of floral nectaries of *Chamelaucium uncinatum* (Myrtaceae). Annals of Botany 78: 189-196.

Olsson K., and Ågren J. (2002) Latitudinal population differentiation in phenology, life history and flower morphology in the perennial herb *Lythrum salicaria*. Journal of Evolutionary Biology 15: 983-996.

O'Neil P. (1997) Natural selection on genetically correlated phenological characters in *Lythrum salicaria* L. (Lythraceae). Evolution 51: 267-274.

O'Neil P., and Schmitt J. (1993) Genetic constraints on the independent evolution of male and female reproductive characters in the tristylous plant *Lythrum salicaria*. Evolution 47: 1457-1471.

Pearsall W.H. (1918) The aquatic and march vegetation of Esthwaite water. Journal of Ecology 6: 53-74.

Percival M.S. (1961) Types of nectar in angiosperms. New Phytologist 60: 235-281.

Philip L.J., Posluszny U., and Klironomos J.N. (2001) The influence of mycorrhizal colonization in the vegetative growth and sexual reproductive potential of *Lythrum salicaria* L. Canadian Journal of Botany 79: 381-388.

Razem F.A., and Davis A.R. (1999) Anatomical and ultrastructural changes of the floral nectary of *Pisum sativum* L. during flower development. Protoplasma 206: 57-72.

Reynolds E.S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology 17: 208-212.

Ronse Decraene L.-P., and Smets E. (1991) The impact of recetacular growth on polyandry in the Myrtales. Botanical Journal of the Linnaean Society 105: 257-269.

Sazima M., Vogel S., do Prado A.L., de Oliveira D.M., Franz G., and Sazima I. (2001) The sweet jelly of *Combretum lanceolatum* flowers (Combretaceae): a cornucopia resource for bird pollinators in the Pantanal, western Brazil. Plant Systematics and Evolution 227: 195-208.

Schill R., Baumm A., and Wolter M. (1985) Vergleichende Mikromorphologie der Narbenoberflachen bei den Angiospermen; Zusammenhange mit Pollenoberflachen bei heterostylen Sippen. Plant Systematics and Evolution 148: 185-214.

Schoch-Bodmer H. (1936). The proportion of long-, mid-, and short-styled plants in natural populations of *Lythrum salicaria* L. Journal of Genetics 36: 39-43.

Scribailo R.W. and Barrett S.H. (1991) Pollen-pistil interactions in tristylous *Pontederia sagittata* (Pontederiaceae). II. Patterns of pollen tube growth. American Journal of Botany 78: 1662-1682.

Shamsi S.R.A., and Whitehead F.H. (1974) Comparative eco-physiology of *Epilobium hirsutum* L. and *Lythrum salicaria* L. 1. General biology, distribution, and germination. Journal of Ecology 62: 279-290.

Shivanna K.R., and Rangaswamy N.S. (1992) Pollen biology: a laboratory manual. Springer-Verlag, New York, NY, U.S.A.

Steel R.G.D., and Torrie J.H. (1980) Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill. U.S.A.

Stout A.B. (1925) Studies of *Lythrum salicaria* II: A new form of flower in this species. Bulletin of the Torrey Botanical Club 52: 35-80.

Tobe H., Hakki M.I., Langhammer L. (1989) Floral nectary in *Medinilla magnifica*, an Old World Melastomataceae. Botanische Jahrbücher Für Systematik Pflanzengeschichte und Pflanzengeographie 111: 57-62.

Ture C., Bingol N.K., and Middleton B.A. (2004) Characterization of the habitat of *Lythrum salicaria* L. in floodplain forests in western Turkey: Effects on stem height and seed production. Wetlands 24: 711-716.

USDA Plant Database (2008): http://plants.usda.gov/java/nameSearch?keywordquery=lythrum&mode=sciname (referenced January, 2008).

Vockeroth J.R. (1992) The Insects and Arachnids of Canada. Part 18. The Flower Flies of the Subfamily Syrphinae of Canada, Alaska, and Greenland. Ottawa, Ontario: Research Branch, Agriculture Canada.

Vogel S. (1997) Remarkable nectaries: structure, ecology, organophyletic perspectives I. Substitutive nectaries. Flora 192: 305-333.

Vogel S. (1998) Remarkable nectaries: structure, ecology, organophyletic perspectives IV. Miscellaneous cases. Flora 193: 225-248.

Waites A.R., and Ågren J. (2004) Pollinator visitation, stigmatic pollen loads, and among-population variation in seed set in *Lythrum salicaria*. Journal of Ecology 92: 512-52.

Wist T.J. (2005) Pollination Biology of *Echinacea angustifolia* and *Echinacea purpurea* (Asteraceae) in Saskatchewan. M.Sc. Thesis, University of Saskatchewan, Saskatoon, Canada.

Wist T.J., and Davis A.R. 2006. Floral nectar production and nectary anatomy and ultrastructure of *Echinacea purpurea* (Asteraceae). Annals of Botany 97: 177-193.

Wolff G.P. (1924) Zur vergleichenden Entwicklungsgeschichte und biologischen Bedeutung der Blütennektarien. Botanical Archives 8: 305-344.