Development, growth and ultrastructure of the floral nectar spur of *Centranthus ruber* (L.) DC (Valerianaceae)

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ABSTRACT

The main objective of this research project was to study the growth and development of the floral nectar spur of *Centranthus ruber* (L.) DC. Nectar spurs are tubular floral outgrowths, generally derived from the perianth organs, which typically contain secreted floral nectar. The morphological characteristics of the spur, particularly the length, determine which floral visitors will be able to access the nectar reward pooled at the spur tip. Therefore, nectar spurs are ecologically important for the development of specialised pollinator interactions and have been demonstrated to act as key innovations in the evolution of some taxa.

Morphological and anatomical characteristics of the spur and floral nectary were investigated using light and scanning electron microscopy. Ultrastructural features of the nectar spur, particularly the floral nectary within, were assessed using transmission electron microscopy. Nectar in *C. ruber* is produced by a trichomatous nectary which runs along the entire, inner abaxial surface of the spur. The nectary is aligned with the single vascular bundle which runs along the abaxial side of the spur, through the sub-nectary parenchyma, and back up the adaxial side. The secretory trichomes are unicellular and, in late development, they develop a thick layer of secondary wall ingrowths which vastly increases the surface area of the plasma membrane for nectar secretion. Elongate, non-secretory trichomes occupy the entire remaining circumference of the spur's inner epidermis, but their density is reduced compared to the secretory trichomes.

The cellular basis for spur growth is poorly characterized in the literature. Until recently, it was assumed that all nectar spurs grow by the constant production of new cells via up to three potential meristematic regions (the meristem hypothesis, Tepfer 1953). The cellular basis for spur growth in *C. ruber* was investigated by cell file counts and cell length and width measurements along the lateral side of nectar spurs in each of the developmental stages. DAPI

stained spurs were also examined with Confocal/Apotome microscopy to determine the timing and position of cell division activity throughout spur development. It was determined that elongation of the spur epidermal cells contributes much more to spur growth than cell division. In early development, division is the primary driver of spur growth and the cells are isotropic. However, as development progresses, cell division activity slows down and the spur cells become increasingly anisotropic until anthesis.

The patterns of nectar secretion were determined by assessing the volume, solute concentration and carbohydrate composition of the nectar throughout flowering phenology in two *C. ruber* plants. Nectar volumes and solute amounts rose initially, followed by an eventual decline in both as phenology progressed towards senescence. Because this study was conducted on greenhouse grown plants, it can be assumed that nectar was not removed by insects, suggesting that it is likely reabsorbed following secretion. High performance liquid chromatography (HPLC) analysis determined that *C. ruber*'s nectar is sucrose dominant and that nectar composition remains stable following anthesis throughout floral phenology.

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TABLE OF CONTENTS

Chapter 4: NECTAR SECRETION DYNAMICS OF *CENTRANTHUS RUBER*

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

DAPI – 4',6-diamidino-2-phenylindole

ER – Endoplasmic reticulum

GA – Glutaraldehyde

HPLC – High performance liquid chromatography

LM – Light microscopy

NCV – Nectar concentration by volume

NCW – Nectar concentration by weight

SEM – Scanning electron microscopy

SFG – Sucrose, fructose and glucose balanced nectar

S/F+G – Ratio of sucrose concentration to the combined concentrations of fructose and glucose

TBO – Toluidine blue O

TEM – Transmission electron microscopy

Chapter 1: INTRODUCTION

1.1 Floral modifications and diversity in angiosperms

The Darwinian perspective on the numerous floral modifications which have occurred throughout angiosperm evolution is that many represent adaptations imposed by selective pressures from animal pollinators (Stebbins 1970, Herrera 1996, Harder and Johnson 2009). These floral modifications include changes in floral colour, floral symmetry, and the degree of floral fusion. However, with quantitative data somewhat scarce, it remains largely unknown what percentage of animal-pollinated plants have traits which were selected by their pollinators and to what degree each phenotypic trait has been modified due to pollinator imposed selection pressures (Herrera 1996). It is clear from the literature, however, that pollinators do discriminate and preferentially visit flowers with specific floral morphologies, thereby potentially exerting selective pressure on specific traits.

It is suggested that transitions in floral colour throughout angiosperm evolution could have been caused by pollinator selection (Rausher 2008). Pollinators have been shown to preferentially visit certain coloured flowers in numerous genera (Waser and Price 1981, Stanton *et al*. 1989, Schemske and Bradshaw 1999, Odell *et al*. 1999, Omura and Honda 2004). As part of the pollination syndrome concept (Fenster *et al*. 2004), particular floral colours tend to be associated with particular pollinator types, such as red with hummingbirds (Cronk and Ojeda 2008), blue with bumblebees (Hodges and Derieg 2009) and white with hawkmoths (Miller 1981).

During late angiosperm evolution, multiple instances of changes in floral symmetry from actinomorphic (polysymmetric) to zygomorphic (monosymmetric) (Donoghue *et al*. 1998, Rudall and Bateman 2004, Cubas 2004), have promoted rapid radiation (Endress 1999).

Lineages with bilaterally symmetrical flowers tend to be more species rich than those with radially symmetric flowers (Sargent 2004). Zygomorphic flowers force pollinators to approach from a specific orientation which can aid in optimal contact between the pollinator's body and the pollen (Cubas 2004). The evolution of zygomorphy is also often associated with indeterminate inflorescences which do not have single, terminal flowers, but numerous flowers borne laterally (Coen *et al*. 1995). Zygomorphic flowers borne on inflorescences such as racemes or spikes often have one or more petals modified into a platform, allowing the floral visitors to land before entering the flowers, a manoeuvre which would not be possible on actinomorphic flowers with their petals arranged vertically (Stebbins 1970).

The fusion of individual floral parts into compound organs has also contributed greatly to increased diversity in floral morphology (Verbeke 1992). The ancestral condition in angiosperms appears to be polypetalous flowers, with fused petals (sympetalous) representing a more derived form (Wernham 1912). The evolution of tubular corollas, formed by the fusion of petals, was caused by interactions with pollinators and is closely associated with reproductive success (Verbeke 1992). A corolla tube tends to restrict short-tongue pollinators from accessing the nectar reward at its base, causing the development of more specialized plant-pollinator interactions.

1.2 Nectar spurs and species diversification

Spurs appear to promote greater specificity in the coevolutionary interactions between a floral species and its pollinators (Hunter 1998). A spur can be regarded as a barrier between the potential pollinator and the contained nectar reward, which can only be overcome if the length of the pollinator's tongue is sufficient to reach the nectar accumulated within the spur. This specialized interaction results in the possibility of reproductive isolation and speciation (Koopman and Ayers 2005). Although it has been suggested that plants which form specialized,

mutualistic relationships with their pollinators may be at increased risk of extinction compared to generalist species (Johnson and Steiner 2000), specialization and generalization do not represent strict categories; instead, they are the ends of a continuum which ranges from obligate specialization to extreme generalization (Waser 2006). Species may be specialized to a specific type of pollinator without being drastically limited in the number of pollinator species which visit, therefore avoiding the risks which are associated with obligate specialization (Johnson and Steiner 2000).

Key innovations are morphological adaptations which promote diversification and rapid speciation by allowing available resources to be used in a novel way and to enhance competitive ability (Hodges 1997, Hunter 1998). Nectar spurs are proposed to be key innovations in the evolution of some species, including those of *Aquilegia* L. (Ranunculaceae), a genus which has recently undergone a rapid radiation closely coupled with its evolution of nectar spurs (Hodges 1997), and possibly *Euphorbia* L. (Euphorbiaceae) (Cacho *et al*. 2010). Nectar spurs may also have been a key innovation in *Halenia* Borkh. (Gentianaceae), and if not, they were at least a preadaptation which may have allowed speciation rates to increase when *Helenia* moved from temperate to subtropical and tropical areas where potential pollinators awaited (von Hagen and Kadereit 2003). Clades with spurred members generally have greater numbers of species than their closely related unspurred relatives due to the premating isolation (Hodges and Arnold 1995) which can be induced by minor changes in spur morphology (Cacho *et al*. 2010, Hodges and Arnold 1995). It is proposed that the evolution of nectar spurs in *Aquilegia* has allowed those species to specialise to different pollinators (Hodges and Arnold 1994). Spur length has been acknowledged as a key trait for floral isolation in many orchid species (Schiestl and Schluter 2009) and in *Aquilegia* species (Hodges and Derieg 2009).

1.3 Morphological characteristics of nectar spurs

Nectar spurs are slender, tube-like, perianth outgrowths, commonly associated with the production and/or containment of nectar, which are only found in approximately 0.62% of angiosperm genera [84 spurred genera (Table 1.1) of a total 13,500 angiosperm genera (Renner and Ricklefs 1995)]. The majority of spurred species have zygomorphic (monosymmetric) flowers with a single spur per flower, however some actinomorphic (polysymmetric) flowered species, such as *Aquilegia* (Ranunculaceae) species, have a spur on each petal (Endress 2001). Spurs are most commonly derived from the calyx or corolla, but they can also result from the complex fusion of multiple floral parts, including, in addition to perianth organs, portions of the androecium and gynoecium in some species (Hodges 1997, Koopman and Ayers 2005). It is suggested that because spurs form from various floral organs, the mechanisms for their formation are not likely to be broadly conserved across plant families (Golz *et al*. 2002). However, in several different species, both monocot and dicot*, KNOTTED1-like homeobox* (*KNOX*) gene expression has been implicated in spur development (Golz *et al*. 2002, Box *et al.* 2011, 2012).

Spur morphology can be related to an association with particular pollinators, with the physical characteristics of the spur and its position showing significant variation between species. Important characteristics which help to determine potential interactions with pollinators include spur colour, position, length and curvature (Hodges and Arnold 1995, Hodges 1997). Variation in these traits allows for the establishment of high degrees of pollinator specificity and fidelity.

As with other floral parts, different coloured spurs help to attract different pollinators (Hodges and Kramer 2007). For example, blue flowers tend to attract bumblebee pollinators

Table 1.1 Taxonomic distribution of floral nectar spurs, modified from Hodges (1997). Clade and order designations follow APGII (2003).

Class	Clade	Order	Family	Genera with spurs (or examples)
Mono-cotyledonae	Monocots	Asparagales	Orchidaceae Juss.	*Aerangis* Rchb.f., *Anacamptis* Rich., *Anagraecum* Schltr., *Anthosiphon* Schltr., *Ascocentrum* Schltr., *Bonatea* Willd., *Christensonia* Haager, *Cryptocentrum* Benth., *Dactylorhiza* Neck., *Dendrophylax* Rchb.f., *Galearis* Raf., *Gymnadenia* R.Br., *Habenaria* Willd., *Limodorum* Boehm., *Mystacidum* Lindl., *Neofinetia* Hu, *Orchis* L., *Piperia* Rydb., *Platanthera* Rich., *Pseudomaxillaria* Hoehne, *Pseudorchis* Ség, *Rangaeris* (Schltr.) Summerh., *Sepalosaccus* Schltr.
		Liliales	Liliaceae Juss.	*Tricyrtis* Wall.
Eudi-cotyledonae	Eudicots	Ranunculales	Berberidaceae Juss.	*Epimedium* L.
			Fumariaceae Bercht. & J.Presl	*Corydalis* DC., *Dicentra* Bernh., all other genera in family
			Ranunculaceae Juss.	*Aconitum* L., *Aquilegia* L., *Delphinium* L.
	Asterids	Ericales	Balsaminaceae A.Rich.	*Impatiens* L., *Hydrocera* Blume
	Euasterids I	Gentianales	Gentianaceae Juss.	*Halenia* Borkh.
		Lamiales	Lamiaceae Martinov	*Plectranthus* L'Hér.
			Lentibulariaceae Rich.	*Utricularia* L., *Centrosema* (DC.) Benth., *Genlisea* A.St.-Hil., *Pinguicula* L.
			Scrophulariaceae Juss.	*Chaenorhinum* (DC.) Rchb., *Cymbalaria* Hill, *Diascia* Link & Otto, *Kickxia* Dumort., *Linaria* Mill., *Nuttallanthus*D.A.Sutton
	Euasterids II	Asterales	Campanulaceae Juss.	*Heterotoma* Zucc., some *Lobelia* L.
		Dipsacales	Caprifoliaceae Juss.	Some *Lonicera* L.
			Diervillaceae (Raf.) N.Pyck	*Diervilla* Mill.
			Valerianaceae Batsch	*Centranthus* (L.) DC., *Plectritis* (Lindl.) DC.
			Linnaeaceae (Raf.) Backlund	*Kolkwitzia* Graebn.
	Rosids	Geraniales	Geraniaceae Juss.	*Pelargonium* L'Hér. Ex Aiton
		Myrtales	Vochysiaceae A.St.-Hil.	All genera except *Amphilochia* Mart. and *Euphronia* Mart.
	Eurosids I	Fabales	Fabaceae Lindl.	*Amherstia* Wall., *Bauhinia* L.
		Malpighiales	Euphorbiaceae Juss.	*Euphorbia* L.
			Violaceae Batsch	*Anchietea* A.St.-Hil., *Corynostylis* Mart., *Noisettia* Kunth, *Viola* L.
	Eurosids II	Brassicales	Tropaeolaceae Juss. Ex DC.	*Tropaeolum* L.

(Hodges and Derieg 2009), whereas red flowers tend to attract hummingbirds (Cronk and Ojeda 2008). Several shifts in the types of anthocyanin pigments produced in *Aquilegia* species have led to colour changes, causing major pollinator shifts from bee to hummingbird and from hummingbird to hawkmoth pollination (Hodges and Derieg 2009). Contrast is also an important factor, as observed in *Delphinium nelsonii* Greene (Ranunculaceae), in which flowers with more pronounced colour differences between their guard and spurred nectariferous petals are preferentially pollinated by bumblebees and hummingbirds (Waser and Price 1985).

Whereas colour attracts pollinators, spur shape and length are important determinants of the feasibility of an effective interaction because the spur acts as a barrier between pollinator and the enclosed nectar reward. There exists immense variation in the spur curvature (0° to 297°) within and between populations of *Impatiens capensis* Meerb. (Balsaminaceae) possibly in part due to its interactions with both insect and bird pollinators which each prefer different degrees of spur curvature (Travers *et al.* 2003). Spur length is correlated with the proboscis length of the pollinator species and has the ability to directly affect reproductive success (Hodges 1997). One of the longest spurs known, which can measure over 30cm, is found on the orchid (*Angraecum sesquipedale* Thouars) flowers made famous by Darwin's prediction of a hawkmoth pollinator (now known to be *Xanthopan morgani praedicta* R. & J.) with an exceptionally long proboscis (Little *et al*. 2005). By contrast, the spur of *Lobelia cordifolia* Hook. & Arn. (Campanulaceae) is miniscule, measuring just 1.0-2.2mm on average, suggesting association with short-tongued pollinators (Ayers 1990). Even closely related species, such as several different groups in the *Disa draconis* Sw. complex (Orchidaceae), which live in different habitats (southern vs. northern mountain regions) and rely on different pollinators, show marked differences in their average spur lengths (32-38mm vs. 57-72mm) (Johnson and Steiner 1997). Experimentally-shortened

Platanthera bifolia (L.) Rich. and *P. chlorantha* Cust. Ex Rchb. (Orchidaceae) spurs, when exposed to pollinators, have less pollen removed and deposited, resulting in reduced fruit set (Nilsson 1988). Similarly, shortened spurs of *P. mandarinorum* Rchb. subsp. *hachijoensis* Honda (Murata) have reduced reproductive success, particularly the male aspect (pollen removal) (Inoue 1986). Reduction in fruit and seed set is also seen in similar experiments with *Disa draconis,* although shortening the spur only affects the female aspect of reproductive success (pollen receipt) in this species (Johnson and Steiner 1997).

1.4 Anatomy of spurs and nectar secretion

Nectaries are made up of three main tissues: the nectary epidermis, the nectary parenchyma and the subnectary parenchyma, which commonly contains a vascular connection (Fahn 1979, Nepi 2007). The nectariferous tissue inside the spur is usually closely associated with the vascular tissue, as the phloem sap is considered to be the source of the pre-nectar (Fahn 1979). Generally, several layers of parenchyma cells separate the nectariferous tissue from the vascular tissue (Figueiredo and Pais 1992, Stpiczynska 2003a, Stpiczynska *et al*. 2011) and the number and structure of the vascular bundles serving the nectary is variable (Nepi 2007). In *Aquilegia*, each spurred petal has one major trace and the vascular bundles resemble the pattern seen in the foliage leaves (Tepfer 1953). In many Aeridinae (Orchidaceae) species, the spurs each have two large and several smaller vascular bundles, with the total number of bundles ranging from 2-22 (Stpiczynska *et al*. 2011). Nectar is generally composed primarily of glucose, fructose and sucrose (Brandenburg *et al*. 2009) and the carbohydrate component of the nectar is directly transported from the phloem or, alternatively, produced by the hydrolysis of starch accumulated in the nectariferous cells (Heil 2011).

The location of nectar secretion varies between different spurred species. The spur itself

can be the site of nectar production, such as in *Impatiens capensis* Meerb. (Balsaminaceae), where the nectar is secreted at the distal tip of the spur from a thin strip of vascular tissue on the spur's ventral side (Marden 1984), or the nectar can be produced elsewhere (Pacini *et al*. 2003), such as in *Linaria vulgaris* (L.) Mill. (Scrophulariaceae), in which nectar produced beneath the base of the ovary flows into and collects within the base of the spur (Nepi *et al.* 2003).

Differences also exist in the types of secretory cells responsible for the release of nectar. In some cases, such as in *Limodorum abortivum* (L.) Sw. (Orchidaceae), a cuticle layer is present along the inner epidermal surface of the spur, which ruptures to allow the nectar to be released into the nectariferous cavity (Figueiredo and Pais 1992). In other species, such as *Tropaeolum majus* L. (Tropaeolaceae), stomata on the inner surface of the spur are responsible for the release of nectar (Fahn 1979) which is secreted mainly by parenchyma cells between the vascular bundles and the inner epidermis of the spur (Rachmilevitz and Fahn 1975). The spurs of several taxa of *Habenaria* (Orchidaceae) do not have defined nectariferous tissue or distinct secretory cells (Galetto *et al*. 1997). In these taxa, the inner epidermis has one-celled papillae, concentrated mostly at the base of the non-vascularised spur (Galetto *et al*. 1997).

Trichomes, either unicellular or multicellular, can also be present as the secretory cells, and their position within the spur is quite variable. Trichomatous nectaries represent a more recent evolutionary form than other nectary types (Smets 1986) and they also represent the most common type of epidermal nectaries in angiosperms (Bernardello 2007). In some species with trichomatous nectaries, such as *Kolkwitzia amabilis* Graebn. (Caprifoliaceae; listed as Linnaeaceae by APGII (2003) in Table 1.1), the secretory trichomes are dispersed amongst smaller papillae which also contribute to nectar secretion (Dmitruk 2012). The cuticle of nectary trichomes tends to be pushed upwards as nectar is secreted into the subcuticular space prior to its

release, either by cuticle breakage or the permeation of the nectar through thin sections of the cuticle (Nepi 2007). The secretory trichomes of *Platanthera chlorantha* (Orchidaceae) have cell wall fibrillar outgrowths through their cuticles, likely allowing nectar to flow through (Stpiczynska *et al*. 2005, Nepi 2007). In *Ascocentrum* (Orchidaceae) species, unicellular trichomes are located near the middle of the nectar spur, forming a complete ring around it, with the longest trichomes positioned closest to the vascular tissue (Stpiczynska *et al*. 2011). In *Papilionanthe vandarum* (Rchb.f.) Garay (Orchidaceae), secretory trichomes (mainly unicellular, sometimes bicellular) are present all over the inner spur surface, but concentrated in a vertical strip aligned with the vascular tissue (Stpiczynska *et al*. 2011).

The ultrastructural features of the secretory cells change during the different stages of nectar secretion and can provide some indication of the secretion process (Stpiczynska *et al*. 2005). Nectary parenchyma is generally made up of relatively small, isodiametric cells with densely staining cytoplasm, whereas the subnectary parenchyma has larger cells with larger intercellular spaces (Nepi 2007). The nectary parenchyma cells tend to have small vacuoles prior to secretion, which increase in size during and after secretion (Nepi 2007). Ribosomes and numbers of mitochondria are also high in these cells, increasing during secretion (Nepi 2007). In *Limodorum abortivum*, the endoplasmic reticulum (ER), dictyosomes and plastids are evident in the secretory cells during the secretion phase and are suspected to be involved in nectar secretion (Figueiredo and Pais 1992). The trichomes and epidermal cells of the nectary of *P. chlorantha* contain many secretory vesicles and dictyosomes during the secretory stage (Stpiczynska *et al*. 2005). The epidermal cells inside the spurs of several *Habenaria* species (Galetto *et al*. 1997) and the plastids in the unicellular secretory trichomes and secretory parenchyma of *P. chlorantha* species (Stpiczynska *et al*. 2005) are both dense with starch grains prior to nectar secretion. Both

are virtually emptied of starch after nectar secretion begins, strongly suggesting that the starch acts as a source of the nectar. It is also possible that the starch is used as an energy source to fuel the secretion process (Nepi 2007).

Spurs vary in the amount of nectar they produce, likely for numerous different reasons including flower size, nectary size, pollinator type and available energy. Some aerangoid orchids nearly fill their long spurs with nectar, but keep nectar sugar concentration higher near the tip of the spur, encouraging hawkmoth visitors to probe deeply to access the most valuable food resources and thereby increasing the likelihood of pollinia deposition (Martins and Johnson 2007). Additionally, some spurs are deceptive and nectarless, such as those of *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase*, A. pyramidal* (L.) Rich., *Dactylorhiza sambucina* (L.) Soó, *D. fuchsii* (Druce) Soó, and several *Orchis* species (Orchidaceae) (Bell *et al*. 2009). It is suggested that *Viola cerasifolia* A. St.-Hil and *V. subdimidiata* A. St.-Hil, which have small spurs and produce very little nectar, there has been a shift away from being nectar flowers towards using pollen as the main floral resource available to flower visitors (Freitas and Sazima 2003).

Prior to pollination, the spur generally serves to protect the nectar from extensive evaporation, although it is still susceptible to nectar robbery by some insects that pierce the spur tissue externally to access the nectar without engaging in pollination (Pacini *et al*. 2003). An example of nectar robbery occurs in *Corydalis cava* (L.) Schweigg. & Koerte (Fumariaceae), in which queen bumblebees (*Bombus terrestris*) are able to pierce the petal spur to access the nectar, unlike *C. cava*'s usual long-tongued specialist pollinators which access the nectar without damaging the spur, contributing to pollination of this self-incompatible species (Olesen 1996). In some species such as *Platanthera chlorantha* (Stpiczynska 2003a) and *Linaria vulgaris* (Nepi *et*

al. 2003), it has been demonstrated that some of the nectar is reabsorbed following pollination. According to Nepi *et al*. (2003), the purposes of nectar reabsorption in *L. vulgaris* are energy recovery and the maintenance of low sugar concentrations during evaporation.

1.5 Taxonomic distribution of nectar spurs

Nectar spurs have independently evolved in at least 15 angiosperm families (Table 1.1) (Hodges 1997). For example, phylogenetic analyses suggest that spurs arose independently four times and in two different ways in the Ranunculales order, resulting in species with single spurs (some Papaveraceae, Berberidaceae and Ranunculaceae) and others with multiple spurs (some Papaveraceae, and Ranunculaceae) (Damerval and Nadot 2007). Certain angiosperm families (Balsaminaceae, Fumariaceae, Lentibulariaceae and Tropaeolaceae) are characterized by the presence of spurs, whereas others (Campanulaceae, Caprifoliaceae, Fabaceae, Gentianaceae, Geraniaceae, Orchidaceae, Ranunculaceae, Scrophulariaceae, Valerianaceae, Violaceae and Vochysiaceae) include both spurred and non-spurred members (Hodges 1997). Two spurred families absent from Hodges' (1997) list are the Liliaceae, which has a few spurred members, including species of *Tricyrtis* (Takahashi 1984, 1989, Peng *et al*. 2007) and the Euphorbiaceae which include a few spurred species in the genus *Euphorbia* (Cacho *et al*. 2010). These 21 families (Table 1.1) with spurred members are relatively spread out taxonomically, belonging to 13 different angiosperm orders. Only two families, the Orchidaceae and the Liliaceae, belong to the Monocotyledonae, with the remaining 19 belonging to the Eudicotyledonae (Table 1.1).

1.5.1 Introduction to the Dipsacales

The Asterids are one of the largest groups of flowering plants, consisting of ten orders which include approximately 80 000 species (Bremer *et al*. 2002). Eight of these ten orders make

up the euasterid group, which is further subdivided into two major groups: euasterids I (Lamiids) and euasterids II (Campanulids) (Bremer *et al*. 2002). The campanulids include three orders, the Apiales, Asterales and Dipsacales which are very well defined and supported by chloroplast DNA analysis, as well as some other families without clear orders designated (Bremer *et al*. 2002). The Dipsacales includes approximately 1100 species (Bell and Donoghue 2005).

Traditionally, the Dipsacales included the families Adoxaceae, Caprifolicaeae, Dipsacaceae, Valerianaceae and sometimes Morinaceae and Triplostegiaceae (Bell *et al*. 2001). Other sources also included the Collumelliaceae (Backlund 2002), Diervillaceae and Linnaeaceae (Simpson 2006). Sometimes the order Dipsacales is restricted to include just four families: Caprifoliaceae, Dipsacaceae, Morinaceae and Valerianaceae, excluding the Adoxaceae (Backlund and Bremer 1997). Roels and Smets (1996) present three "core" families in the Dipsacales: Caprifoliaceae, Dipsacaceae and Valerianaceae, which are all characterized by two or more autapomorphies including an amoeboid tapetum and unicellular trichomes associated with the corolla. Recent revisions of the order (Bell and Donoghue 2005, Judd *et al*. 2008) have divided the Dipsacales into just two families: (1) the Adoxaceae defined by radial flowers, non-spiny pollen, a short style with a lobed stigma and a glandular nectary (if present) atop the ovary, and (2) the Caprifoliaceae defined by the presence or absence of bilateral flowers, spiny pollen, an elongate style with a capitate stigma and a nectary on the inner corolla surface, consisting of densely packed hairs. Under this classification, the Caprifoliaceae is circumscribed broadly to include the Dipsacaeae and Valerianaceae (Judd *et al*. 2008).

1.5.1.1 Nectary structure and function within the order

The Dipsacales is characterized by petal nectaries (Bernardello 2007). The nectaries of the Dipsacales *sensu stricto* (excluding *Adoxa*, *Sambucus*, and *Viburnum*) are found in various

positions on the corolla tubes and are composed of unicellular trichomes atop nectary parenchyma (Wagenitz and Laing 1984). To establish a comparison of floral nectaries in taxa closely related to *Centranthus ruber* (Valerianaceae), knowledge of these floral glands in originally designated families of the Dipsacales which are all, apart from the Adoxaceae, now included within the Caprifoliaceae, is presented (Table 1.2). The basal Adoxaceae members lacked distinct nectaries, whereas the basal members of Caprifoliaceae had nectaries with unicellular hairs at the bases of their corolla tubes (Donoghue *et al*. 2003). Of all families in the Dipsacales, the nectaries within the Adoxaceae show the most variation, ranging from multicellular, trichomatous, corolla nectaries, to disk-shaped, gynoecial nectaries, to an absence of any nectaries at all (Table 1.2). The trend in the remaining families (Dipsacaceae (including Triplostegiaceae), Morinaceae, Diervillaceae, Linnaeaceae and Caprifoliaceae), which are all now included in the Caprifoliaceae, is towards unicellular, trichomatous corolla nectaries (Table 1.2).

Table 1.2 – Floral nectaries within the Dipsacales

Family	Taxon	Nectary type	Location
Adoxaceae	*Adoxa* L.	Trichomatous, multicellular (Sprague 1926, Wagenitz and Laing 1984)	Multiple, at the bases of the lobes of the inner perianth whorl (Sprague 1926, Wagenitz and Laing 1984)
	Sinadoxa C.Y.Wu, Z.L.Wu & R.F.Huang		
	Tetradoxa C.Y.Wu		
	Viburnum L.	Disk-like (*Viburnum farreri* Stearn; Erbar 1994, as cited in Donoghue *et al*. 2003), gynoecial (Erbar and Leins 2010). Extrafloral nectaries present on the leaves (Mabberley 1997)	Atop the inferior ovary
	Sambucus L.	Lacks floral nectaries (Erbar and Leins 2010). Extrafloral nectaries present on the leaves (Mabberley 1997)	
Dipsacaceae (including Triplo-stegiaceae)	*Dipsacus* L.	Trichomatous, caducous (Roels and Smets 1996)	At the base of the corolla tube (Wagenitz and Laing 1984, Davis 2003)
	Succisa Haller		
	Scabiosa L.		
	Cephalaria Schrad.		
	Knautia L.		
	Pterocephalus Vaill. Ex Adans.		
	Succisella Beck		
	Tremastelma Raf.		
	Triplostegia Wall. Ex DC.		
Morinaceae	*Acanthocalyx* (DC.) M.J.Cannon		
	Cryptothladia (Blume) M.J.Cannon	Two-lobed (Cannon and Cannon 1984, as cited in Bell and Donoghue 2003)	At the base of the corolla tube, each associated with one of the two sterile stamens (Cannon and Cannon 1984, as cited in Bell and Donoghue 2003)
	Morina L.		
Diervillaceae	*Diervilla* Mill.	Trichomatous, club-shaped (Backlund and Pyck 1998).	At the base of the corolla tube (Backlund and Pyck 1998).
	Macrodiervilla Nakai		
	Weigela Thunb.		
	Weigelastrum (Nakai) Nakai		
Linnaeaceae	*Abelia* R.Br.	Trichomatous, cushion shaped	Nectariferous petal (Backlund and Pyck 1998)
	Dipelta Maxim.		
	Kolkwitzia Graebn.		
	Linnaea L.	Trichomatous (Weberling 1989)	
	Zabelia (Rehder) Makino		
Caprifoliaceae (*sensu lato*)	*Lonicera* L.	Trichomatous (*L. kamtschatica* (Sevast.) Pojark.; Weryszko-Chmielewska and Bozek 2008)	At the base of the corolla tube, in the corolla spur where present (Weryszko-Chmielewska and Bozek 2008). In some species, the nectary is restricted to the base of just one petal (Wilkinson 1949).
	Leycestria Endl.	Trichomatous	At the bases of each of the five petals (Wilkinson 1949).
	Symphoricarpos Duhamel	Trichomatous, unicellular trichomes (*S. rivularis* Suksd.; Smets 1986)	Inner corolla surface (*S. rivularis* Suksd.; Smets 1986)

1.5.1.2 Introduction to the Valerianaceae

The Valerianaceae is an angiosperm clade with approximately 300 (Simpson 2006) to 350 (Bell 2004) or 400 (Hidalgo *et al*. 2004) species. The defining characteristics of the group are (1) sympetalous, asymmetric flowers, (2) inferior ovaries with three carpels, (3) a single fertile carpel with one anatropous ovule, (4) an achene type fruit and (5) the absence of endosperm in the ripe seed (Bell 2004). The corolla has five lobes and the calyx can be absent, toothed or form a pappus (Simpson 2006).

The following genera are usually included within the Valerianaceae family: *Centranthus*, *Nardostachys* DC*., Patrinia* Juss., *Phyllactis* Pers*., Plectritis*, and *Valeriana* L. (Bell *et al*. 2001, Donoghue *et al*. 2001). The position of another genus, *Triplostegia*, has been debated, and it is sometimes placed in the Valerianaceae and sometimes into the Dipsacaceae when it is not assigned to its own family (Zhang *et al*. 2002). Recent nuclear and chloroplast sequence data suggest that *Triplostegia* fits more closely with Dipsacaceae than Valerianaceae (Bell 2004, Hidalgo *et al*. 2004), however morphological characteristics and the presence of valepotriates, chemical compounds only known within the Valerianaceae, suggest inclusion within the latter. Additional studies must be done with *Triplostegia* to resolve this uncertainty (Hidalgo *et al*. 2004).

Relationships between species in the Valerianaceae are not fully understood, with various interpretations presented in the literature. According to recent plastid and nuclear data, the group is monophyletic (excluding *Triplostegia*) and includes four groups (Asian, Mediterranean, Eurasian and American) which coincide with the Valerianaceae's biogeography (Hidalgo *et al*. 2004). The Asian group includes *Patrinia*, *Nardostachys* and *Valeriana hardwickii* Wall. (Hidalgo *et al*. 2004). *Patrinia* and *Nardostachys* are the basal most lineages (Bell 2004, Caputo

and Cozzolino 1994), together forming a well-supported clade (tribe Patrinieae) (Hidalgo *et al*. 2004). Two clades make up the Mediterranean group, one including *Fedia* Gaertn. and *Valerianella* Mill. and the other including *Centranthus* and *Valeriana longiflora* Willk. (Hidalgo *et al.* 2004). The Eurasian group also forms two clades, each comprising several different *Valeriana* species (Hidalgo *et al*. 2004). The American group includes other *Valeriana* species and *Plectritis*, although it is not strongly supported (Hidalgo *et al*. 2004). *Plectritis*, like *Centranthus*, possesses a corolla nectar spur (Ganders *et al*. 1977a), however, in some populations the spur is reduced to a small bump at the base of the corolla tube (Ganders *et al*. 1977b). These are the only spur-bearing members of the Valerianaceae (Table 1.1).

1.5.1.2.1 *Centranthus ruber* (L.) DC.

The genus *Centranthus* originates from the Mediterranean region and is known to comprise nine (Richardson 1975) to >12 species (Larsen 1958). It has some commercial value as a cultivated ornamental species (Simpson 2006), however it is not extremely commercially important and has therefore not been studied in much detail (Richardson 1975). Linnaeus originally designated *C. ruber* as *Valeriana rubra* L., assuming it was closely related to *Valeriana officinalis* L., however it was later given its own genus *Centranthus* which comes from the Greek for 'spur flower' (Shenton 2006). Therefore, although *C. ruber* is an ornamental species, it was not likely to have been bred for its nectar spur.

Centranthus ruber has asymmetric flowers, each with a single lateral stamen which is situated beside its fertile carpel in the dorsal part of the flower (Donoghue *et al*. 2003). The genus is also characterized by a calyx which develops into a plumose pappus during fruiting (Richardson 1975). The five-petalled flowers each possess a single spur (Fig. 2.1D) located on the lower abaxial surface of the corolla tube (Roels and Smets 1996, Simpson 2006). Early in

development, the spur appears as a small bump at the base of the corolla tube (Roels and Smets 1996) which continues to lengthen until anthesis. The spur is quite small and delicate, measuring on average less than a half centimetre in length at maturity; however, in relation to the flower size itself, the spur is considered to be quite long (Weberling 1989). Despite the evolutionary significance of nectar spurs, very little research has been done on the floral spur, particularly regarding the anatomical features of nectary tissue within the spur and to its growth and development.

Centranthus ruber was selected for this study for several reasons. Firstly, *C. ruber* produces many flowers per inflorescence, therefore plant material of all developmental stages is readily available at all times. Secondly, very little information is currently available in the literature about the anatomy, ultrastructure and growth pattern of *C. ruber*'s spur; this study will contribute new findings to the limited literature available on nectar spurs. Thirdly, TEM investigation of nectaries within the Dipsacales appears to be limited to *Lonicera japonica* Thunb. ex Murray (Fahn and Rachmilevitz 1970, Fahn 1979) and *L. kamtschatica* (Weryszko-Chmielewska and Bozek 2008), providing another opportunity to contribute new information to the field. Finally, there have been few studies of nectar carbohydrate composition throughout flowering phenology in any spurred species, to date.

The ecological importance of nectar spurs is evidenced by their ability to be involved in the evolution of mutualistic, specialized pollinator interactions, which can sometimes lead to speciation. Since nectar spurs represent an under-studied segment of floral research, there are many unanswered questions and many opportunities for novel studies which could add valuable information to this field.

1.6 Objectives

1.6.1 Objective 1: Investigation of the morphological, anatomical and ultrastructural characterisitcs of the spur and floral nectary of *Centranthus ruber*

The first objective was to investigate the morphological, anatomical and ultrastructural characteristics of *C. ruber*'s spur and nectary at different developmental stages, using LM, SEM and TEM. Determination of the nectary position and type were key objectives, along with describing the features of the nectariferous tissues, including any associated vascular tissue.

1.6.2. Objective 2: Analysis of the spur growth pattern in *Centranthus ruber*

The second objective was to assess the patterns of cellular division and growth which cause the outgrowth of the spur from the corolla tube in order to understand how spur growth occurs in *C. ruber*. SEM micrographs were used to carry out single cell file counts and cell length and width measurements along the spur's outer epidermis. Confocal and Apotome microscopy were used to assess the timing and location of cell division activity in developing spurs stained with 4',6-diamidino-2-phenylindole (DAPI), a nuclear fluorochrome.

1.6.3. Objective 3: Analysis of nectar secretion dynamics and nectar composition in *Centranthus ruber*

The third objective was to analyze the nectar of *C. ruber*, to determine the volume, concentration and carbohydrate composition throughout development in order to complement the structural investigation of *C. ruber*'s nectary with qualitative and quantitative nectar data. Nectar carbohydrate composition was determined using HPLC.

Chapter 2: MORPHOLOGICAL, ANATOMICAL AND ULTRASTRUCTURAL INVESTIGATIONS OF THE NECTAR SPUR AND FLORAL NECTARY OF *CENTRANTHUS RUBER*

2.1 Introduction

All nectar spurs share a hollow, tubular morphology because they all serve to house secreted nectar, thereby creating a barrier between this valuable resource and potential pollinators. However, because nectar spurs have independently arisen in at least 15 angiosperm families (Hodges 1997), particular morphological characteristics vary significantly between groups. As discussed in Chapter 1, variations in spur length, shape, position, colour and curvature can be related to the preferences, morphologies and habits of their pollinators. Particularly, the length of the spur tends to correlate with the proboscis length of its pollinators. Flowers with exceptionally long spurs such as those of the flower commonly known as Darwin's Orchid (*Angraecum sesquipedale*) from Madagascar can measure upwards of 30cm and are pollinated by hawkmoths with extraordinarily long proboscides (Little *et al*. 2005), whereas some spurs, such as those of *Lobelia cordifolia*, measure mere millimetres in length, suggesting association with much shorter-tongued pollinators (Ayers 1990).

The tissues which synthesize and secrete nectar are known as nectaries and, throughout the literature, various terminology has been used to describe the different tissue types of which they are composed. The terminology which is most generally accepted, and will be used to describe the nectary of *Centranthus ruber*, was proposed by Nepi (2007), who divides the nectary into three different regions: the nectary epidermis, the nectary parenchyma and the sub-nectary parenchyma. If the nectary has a vascular connection, it generally arises from the sub-nectary parenchyma, a tissue which is composed of more loosely packed parenchyma cells than the nectary parenchyma and which is not directly involved in nectar secretion (Fahn 1979, Nepi

2007).

The morphological characteristics of the inner spur surfaces and the anatomical characteristics of the spur tissues can also vary because, in some species, the nectar producing tissues are located within the spur, e.g., *Impatiens capensis* (Marden 1984), *Aquilegia* spp. (Tucker and Hodges 2005), *Platanthera chlorantha* (Stpiczynska 2003a, 2003b), and many others, whereas a smaller proportion of spurs are merely a reservoir for nectar secreted elsewhere, e.g., *Linaria vulgaris* (Nepi *et al*. 2003), *Corydalis, Dicentra* and their relatives (Endress and Matthews 2006). If the spur houses the nectary, there is commonly a vascular connection within the subnectary parenchyma which provides nectar components from the phloem (Fahn 1979). Spurs with nectaries located within can have nectar secreting structures such as modified stomata (e.g. *Tropaeolum majus*, Rachmilevitz and Fahn 1975) or trichomes, e.g., *Ascocentrum curvifolium* (Lindl.) Schltr. (Stpiczynska *et al*. 2011) *Platanthera chlorantha* (Stpiczynska 2003a, 2003b), for secretion. Other species do not have specialized nectar secreting structures, simply possessing a secretory epidermis with a permeable cuticle through which nectar exudes, e.g., *Limodorum abortivum* (Figueirdo and Pais 1992), *Sedirea japonica* (Linden & Rchb.f.) Garay & H.R.Sweet (Stpiczynska *et al*., 2011). Although many spurs contain nectar, numerous spurred species, particularly within the Orchidaceae, are deceptive and do not house nectar (Boyden 1982, Lammi and Kuitunin 1995, Sletvold *et al*. 2010).

The ultrastructure of the nectariferous tissues inside of the nectar spur will vary based on the type of nectary and the method of nectar secretion. Ultrastructural changes can be difficult to study because there is constant flux occurring in multiple tissues which are involved in the secretion process (Pacini and Nepi 2007). However, by observing multiple samples, observations of ultrastructural differences throughout development give an indication of the secretion process

(Stpiczynska *et al*. 2005). For example, if there are plastids within the nectariferous tissues which contain a large number of starch grains prior to secretion, but fewer or none as secretion progresses, it can be deduced that some of the starch was hydrolyzed to produce nectar sugar components or used as an energy source for the secretion process (Nepi *et al*., 1996). The storage of starch for use in nectar production is common in nectaries which have a high rate of nectar production (Belmonte *et al.* 1994, Nepi *et al.* 2001). Increased numbers of one of more of the following: mitochondria, Golgi bodies and ER profiles (Kronestedt *et al*. 1986, Figueriedo and Pais 1992, Stpiczynska *et al*. 2005, Wist and Davis 2006) were also reported during nectar secretion. These organelles are presumably involved in the production and modification of nectar components or in the production of energy for fueling the secretion process.

This study will analyze the morphological, anatomical and ultrastructural changes in *C. ruber*'s spur and nectary tissues at seven developmental stages, from immature bud to mature flower at anthesis (Table 2.1). Scanty morphological or anatomical information is available in the literature regarding the spur and floral nectary of *C. ruber.* Additionally, no ultrastructural studies on members of the family Valerianaceae are currently available in the literature. However, work on *Lonicera japonica* and *Lonicera kamtschatica* (family Caprifoliaceae, order Dipsacales) has been done and may provide the best source for comparison with *C. ruber* (Fahn and Rachmilevitz 1970, Weryszko-Chmielewska and Bozek 2008). Both *Lonicera* species have short spurs which house trichomatous nectaries, with unicellular trichomes comparable to those of *C. ruber*.

Table 2.1 - Stages of floral development of *Centranthus ruber* investigated. Bud and spur dimensions are given as mean ± s.e.

Stage	Bud/flower length (mm)	Spur length (mm)	Physical characteristics
1	1.05 +/- 0.16, n=10	0.06 +/- 0.02, n=10	Immature buds, based on bud length (0.52-1.53 mm)
2	2.15 +/- 0.38, n=29	0.14 +/- 0.06, n=29	Immature buds, based on bud length (1.56-2.99 mm)
3	3.86 +/- 0.68, n=18	0.54 +/- 0.23, n=18	Immature buds, based on bud length (3.01-5.19 mm)
4	6.45 +/- 0.81, n=10	1.40 +/- 0.23, n=10	Closed, immature flowers, pale green petals
5	10.20 +/- 1.29, n=15	2.93 +/- 0.59, n=15	Closed, nearly mature flowers, petals still pale green
6	15.65 +/- 1.75, n=10	4.30 +/- 0.71, n=10	Closed, nearly mature flowers, white petals
7	16.70 +/- 1.25, n=10	4.50 +/- 1.08, n=10	Fully opened flowers at anthesis, white petals.

2.2 Materials and methods

This study used plants of *Centranthus ruber* (var. "Snowcloud") (catalogue code #8281). Seeds obtained from Thompson & Morgan (Poplar Lane, Ipswich, England) were sown in Sunshine Mix 1® (Sun Gro Horticulture Canada Ltd, Vancouver, B.C.) and the plants were watered as required on a greenhouse bench. Fertilizer (N-P-K = 20-20-20) was supplied weekly. Over the course of the project, four different *C. ruber* plants were used. A single plant was used for morphometric measurements, but they were confirmed with a second plant which showed no significant differences from the first.

2.2.1 Designation of floral development stages

Seven stages of floral development (Table 2.1) were designated based on morphological characteristics (Stages 4-7) and bud size (Stages 1-3). Lengths of the bud and spur at Stages 1-3 were measured under the compound microscope equipped with a micrometer. Lengths at Stages

4-7 were measured using fresh material under the dissecting microscope using a ruler with 0.5mm increments. The spur was measured from the point of attachment above the ring of sepals situated above the ovary (Fig 2.1F). The flower length was measured from the corolla tip to the base of the ovary. One plant was used for all of the morphometric measurements, which were confirmed with a second plant, showing no significant differences from the first plant. Stages 1-3 are early stages of development in which the buds are still tightly packed and partially concealed due to the tight association of the subtending bracts and surrounding buds (Figs. 2.1B, C). Stages 4-5 are mid-developmental stages in which the buds become fully visible, but the corolla remains pale green. These two stages are distinguished by bud size, with Stage 5 buds being closer in size to Stage 6 buds. Stages 6-7 are the later stages of floral development. At Stage 6, the petals are still closed, but they have turned the mature, white colour. At Stage 7, the flowers have reached anthesis (Figs. 2.1C-E) and nectar begins to accumulate in the floral nectar spur.

2.2.2 Light microscopy

Flowers from Stages 1-7 were prepared following a protocol similar to that described in Wist and Davis (2006). Flowers were fixed with 2.5% glutaraldehyde (GA) in 25mM sodium phosphate buffer, pH 6.8, post-fixed with 1% osmium tetroxide in 25mM sodium phosphate buffer and then dehydrated in a graded ethanol series. Propylene oxide was added dropwise over several hours, followed by two changes of 100% propylene oxide. The samples were left overnight on ice and then Araldite resin was added dropwise over several hours. The vials were covered with pin-poked tinfoil and left in the fume hood for several days, allowing the propylene oxide to evaporate. Once all of the propylene oxide had evaporated, the samples were put into TEM molds and the resin was allowed to harden overnight at 60ºC. Samples were sectioned into semi-thin (1.0-1.5 microns) sections with a Reichert-Jung Ultracut microtome. A drop of 2.5%

Toluidine Blue O (TBO) stain in sodium carbonate buffer at pH 11.1 was placed on top of each section and the slides were slowly moved over a flame, without allowing the stain to boil. The stain was rinsed off with distilled water and the slides were left to dry. Coverslips were applied using Permount® (Fisher Scientific) mounting solution and left to dry on slide-warming trays under metal weights. Slides were observed using a Zeiss Axioplan Fluorescence microscope equipped with a AxioCamICc1 CCD digital camera. Micrographs of the spur at different stages of development were taken using AxioVision 4.7 imaging software to examine the anatomical changes of the spur which occur as development progresses.

Some additional Stage 7 flowers were cleared with heat and lactic acid (85%, left to incubate at 60°C for 4 hours) and then stained with Neutral Red to stain the vascular bundles of the flower, including the spur.

2.2.3 Scanning electron microscopy

Spurs of all stages, both intact and pre-bisected or cross-sectioned to reveal the nectary trichomes within, were prepared for SEM by fixation in 2.5% GA in 25mM sodium phosphate buffer, pH 6.8, followed by post-fixation in 1% osmium tetroxide in the same buffer and then rinsed with distilled water before dehydration with a graded acetone series. Samples were critical point dried, mounted onto SEM stubs, gold coated and viewed with a Philips 505 scanning electron microscope at 30kV. The film used to capture the images was Fuji FP-100B black-and-white instant film.

2.2.4 Transmission electron microscopy

Samples were prepared for TEM using a protocol similar to that described in Wist and Davis (2006). Flowers from all seven developmental stages were embedded in Araldite resin as

in section 2.2.1.1. Ultrathin sections (60-80nm) were prepared using a Reichert-Jung Ultracut microtome. The sections were mounted onto formvar-coated slot grids, stained with uranyl acetate (2% aqueous) and lead citrate and then rinsed with 0.02M NaOH. Micrographs were taken using a Philips CM10 transmission electron microscope. An accelerating voltage of 100kV and an emission setting of 4 were used to capture images.

The TEM micrographs were analyzed to determine the ultrastructural changes occurring during spur development and the nectar secretion process. Changes in the organelles present in the nectariferous tissues and the extent of wall ingrowth formation along the outer wall of the secretory trichomes were assessed. The number of plastid profiles (with and without starch grains) present in sections through the secretory trichomes, nectary epidermal cells and nectary parenchyma cells at different developmental stages, were compared. The magnifications of the images used to obtain the plastid profile and starch grain counts ranged from 1200x to 8900x. Cell wall and cuticle thicknesses were compared between secretory trichomes, nectary epidermal cells and epidermal cells of the outer spur surface, throughout development. The magnifications of the images used to obtain the cell wall and cuticle measurements ranged from 1200x to 15500x. Image brightness and contrast were corrected using Adobe Photoshop CS3 Extended.

2.3 Results

2.3.1 Inflorescence characteristics

C. ruber is a perennial plant which produces numerous showy, cymose inflorescences on spreading stems which have woody bases (Fig. 2.1A). *C. ruber* inflorescences are composed of terminal clusters of small, five-petalled flowers, arranged in compound cyme formations (Figs. 2.1B, C).

2.3.2 Flower and nectar spur morphology

2.3.2.1 Light and scanning electron microscopy

Flowers of *C. ruber* (var. "Snowcloud") have five white petals and are asymmetrical, each with a single lateral stamen and single carpel (Figs. 2.1D, E). Rarely, flowers with three stamens were observed. Each flower has a single nectar spur, located at the base of the corolla tube, running alongside the ovary (Fig. 2.1D, F). In less than 1% of flowers, two nectar spurs were observed, often also displaying six, rather than five, petals (Figs. 2.1G, H). Some two-spurred flowers appear to have the second spur growing off the first, or forking from a common point of growth (Fig. 2.1G), whereas others have the second spur on the other side of the corolla tube, 180 degrees from the first. The first type was seen much more frequently. The point of attachment of the spur is in line with the ring of sepals, which remain coiled up until fruit dispersal when the sepals unfurl into a plumose pappus (Figs. 2.1F, I). A single, non-branching vascular bundle runs down the abaxial and back up the adaxial side of the spur (Figs. 2.2A-D). The nectary is located on the inner, abaxial surface of the spur and is composed of unicellular secretory trichomes (Figs. 2.2B, D-G).

The unicellular, secretory trichomes initiate in Stage 3 as small protrusions from the nectary epidermis, above or interior to the abaxial vascular bundle (Fig. 2.2B). In later development, the trichomes develop a complex labyrinth of wall ingrowths along the inner margins of the primary wall (Fig. 2.2E). As nectar is secreted, the cuticle layer atop the outer cell wall begins to lift upwards, creating a subcuticular space wherein the nectar temporarily accumulates until its release at anthesis (Fig. 2.2E). No pores were observed in the outer cuticle of the secretory trichomes to expedite nectar release. The inner spur epidermal cells in the nectary region are elongated and have a smooth contour (Figs. 2.2F left, G). In addition to

secretory trichomes, the spur also has unicellular, non-secretory trichomes which originate after Stage 3, line the remaining surface of the spur (Fig. 2.2A), and are most commonly directed towards the spur tip (Figs. 2.2A, D, F). These non-secretory trichomes are long, pointed and have distinct cuticular ornamentation that apparently initiates at the trichome base and proceeds acropetally (Fig. 2.2F). The inner spur epidermal cells lining the remainder of the spur, where the non-secretory trichomes are located, have undulating contours typical of epidermal cells (Fig. 2.2 right). The average densities of the secretory trichomes and non-secretory trichomes (10 spurs sampled for each trichome type) at Stage 7 were 3.55/0.1mm² (s.e. =0.096, n=258) and 0.78/0.1mm² (s.e. =0.043, n=294), respectively.

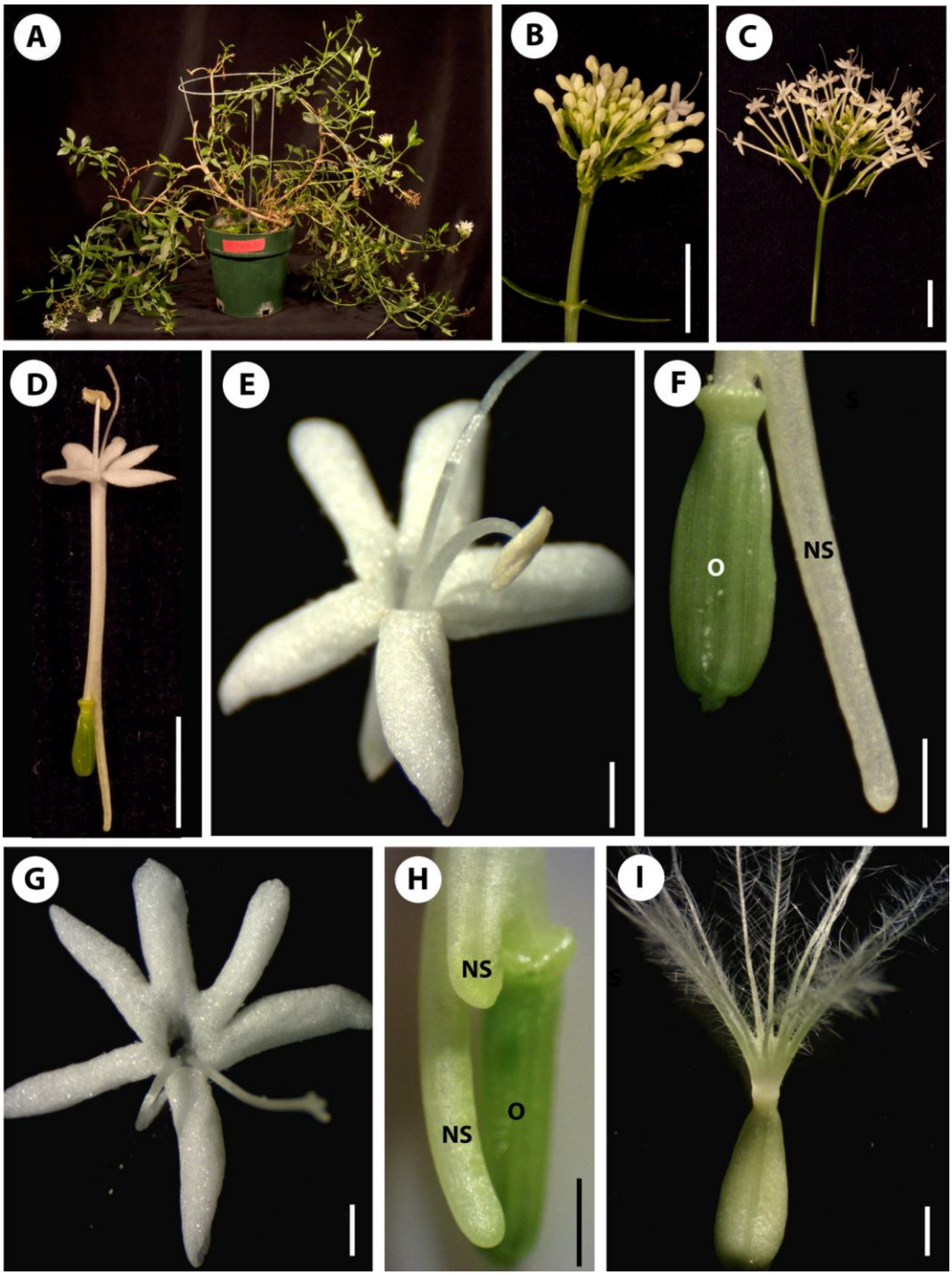

Figure 2.1 - *C. ruber* growth habit and floral morphology. A: Plant showing sprawling growth habit. Numerous stems, which are woody at their bases, give rise to terminal inflorescences. B: Immature cymose inflorescence with closed buds. Buds are packed tightly together in early development and each bud is subtended by a single bract. Until Stage 5, the petals are pale green, turning white at Stage 6. C: Mature inflorescence with flowers at anthesis (Stage 7). D, E: Typical flowers each have 5 petals, a single pistil and a single stamen. F: Floral nectar spur at anthesis, wherein the typical nectar spur measures 4.5mm. G, H: Abnormal flower with six petals and two nectar spurs. I: Wind-dispersed achene. At maturity, the ring of sepals which sits upon the ovary unfurls into a plumose pappus for dispersal. O - ovary, S - sepals, NS - nectar spur. Scale bars B, C=1.0cm, D=0.5cm, E-I=0.1cm.

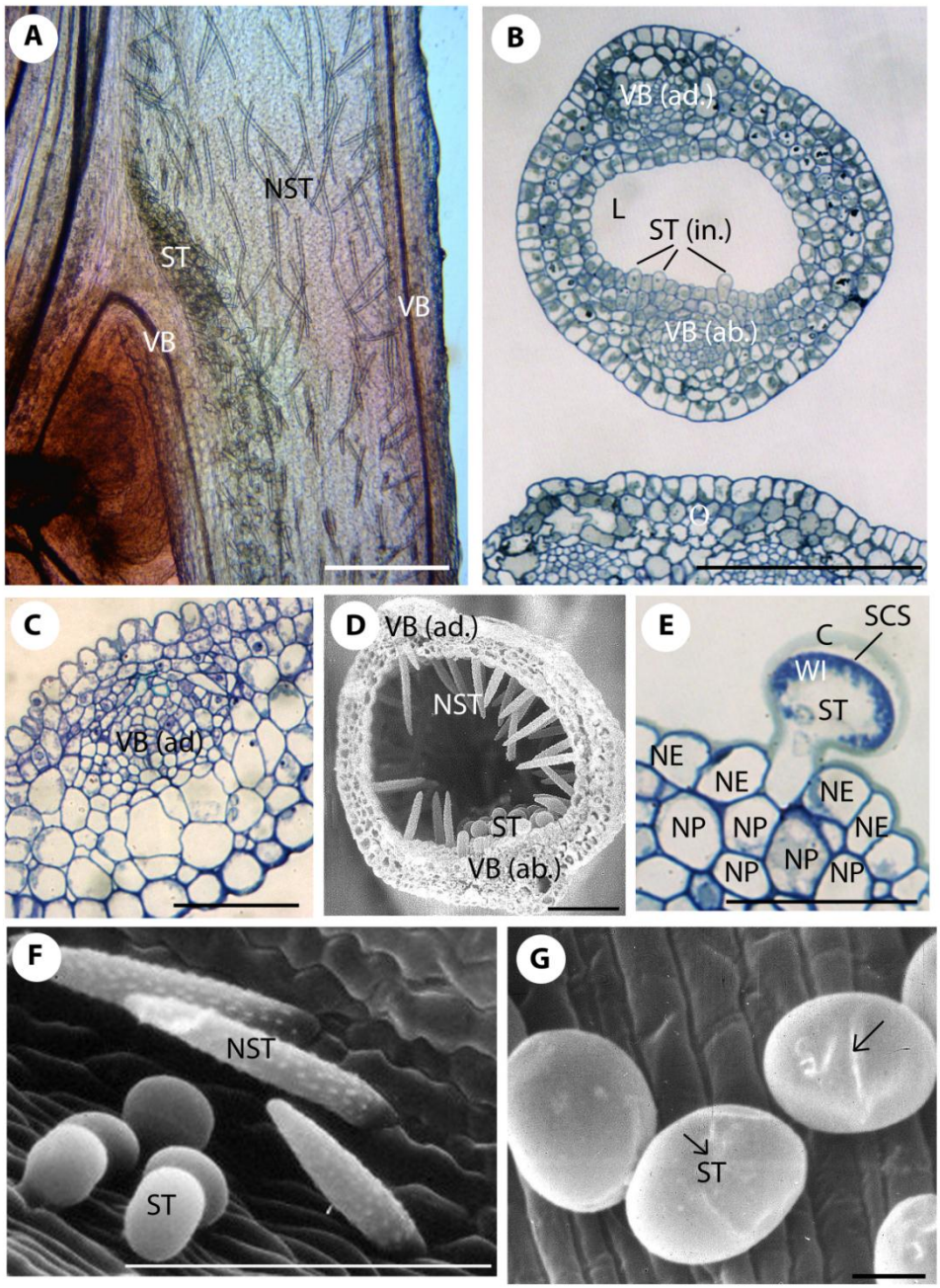

Figure 2.2 – *C. ruber* nectar spur morphology and anatomy. A: Spur stained with Neutral Red. A single vascular bundle runs down the abaxial and back up the adaxial side of the spur. Unicellular secretory trichomes run along the entire length of the abaxial vascular bundle. Non-secretory trichomes sparsely cover the remaining internal surface of the spur. B: Cross section through an immature spur (Stage 3) showing initiating secretory trichomes. C: Cross section through adaxial vascular bundle of the spur, showing both xylem and phloem. D: View down a transverse section of a fully developed nectar spur showing the orientation of the secretory and non-secretory trichomes. E: Cross section through a mature spur (Stage 7) containing a fully developed secretory trichome showing the distended cuticle and wall ingrowths. F: Morphology of the secretory and non-secretory trichomes. G: Morphology of the secretory trichomes showing indentations (arrows). Ab - abaxial, Ad - adaxial, C - cuticle, L – lumen, NE - nectary epidermal cell, NP - nectary parenchyma cell, NST - non-secretory trichomes, O - ovary, SCS – sub-cuticular space, ST (in.) - initiating secretory trichomes, ST - secretory trichomes, VB - vascular bundle, WI - wall ingrowths. Scale bars A, B=0.5mm, C, E= 0.05mm, D, F=0.1mm, G=10µm.

2.3.2.2 Transmission electron microscopy

2.3.2.2.1 Secretory trichomes

The unicellular, secretory trichomes initiate in Stage 3 as small protrusions from the nectary epidermis (Figs. 2.3A, C). At this stage, the nucleus takes up a large portion of the cell and the cytoplasm contains numerous plastids which lack starch grains (Figs. 2.3A, C). Profiles of ER were often observed near the cell wall (Figs. 2.3B, D). Near the top of the developing trichomes, large vacuoles tend to take up most of the cell space and often contain flocculent, osmiophilic material (Figs. 2.3A-C). At Stage 4, the trichomes begin to take on their characteristic shape, with many showing indentations in the middle of their tips (Figs. 2.4A-C). This concavity is only seen on some trichomes and, at maturity, some trichomes have this feature and others do not (Fig. 2.2G). During mid-development, at Stages 4 and 5, the nuclei (Figs. 2.4B-D) and vacuoles (Figs. 2.4A, B, 2.5A-D) still take up a large portion of these cells. Plastids, some with starch grains (Figs. 2.4A-C, 2.5B, C), and mitochondria (Figs. 2.4B, 2.5B, C) were the dominant organelles observed in these stages. In the later stages of development (Stages 6-7), the cytoplasm is filled with numerous organelles and vesicles, often difficult to distinguish, however plastids and mitochondria continue to be present (Figs. 2.6A-C, 2.7C). Wall ingrowths along the outer primary wall of the trichomes begin to appear during Stage 6 (Figs. 2.6A, B). The ingrowths form a very complex network which vastly increases the secretion surface area (Figs. 2.2E, 2.7A, B). At Stage 7, numerous vesicles with a vacuole-like appearance were observed near the tip of the secretory trichomes (Fig. 2.7A). Throughout development, the number of plastid profiles per cell increases dramatically from Stage 4 to Stage 5, peaking at Stages 5-6 and then decreasing by anthesis (Stage 7) (Fig. 2.8). The average number of starch grains per plastid profile shows an initial increase between Stages 3 and 4, followed by a continual decrease in all

developmental stages that followed (Fig. 2.9). Average wall thickness of the outer primary wall varies little throughout development (Fig. 2.10), but the underlying wall ingrowth layer and the cuticle both increase in thickness (Figs. 2.10, 2.11). At Stage 6, the average thickness of the wall ingrowth layer was 0.183µm ± 0.055 (s.e.; n=5), whereas at Stage 7 it had increased to 0.412µm ± 0.198 (s.e.; n=5). In later stages of development, the cuticle shows uneven thickenings (Figs. 2.5B, C, 2.7A). No pores were detected in the outer cuticle with TEM, nor with SEM. Early in trichome development (Stages 3-4), the cuticle remains in contact with the cell wall (Figs. 2.3A-D, 2.4A-D). At Stage 5, the cuticle begins lifting up a small amount creating a subcuticular space on some trichomes (Fig. 2.5D) and in later developmental stages (Stages 6-7), the cuticle continues to lift higher upwards as nectar is secreted beneath (Figs. 2.6A, B, 2.7A).

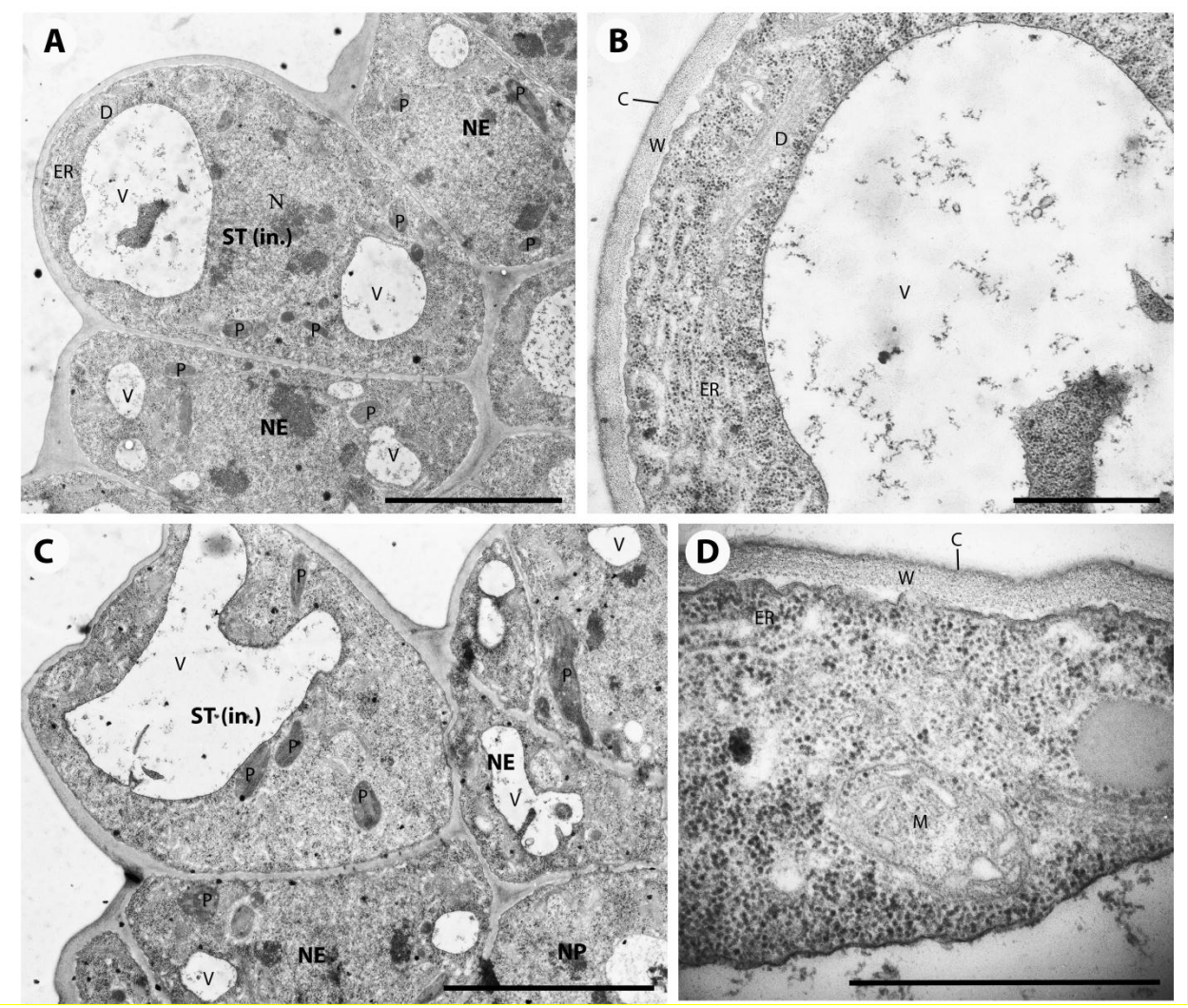

Figure 2.3 - Ultrastructure of the secretory trichomes of the floral nectary in *Centranthus ruber* spurs early in development (Stage 3). A: Initiating secretory trichome (ST (in.)). The trichome begins development as a small mound protruding above the level of the nectary epidermal (NE) cells. A large portion of the vacuole is situated near the trichome tip. Plastids (P) are present in the cytoplasm, but do not contain starch grains. B: Close-up near the tip of the developing trichome in A. Dictyosomes (D) and endoplasmic reticulum (ER) are present near the outer primary wall (W). The cuticle (C) is thin and in direct contact with the outer wall. The vacuole (V) contains some osmiophilic materials. C: Another developing secretory trichome and nectary epidermal cells. The tip of this initiating secretory hair is slightly indented at the top, as is seen in some trichomes, but not all. Again, the vacuole is large and present mainly near the tip of the trichome. D: Close-up at the tip of the trichome in C. A mitochondrion (M) and ER profiles are present near the cell wall. Scale bars A, C=5µm, B, D=1µm.

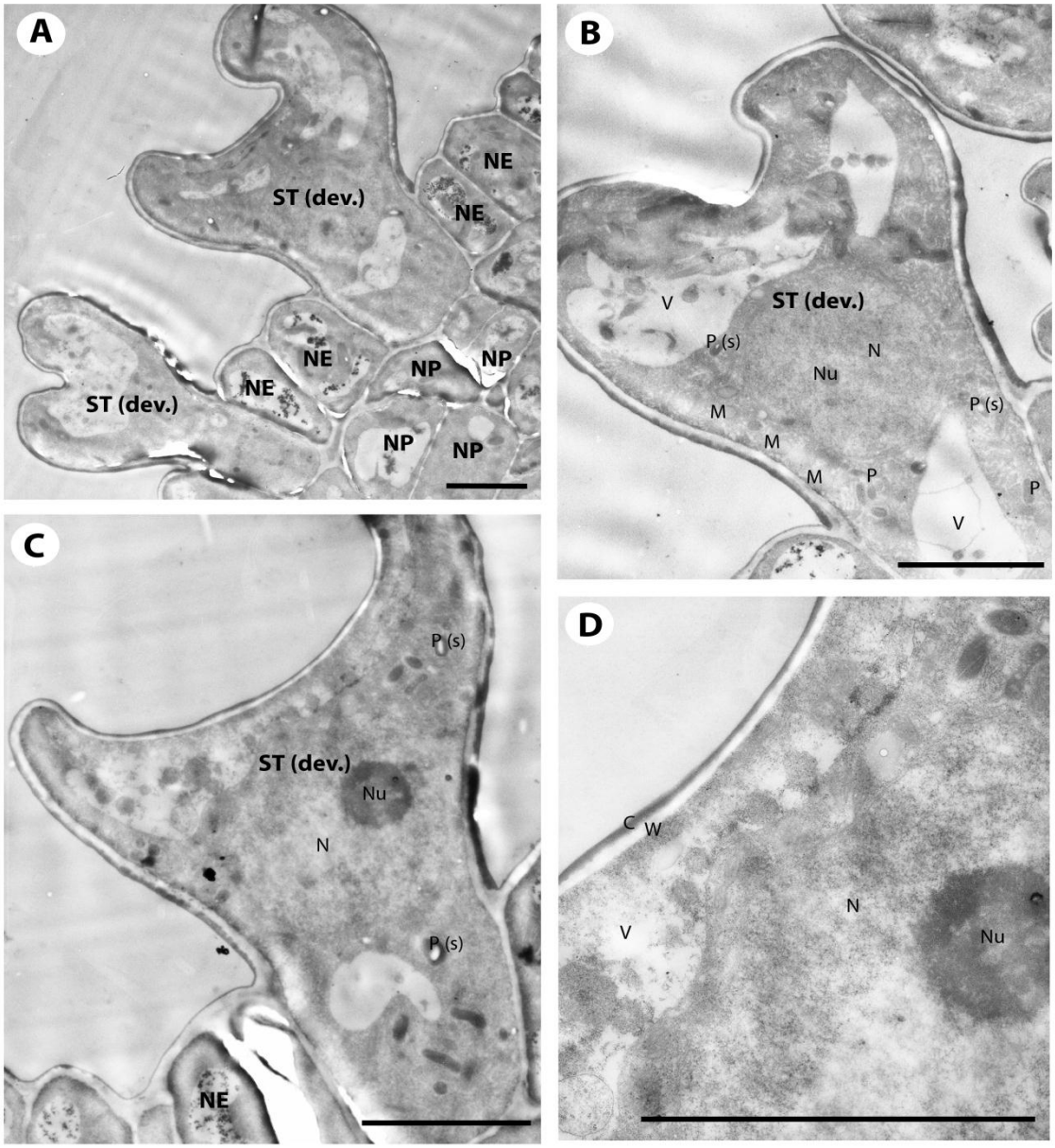

Figure 2.4 - Ultrastructure of the secretory trichomes of the floral nectary in *Centranthus ruber* spurs in mid-development (Stage 4). A: Two developing secretory trichomes (ST (dev.)), nectary epidermal cells (NE) and nectary parenchyma cells (NP). The trichomes show indentations at their tips, a common, but not universal, trait. The majority of the visible vacuoles are still near the tips of the trichomes. B: Developing secretory trichome. Plastids (P), some with starch (P (s)), and mitochondria (M) are present along the periphery of the cell. C: Developing secretory trichome. Numerous plastids are visible, some with starch grains. The nucleus (N), with a prominent nucleolus (Nu), is large and centrally located. D: Close-up near the tip of the secretory trichome in C. Thickness of the outer primary cell wall (W) and cuticle (C) is shown. Cuticle remains in contact with the cell wall at this stage. Scale bars A-D=5µm.

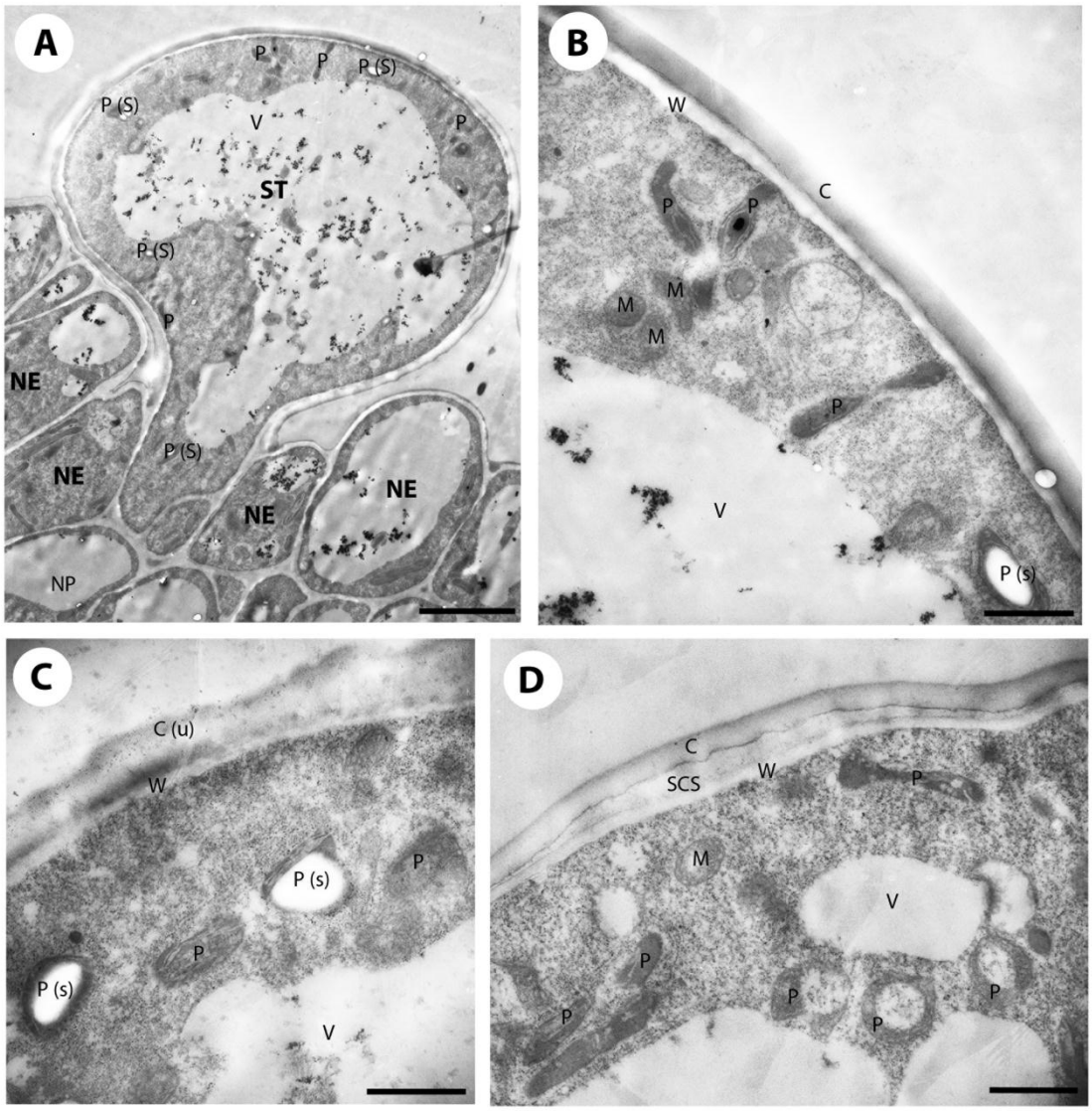

Figure 2.5 - Ultrastructure of the secretory trichomes of the floral nectary in *Centranthus ruber* spurs in mid-development (Stage 5). A: A developing secretory trichome (ST) and nectary epidermal (NE) cells. At this stage, the trichomes generally start to take on their characteristic shape. The vacuoles (V) remain large and take up a large portion of the cell. Plastids (P) are abundant in the cytoplasm and many contain starch grains (P (s)). B: Close-up of the trichome tip from trichome in A. The cuticle (C) has become considerably thicker in comparison to the outer primary cell wall (W) when compared with previous developmental stages. The cuticle shown is still in contact with the outer wall. C: Close-up near the tip of a secretory trichome. The cuticle is starting to have uneven thickenings in this stage. Plastids, some with starch, are located in the cytoplasm near the wall. D. Close-up near the tip of another secretory trichome. The cuticle is pulled away from the cell wall as nectar is presumably beginning to be secreted. Nectar collects in these subcuticular spaces (SCS) until it is released into the lumen of the spur at anthesis. In addition to plastids, several mitochondria (M) are also present near the trichome tip. Scale bars A=5µm, B-D=1µm.

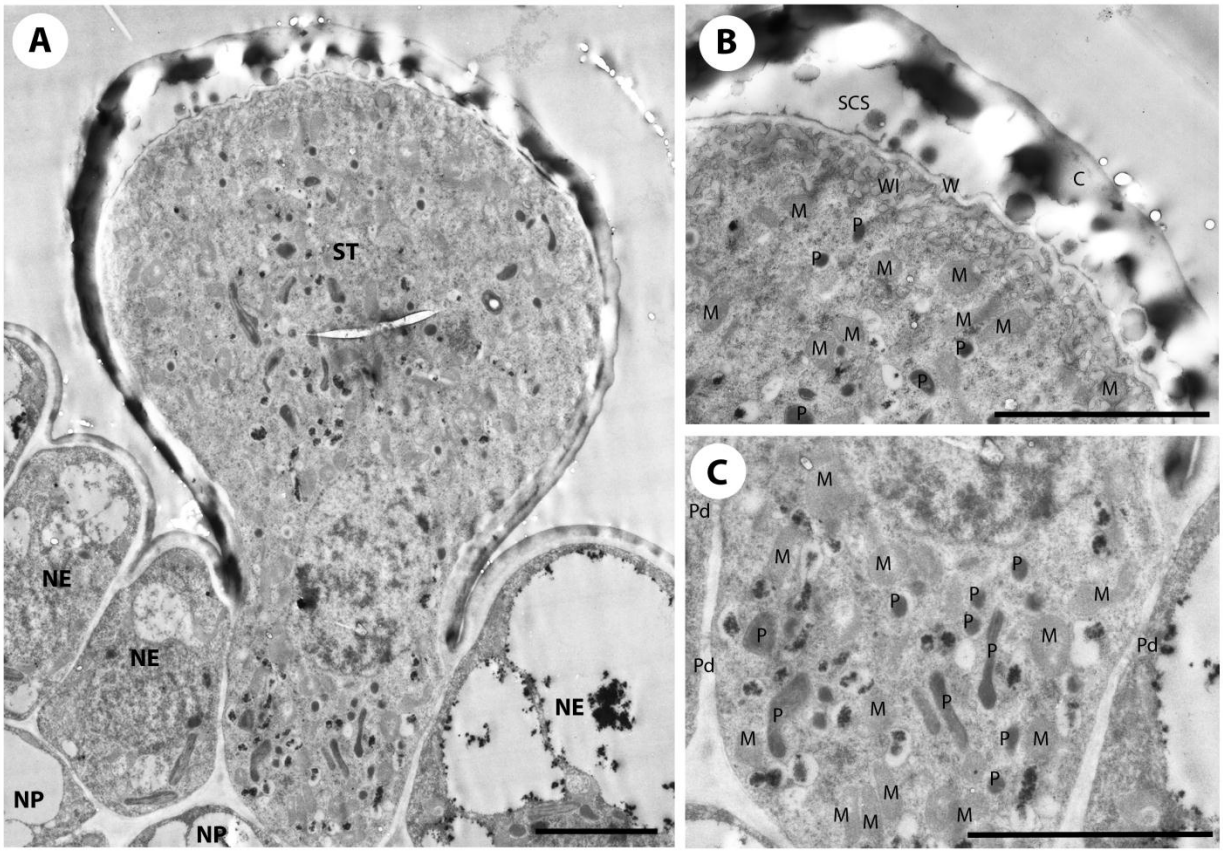

Figure 2.6 - Ultrastructure of the secretory trichomes of the floral nectary in *Centranthus ruber* spurs in later development (Stage 6). A: Secretory trichome (ST) and nectary epidermal (NE) cells during nectar production. At this stage, the vacuole becomes greatly reduced and the cytoplasm becomes dense and filled with organelles, often difficult to distinguish from each other. Numerous plastids (P) and mitochondria (M) are present. B: Close-up near the tip of the secretory trichome in A. The unevenly thickened cuticle (C) continues to be distanced from the cell wall as secretion of nectar occurs into the subcuticular space (SCS). Wall ingrowths (WI) begin to appear which increase the surface area of the plasma membrane for nectar secretion. C: Close-up of base of secretory trichome in A. Mitochrondria (M) and plastids (P) are plentiful. Plasmodesmata (Pd) occur in anticlinal walls adjacent to nectary epidermal cells. Scale bars A-C=5µm.

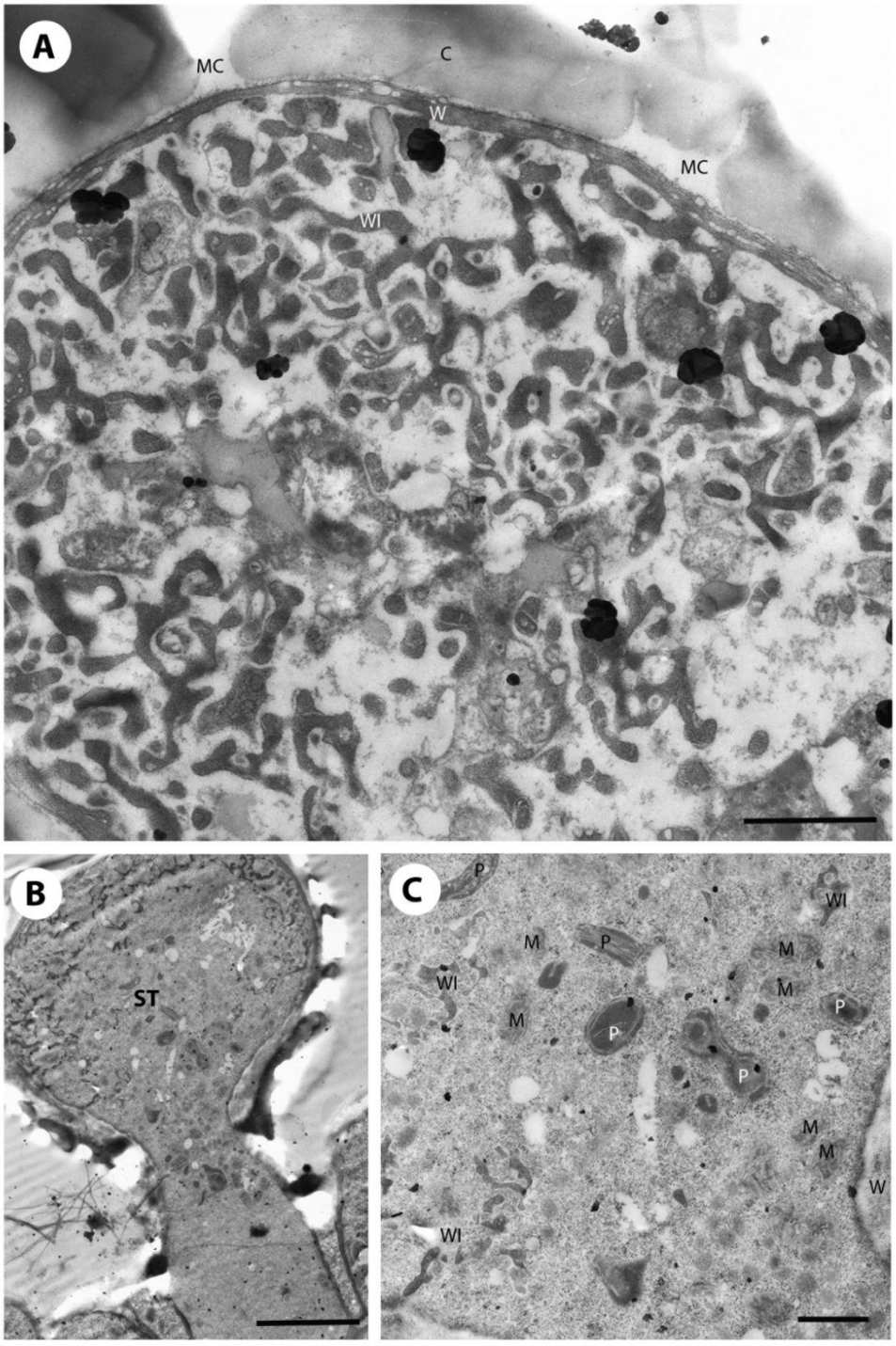

Figure 2.7 - Ultrastructure of the secretory trichomes of the floral nectary in *Centranthus ruber* spurs at anthesis (Stage 7). A: Close up of the thick layer of wall ingrowths (WI) present in the secretory trichome during nectar secretion. Microchannels (MC) are observed running through the cuticle (C), which may serve as a route of nectar passage into the lumen of the spur. B: Secretory trichome at anthesis with distended cuticle and wall ingrowths. C: Close up of the neck region of the secretory trichome in B, showing dense cytoplasm with numerous plastids (P), without starch grains, and mitochrondria (M). The primary wall is thickened at the base of the trichome's head region. Note that the wall ingrowths extend basally to this region. Scale bars A, C=1µm, B=5µm.

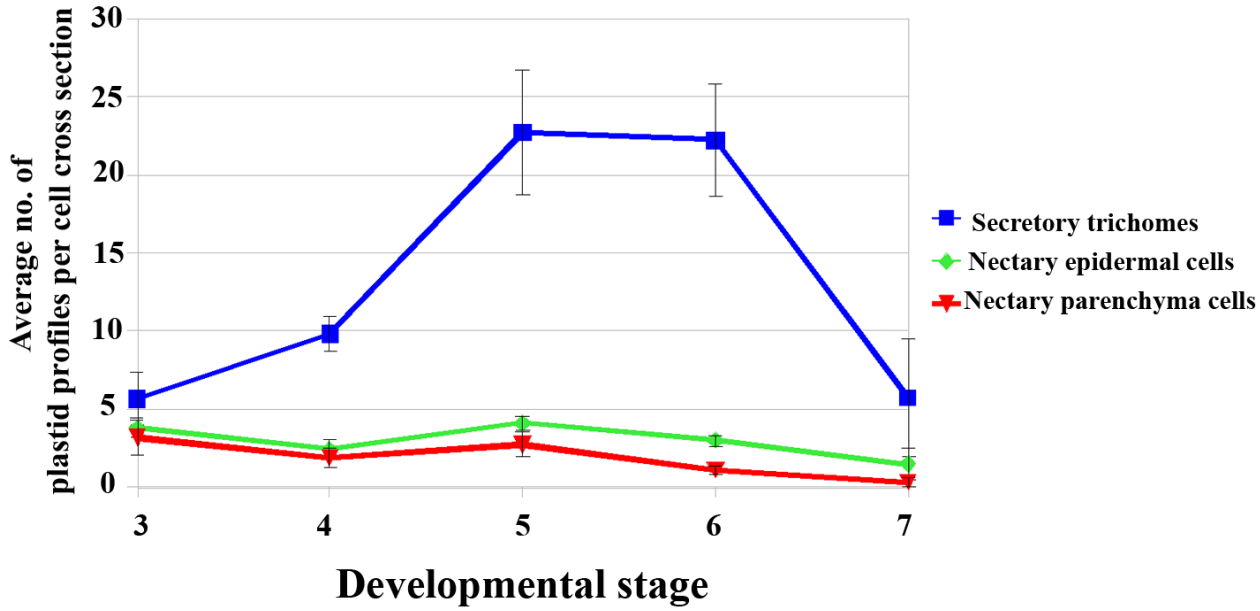

Figure 2.8 - Average numbers of plastid profiles (± s.e.) per cell in nectary tissues of the *Centranthus ruber* spur throughout mid and late development. This graph shows the average number of plastid profiles per cell cross section in the secretory trichomes, nectary epidermal cells and nectary parenchyma cells in spurs at Stages 3-7. The secretory trichomes have the greatest plastid numbers in all developmental stages, although there is no significant difference between the three cell types in Stage 3 and Stage 7. Plastid numbers in the secretory trichomes peak in Stages 5-6 and taper off by anthesis (Stage 7). The plastid numbers in the nectary epidermal and nectary parenchyma cells are significantly less than the secretory trichomes and neither shows any significant differences throughout development. Number of cells observed (n) for Stages 3-7: Secretory trichomes: 4, 6, 10, 5, 3. Nectary epidermal cells: 13, 11, 36, 10, 8. Nectary parenchyma cells: 5, 9, 16, 29, 9.

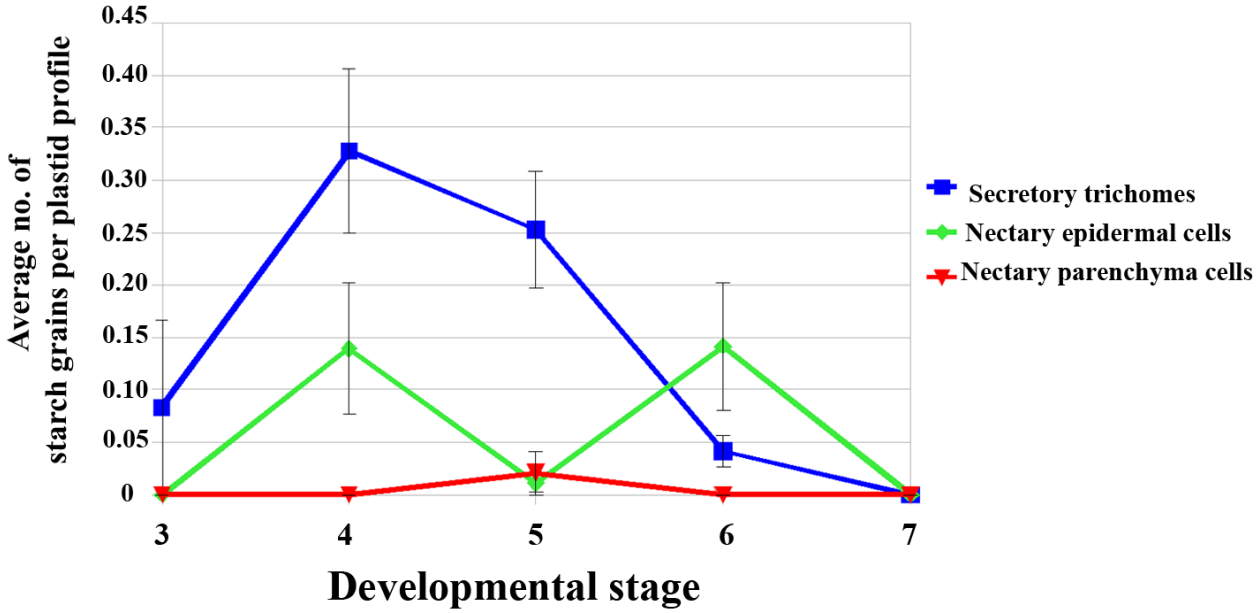

Figure 2.9 - Average number of starch grains per plastid profile observed in the nectary tissues within cross sectioned spurs of *Centranthus ruber* throughout development. This graph shows the average number (± s.e.) of starch grains per plastid in the secretory trichomes, nectary epidermal cells and nectary parenchyma in Stages 3-7. The secretory trichomes had the greatest number of starch grains per profile in Stages 4-5 (pre-secretion). These numbers fell markedly in Stage 6 and Stage 7 (anthesis). The nectary epidermal cells show no clear pattern in numbers of starch grains per profile throughout development. The nectary parenchyma cells also show no clear pattern, but starch is absent at almost every stage. Number of cells observed (n) for Stages 3-7: Secretory trichomes: 3, 6, 10, 5, 3. Nectary epidermal cells: 13, 11, 36, 10, 8. Nectary parenchyma cells: 5, 9, 16, 29, 9.

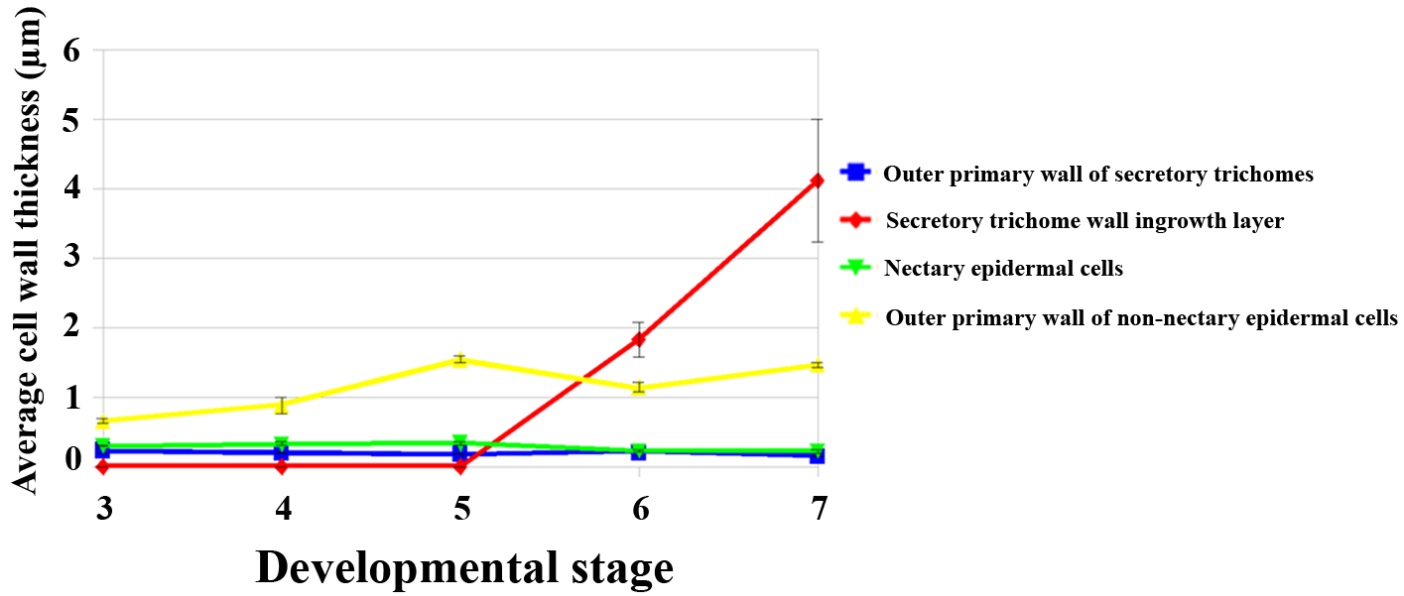

Figure 2.10 - Cell wall thickness in various tissues throughout spur development in *Centranthus ruber*. This graph shows the average (± s.e.) thickness (µm) of the outer primary cell walls of the unicellular secretory trichomes, the wall ingrowth layer of the unicellular secretory trichomes, the primary walls of the nectary epidermal cells and the periclinal walls of non-nectary epidermal cells on the outer surface of the spur throughout development. The outer epidermal cells have a much thicker cell wall than the inner nectary epidermal cells and the secretory trichomes. However, the secretory trichomes produce a layer of wall ingrowths, which is significantly thicker than the wall of the outer epidermal cells in the later stages of development (Stages 6-7). Number of cells observed (n) for Stages 3-7: Secretory trichomes: 3, 6, 13, 3, 7. Secretory trichomes ingrowth layer: 3, 6, 13, 5, 5. Nectary epidermal cells: 12, 20, 55, 15, 13. Outer epidermal cells: 7, 5, 4, 3, 3.

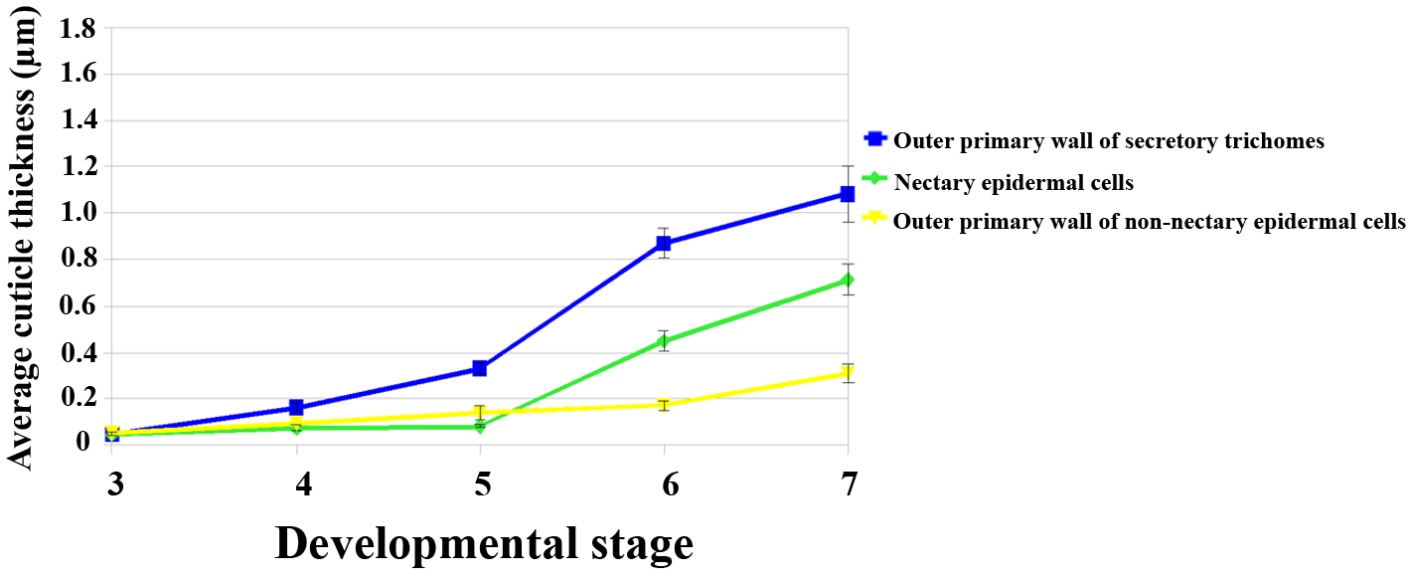

Figure 2.11 - Cuticle thickness in various tissues throughout spur development in *Centranthus ruber*. This graph shows the average (± s.e.) thickness (µm) of the cuticles of the unicellular secretory trichomes, nectary epidermal cells and outer wall of non-nectary epidermal cells on the outer surface of the spur throughout development. In all tissues there is a trend for increasing cuticle thickness as development progresses. Secretory trichomes have the thickest cuticles, followed by the nectary epidermal cells and the outer epidermal cells, which have the thinnest cuticles. Number of cells observed (n) for Stages 3-7: Secretory trichomes: 3, 6, 13, 3, 7. Nectary epidermal cells: 12, 20, 55, 15, 13. Outer epidermal cells: 7, 5, 4, 3, 3.

2.3.2.2.2 Nectary epidermal cells

Throughout development, the nectary epidermal cells are cytoplasmically dense and have small to large vacuoles which often contain osmiophilic materials (Figs. 2.5A, 2.6A, 2.12A, B, 2.13A, B). By anthesis (Stage 7), the vacuole represents most of the volume of each of these epidermal cells. Early in development, numerous plasmodesmata were observed between adjacent nectary epidermal cells (Figs. 2.12A-C) and between the nectary epidermal cells and secretory trichomes (Fig. 2.12D). The plastid numbers are significantly lower than in the secretory trichomes throughout development (Fig. 2.8). Starch grains are rare at Stage 3 (Figs. 2.9, 2.12A, B), but are present in some of the plastids in mid-development (Figs. 2.9, 2.13A, B). No starch grains were observed in any of these cells at anthesis (Stage 7), but the pattern of decreasing starch grains per plastid profile is not a clear trend (Fig. 2.9). The thickness of the outer primary wall is comparable to that of the secretory trichomes (Fig. 2.10), however these cells failed to form the thick layer of wall ingrowths (Figs. 2.6A, 2.10, 2.13A, B) that eventually developed along the outer primary walls of the secretory trichomes (Figs 2.6A, B, 2.7A, B, 2.10). The cuticle is thinner than that of the secretory trichomes, but also shows a trend of increasing thickness throughout development (Figs. 2.11, 2.13A, B).

2.3.2.2.3 Nectary parenchyma cells

Beneath the nectary epidermis there are 2-3 layers of cytoplasmically dense nectary parenchyma cells (Figs. 2.2B, E, 2.14A). Early in nectary development, these parenchyma cells had only a few, relatively small vacuoles per cell (Figs. 2.4A, 2.14A), but typically featured a large, single vacuole as development progressed (Figs. 2.5A, 2.6A), including at anthesis (Fig. 2.2E). Relatively few plastid profiles were observed in this tissue (Figs. 2.8, 2.14A) and the plastids present rarely contained any starch grains at any of the developmental stages

investigated (Figs. 2.9, 2.14A). Plasmodesmata were observed connecting nectary parenchyma cells with nectary epidermal cells (Fig. 2.14B).

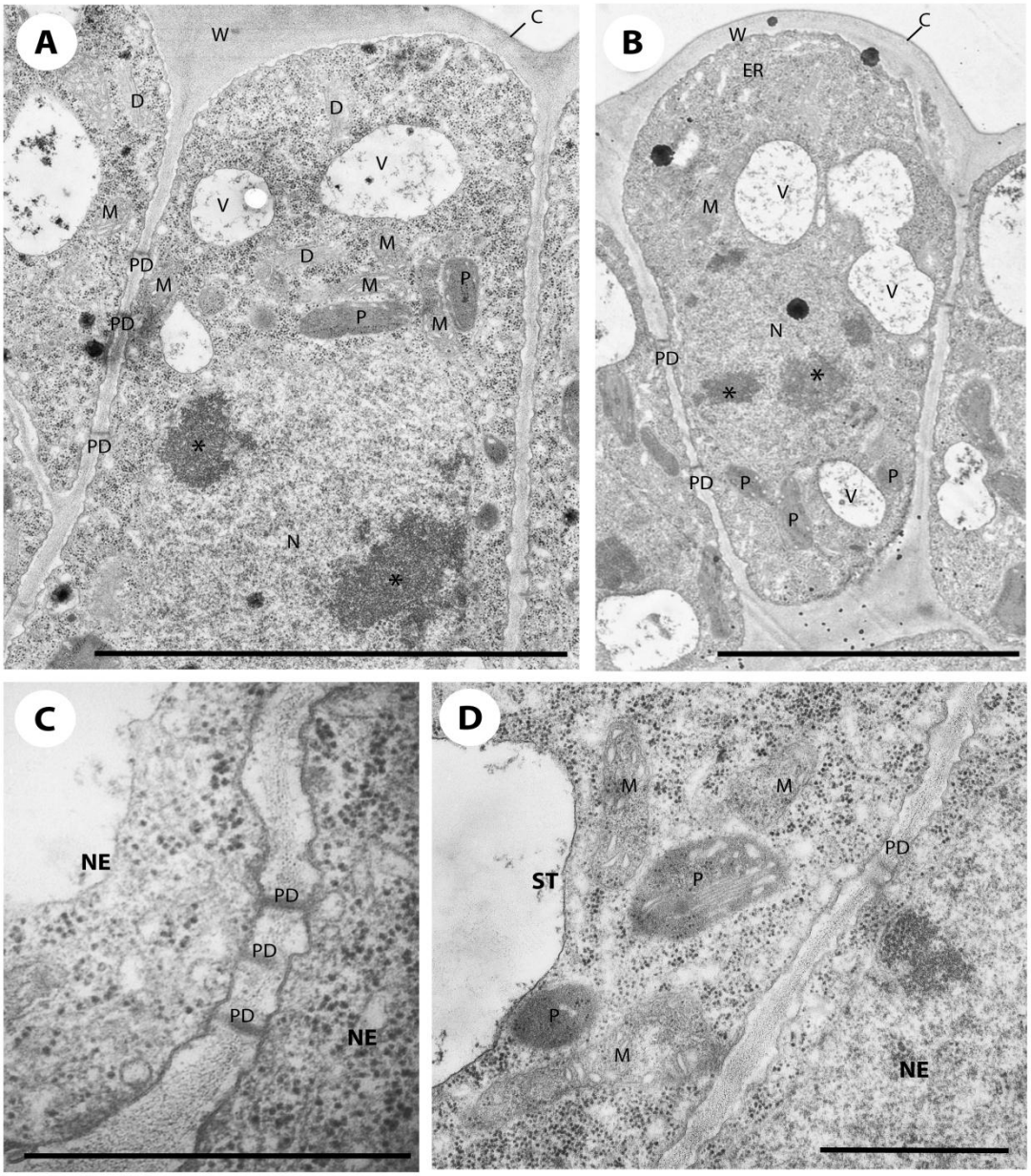

Figure 2.12 – Ultrastructure of the nectary epidermal cells of the floral nectary in *Centranthus ruber* spurs in early development (Stage 3). A, B: Nectary epidermal (NE) cells in early development. A large nucleus (N) with distinct regions of heterochromatin (*) takes up a large portion of the cell. At this stage, nectary epidermal cells are cytoplasmically dense with small vacuoles (V) that often contain osmiophilic materials. Plastids (P), without starch, and mitochondria (M) are present in the cytoplasm. Cuticle (C) layer is very thin in this developmental stage. C: Plasmodesmata (PD) crossing the anticlinal walls between two nectary epidermal cells. D: Plasmodesmata crossing the anticlinal walls between a developing secretory trichome (ST) and nectary epidermal cell. Scale bars A, B=5µm, C, D=1µm.

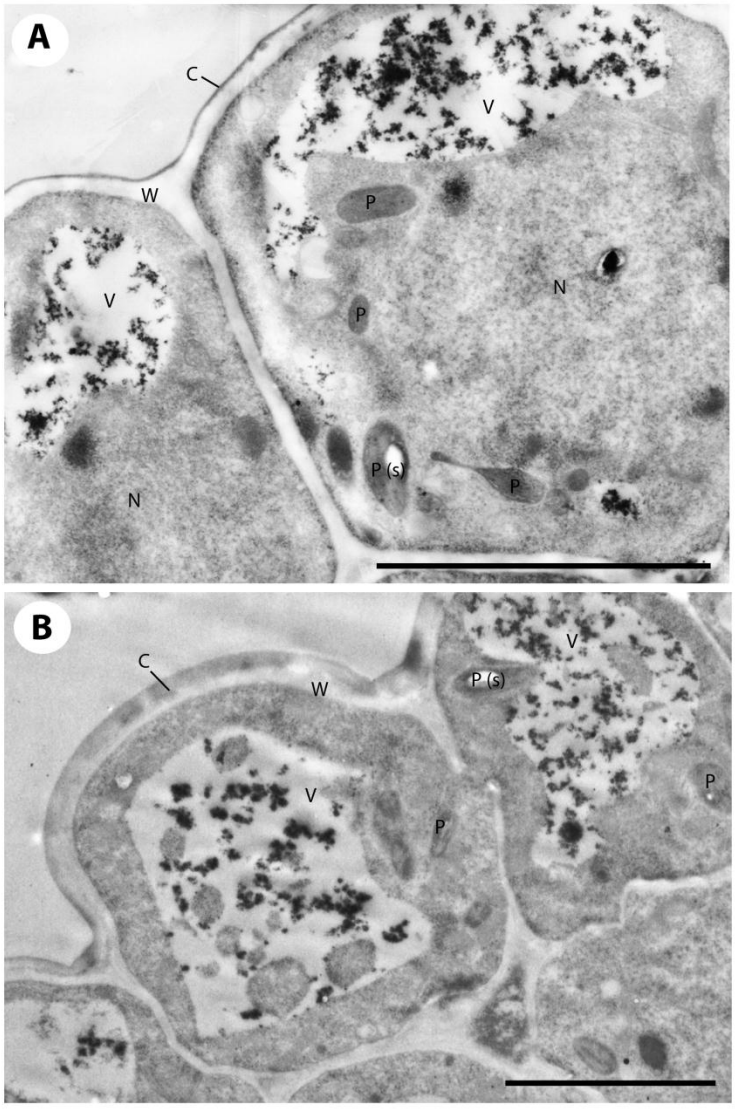

Figure 2.13 – Ultrastructure of the nectary epidermal cells of the floral nectary in *Centranthus ruber* spurs in Stages 4 and 6. A, B: Nectary epidermal cells in Stages 4 and 6. The vacuole (V) in the nectary epidermal cells appears to take up more space in the cell as development progresses. The vacuoles often contain granular, osmiophilic materials. The thickness of the cell wall (W) remains fairly constant, but the cuticle (C) layer becomes increasingly thick throughout development. Plastids (P), some with starch (P (s)), are evident. Scale bars A, B=5µm.

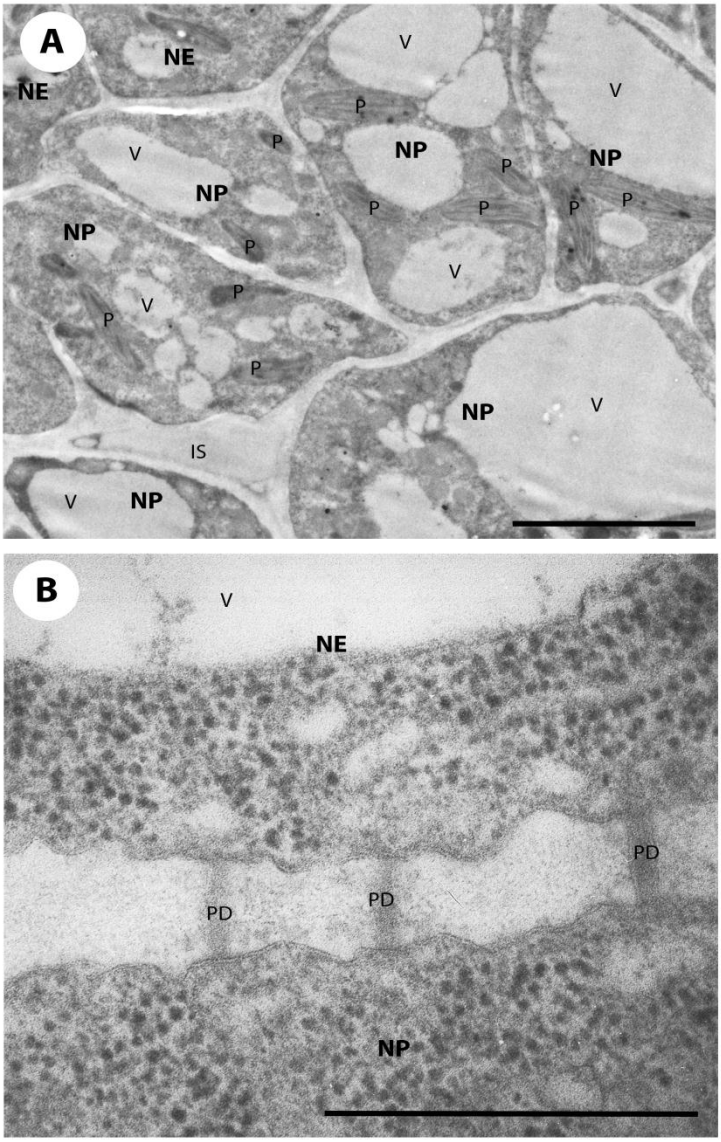

Figure 2.14 – Ultrastructure of the nectary parenchyma cells of the floral nectary in *Centranthus ruber* spurs throughout development. A: Nectary parenchyma cells (NP) and intercellular space (IS) below nectary epidermal cells (NE) (Stage 6). B: Plasmodesmata (PD) within the primary wall between a nectary epidermal (NE) and a nectary parenchyma (NP) cell (Stage 3). Note dense cytoplasm with abundance of ribosomes in both cells. Scale bars A=5µm, B=0.5 µm.

2.3.2.2.4 Vascular tissue

The vascular connection to the nectary's secretory trichomes is a single vascular bundle (Figs. 2.2A, B, D) which contains both phloem and xylem (Fig. 2.15). The xylem cells are found closer to the base of the secretory trichomes, with the phloem tissue below and at the sides of the xylem. Usually 2-3 adjacent xylem vessel elements, with helically-deposited secondary walls, and numerous sieve tube elements were observed (Figs. 2.15A, B). In close proximity to the sieve tube elements, cells of similar size to companion cells with numerous wall ingrowths were observed (Figs. 2.15B-D). The wall ingrowths are unbranched (Figs. 2.15 B-D), and much more sparse in comparison to the labyrinthine ingrowths observed in the mature unicellular secretory trichomes (Fig. 2.7A). These cells appear to be Type A transfer cells (Pate and Gunning 1972) because they have wall ingrowths around the entire periphery of the cell, rather than having ingrowths on only one side (Figs. 2.15 B-D).

2.3.2.2.5 Other spur tissues

Along its lateral sides, where the two vascular bundles are absent, the spur is only 4-5 cells thick (Figs. 2.2B, D), with intercellular spaces becoming increasingly large throughout development. The outer epidermal cells have a very thick outer periclinal wall (Fig. 2.16A) in comparison to the outer primary walls of the inner nectary epidermal cells and secretory trichomes (Fig. 2.10). On the other hand, these outer epidermal cells of the spur have much thinner cuticles than the secretory trichomes and nectary epidermal cells, particularly in Stages 5-7 (Figs. 2.11, 2.16A). The non-secretory trichomes which line the entire circumference along the inner surface of the spur, apart from the abaxial region where the secretory trichomes are present (Figs. 2.2A, D), have a thick and ornamented cuticle (Figs. 2.2F, 2.16 B-D).

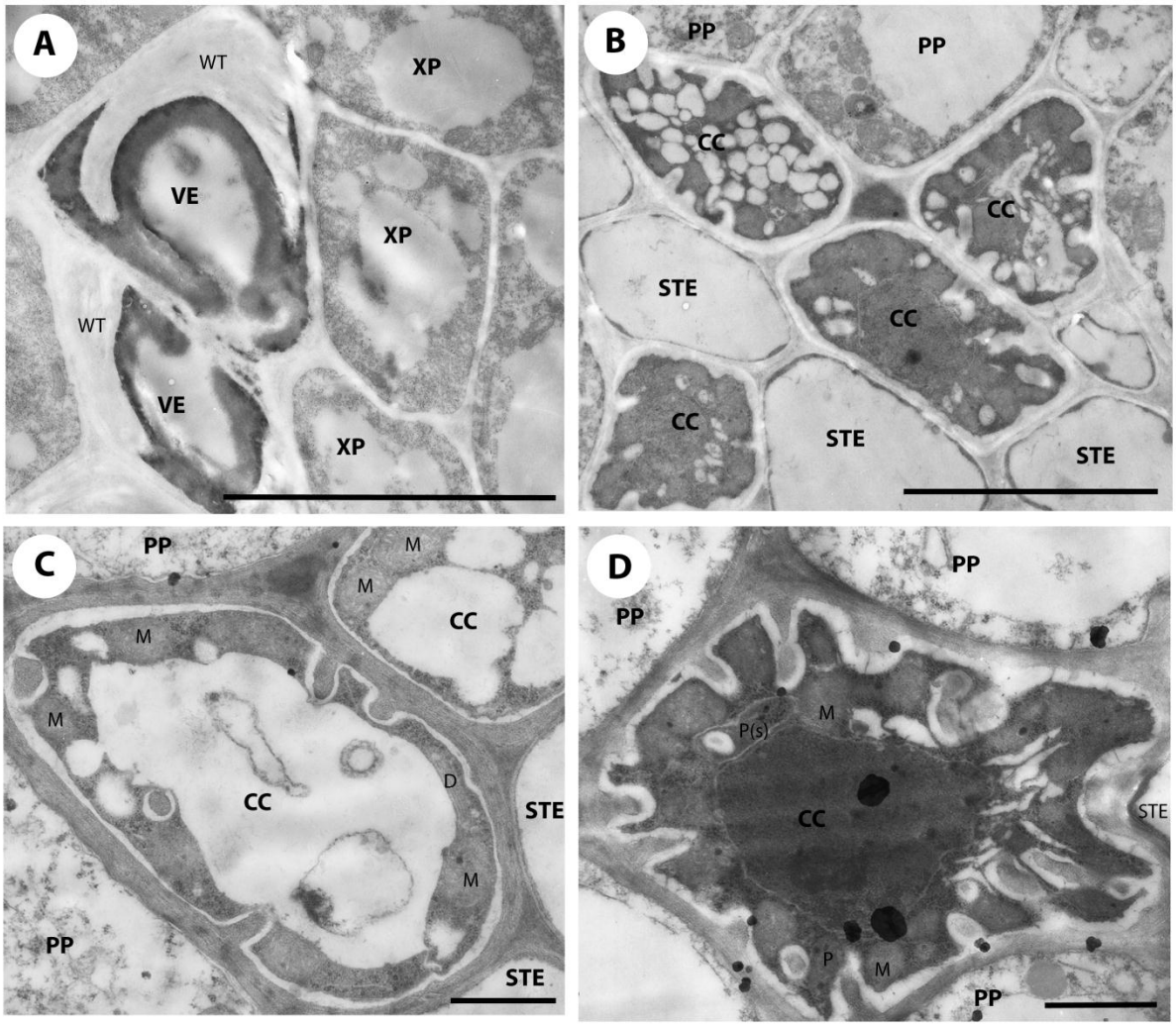

Figure 2.15 – Ultrastructure of the vascular bundle of the floral nectary in *Centranthus ruber* spurs. A: Xylem vessel elements (VE), with helical wall thickenings (WT), and xylem parenchyma cells (XP). B: Phloem consisting of companion cells (CC), sieve tube elements (STE) and phloem parenchyma cells (PP). Companion cells with wall ingrowths on all sides. Vesicles which have a vacuole-like appearance are numerous in one of the companion cells at top left. C, D: Close up of companion cells showing the variability in arrangement of the wall ingrowths. Organelles include dictyosomes (D), mitochondria (M) and plastids (P) that sometimes contain starch (P(s)). Scale bars A, B=5μm, C, D=1μm.

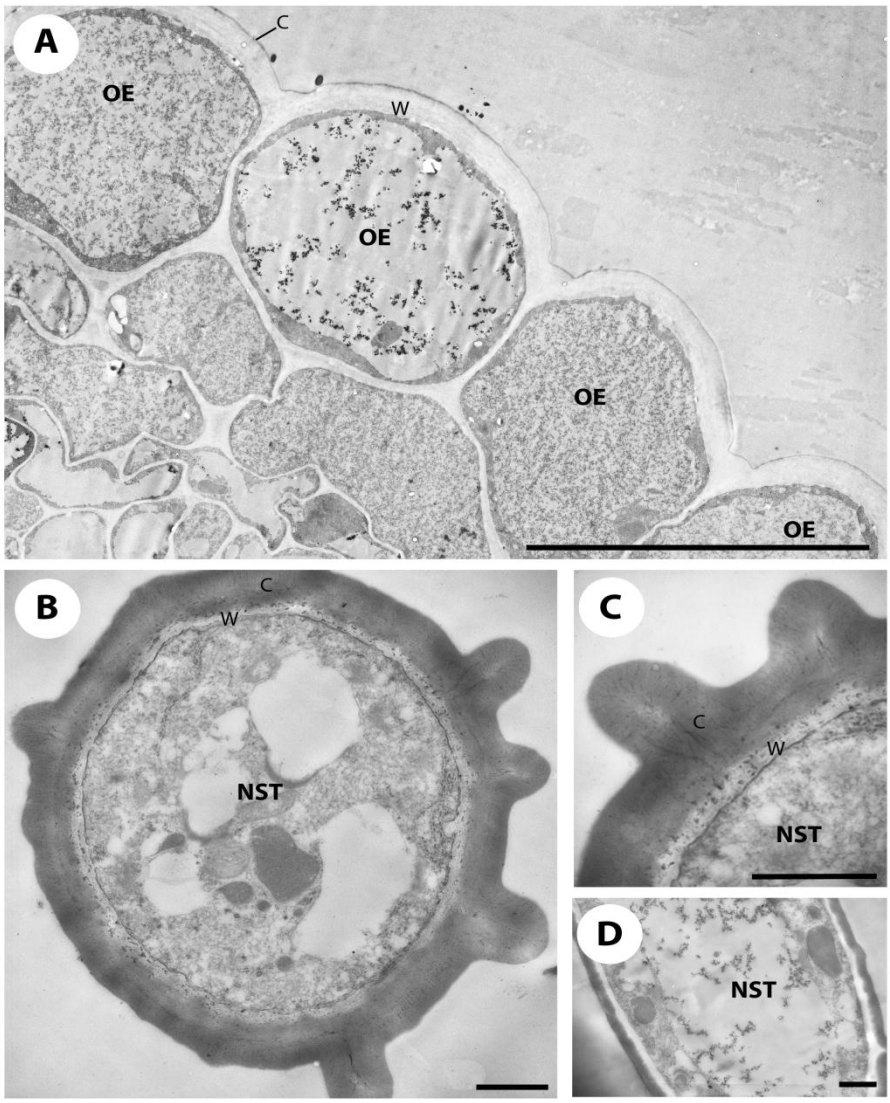

Figure 2.16 – Ultrastructure of outer epidermal cells and non-secretory trichomes of *Centranthus ruber* spurs. A: Outer epidermal cells (OE) (Stage 6) showing their thick outer periclinal walls (W) and thin cuticles (C). The vacuoles of these epidermal cells and subjacent subepidermal calls are large and filled with granular contents. B: Cross section through non-secretory trichome (NST). C: Thick layer of cuticle (C) sits atop the cell wall (W). The thick cuticle is ornamented with striations and has tiny microchannels which run through it. D: Tangential section through non-secretory trichome. Scale bars A=20µm, B-D=1µm.

2.4 Discussion and conclusions

Several morphological characteristics of *C. ruber*'s flowers, such as the long, tubular corolla and the white coloured petals, suggest a moth and/or butterfly pollination syndrome (Miller 1981, Ollerton *et al.* 2009). *C. ruber* has also been previously described as a butterfly-pollinated species based on floral scent composition (Andersson *et al*. 2002). Each flower produces a single achene fruit in which the ring of sepals uncoils into a plumose pappus, a characteristic present in only one other genus in the former Valerianaceae (*Valeriana*; Richardson 1975) and which indicates a wind dispersal mechanism.

The morphological characteristics of *C. ruber*'s corolla are of particular interest in relation to the evolution of different types of floral symmetry within the order Dipsacales. Changes in floral form within the Asterid group, which includes the Dipsacales, have resulted in several common floral types from a radially symmetric ancestor (Donoghue and Ree 2000). Some of these common floral types are: the 2:3 form (2 dorsal and 3 ventral petals), the 4:1 form (4 dorsal and 1 ventral petal) and the 0:5 form (all 5 petals shifted ventrally) (Donoghue and Ree 2000). Floral form within the Dipsacales is highly variable, ranging from radially symmetric to bilaterally symmetric and asymmetric types (Donoghue *et al*. 2003). The Dipsacales can be broken down into two main groups: the families Adoxaceae, which includes only radially symmetric forms, and the Caprifoliaceae (which includes the former Valerianaceae; Judd *et al*. 2008) comprising primarily bilaterally symmetric forms (Boyden *et al*. 2012). The flowers of *C. ruber* are asymmetric at anthesis, which is a rare floral trait, both in the Dipsacales and across angiosperm taxa (Tucker 1999). *C. ruber*'s petals appear to form a 1:4 pattern due to the position of one of the two dorsal petals, which becomes segregated from the other four petals (Donoghue and Ree 2000). *C. ruber* also has only a single stamen, which is a trait found in some other

groups, but is unique within the Valerianaceae (Endress 2001). The stamen is located in the 0300 or 0900 position at anthesis and the nectar spur is in the 0600 position (Tucker 1999). Asymmetry in *C. ruber*'s flowers is a secondarily derived trait (Neal *et al*. 1998). Derived asymmetry, along with the other highly derived symmetry forms (disymmetry and transverse and diagonal zygomorphy), account for only 7.8% of dicot species (Neal *et al.* 1998). Duplications in *CYCLOIDEA*-like and *RADIALIS*-like genes, coding for transcription factors related to those involved in the establishment of floral symmetry in *Antirrhinum majus*, are implicated in generating the changes in floral symmetry which have occurred in the Dipsacales over time (Howarth and Donoghue 2005, Howarth *et al*. 2011, Boyden *et al*. 2012).

Nectary anatomy and ultrastructure have previously been reported for several spurred species, particularly within orchids (Orchidaceae) e.g., *Limodorum abortivum* (Figueiredo and Pais 1992), *Platanthera chlorantha* (Stpiczynska *et al*. 2005), *Anacamptis pyramidalis* f. *fumeauxiana* Marg. & Kowalk. (Kowalkowska *et al*. 2010), *Gymnadenia conopsea* (L.) (Stpiczynska and Matusiewicz 2001), and several *Habenaria* species (Galetto *et al*. 1997). Floral nectaries investigated from spurred, non-orchid species include *Tropaeolum majus* (Tropaeolaceae - Rachmilevitz and Fahn 1975), and two members of the Caprifoliaceae, namely *Lonicera japonica* (Fahn and Rachmilevitz 1970, Fahn 1979) and *L. kamtschatica* (Weryszko-Chmielewska and Bozek 2008). However, no studies on nectary ultrastructure within the Valerianaceae have been found in the literature and TEM investigation of nectaries within the Dipsacales appears to be limited to *L. japonica* (Fahn and Rachmilevitz 1970, Fahn 1979) and *L. kamtschatica* (Weryszko-Chmielewska and Bozek 2008). These two species have trichomatous nectaries, with unicellular secretory trichomes very similar in appearance and structure to those of *C. ruber*, making them suitable species for comparison. Roels and Smets (1996) briefly

mention the presence of unicellular secretory trichomes within the spur of *C. ruber* at maturity, but do not provide any additional information or mention the non-secretory trichomes.

The nectaries of *L. japonica* and *L. kamtschatica* are similar, apart from the added presence of epidermal papillae among the unicellular secretory trichomes in *L. kamtschatica* (Weryszko-Chmielewska and Bozek 2008). These papillae, which are present on almost every epidermal cell in the nectary region, also function in nectar secretion, but to a lesser extent than the larger unicellular trichomes which are interspersed amongst them, each separated by 3-6 papillae (Weryszko-Chmielewska and Bozek 2008). Indentations similar to those seen on some of *Centranthus ruber*'s secretory trichomes in early development were not reported in either of these species. Unlike *L. kamtschatica*, the papillae on the nectary surface were absent in *C. ruber*. Non-glandular trichomes were also reported in *L. kamtschatica*, however, because the spur is so short and secretory trichomes were present along both the abaxial and adaxial inner surfaces, these non-glandular trichomes were mostly found further up the corolla tube and lining the entire remaining corolla surface with very few located beside the secretory trichomes and papillae (Weryszko-Chmielewska and Bozek 2008). These non-glandular trichomes have a similar appearance to those in *C. ruber*, but seem to lack the distinct cuticular ornamentation seen in *C. ruber*. In *C. ruber*, the function of these stiff non-secretory, typically acropetally-directed hairs is not certain; however, the serendipitous discovery by SEM of an immature thrips (Thysanoptera) wedged within the spur lumen suggests a defensive role against potential nectar thieves.

Early during secretory trichome development in *L. japonica*, the cell walls are thin and cuticles are not yet present, whereas later in development the cell walls and cuticle become thicker and the cuticle shows uneven thickenings (Fahn and Rachmilevitz 1970). In *C. ruber*, the

cell wall did not show an increase in thickness between Stages 3-7, but the cuticle continually became thicker throughout development like that of *L. japonica*. In that species, no pores for nectar release were observed in the cuticles covering the secretory trichomes, and it is unknown whether the cuticle eventually breaks or whether the thin areas of the unevenly thickened cuticle were permeable to nectar (Fahn 1979). Cuticular pores were not found in *C. ruber*, nor reported for *L. kamtschatica* (Weryszko-Chmielewska and Bozek 2008) either. However, uneven cuticle thickenings were observed in all three species, suggesting that nectar is exuded through the thinner cuticle regions. The microchannels observed in the cuticle of the secretory trichomes in *C. ruber*, which were also observed in the secretory papillae of *L. kamschatica* (Weryszko-Chmielewska and Bozek 2008), suggest another possible method of nectar secretion. In all three species, nectar secretion and accumulation between the cuticle and the cell causes the cuticle to lift upwards, creating a subcuticular space where nectar resides until it is released into the lumen of the spur (Fahn and Rachmilevitz 1970, Weryszko-Chmielewska and Bozek 2008). Along the outer primary wall beneath the cuticle, the mature secretory trichomes of both *L. japonica* and *L. kamtschatica* have numerous wall ingrowths similar to those of *C. ruber*, indicating that trichomes of all three species are characteristic transfer cells (Fahn and Rachmilevitz 1970, Weryszko-Chmielewska and Bozek 2008). Pate and Gunning (1972) described cells with wall ingrowths as transfer cells because they have vastly increased plasma membrane surface areas, enhancing efficient trans-membrane movement of solutes. In *L. japonica*, vesicles were observed which appear to bud off from the ER cisternae and are suggested to fuse to the plasma membrane associated with the ingrowths (Fahn and Rachmilevitz 1970). ER tubules and cisternae, along with secretory vesicles, were also commonly seen near the wall ingrowths in *L. kamtschatica* (Weryszko-Chmielewska and Bozek 2008). Additionally, autoradiographs of *L. japonica* flowers

treated with tritiated sucrose showed most labelling in these organelles, suggesting that the ER plays a key role in nectar sugar secretion in *L. japonica* (Fahn and Rachmilevitz 1975). In the *C. ruber* trichomes observed, ER profiles were evident, but did not seem as prominent, suggesting that ER has a less significant role in nectar secretion in this species.

The presence of starch in plastids was quantified for the secretory trichomes of *C. ruber*. Starch content peaked in Stage 4 spurs, but then declined by anthesis (Stage 7) when nectar secretion commenced. Similarly, in *L. japonica*, starch grains which were present in the plastids prior to secretion were no longer present once secretion reaches maximal levels (Fahn and Rachmilevitz 1970). This hydrolysis of starch may, in part, contribute to the nectar production in *C. ruber*; however, the majority of the nectar solutes are likely derived from the the phloem sap. The numerous wall ingrowths observed in the phloem cells, indicative of a transfer cell function, implies that many solutes are being transferred from the sieve tube elements to the rest of the nectary tissues. It is interesting to note the striking difference in the wall ingrowth morphology between those in *C. ruber*'s secretory trichomes and those located in the vascular tissue of the nectar spur. This immense diversity in wall ingrowth patterns occurs between different types of transfer cells, and also among cells of the same transfer cell type (Offler *et al.* 2002). The wall ingrowths located on cells adjacent to the sieve tube elements suggest a function in the movement of solutes from the sieve tube elements to nectariferous tissue nearby. In the minor vein of leaves, Pate and Gunning (1972) described four different types of transfer cells (Types A, B, C and D). The transfer cells present in *C. ruber*'s vascular bundle resemble Type A transfer cells, but could possibly be Type B. Type A transfer cells are modified companion cells, which are characterized by the presence of wall ingrowths along the entire inner surface of the cell (Pate and Gunning 1972). Type B transfer cells are phloem parenchyma cells, which have wall

ingrowths along only one side of the cell. The transfer cells in the phloem of *C. ruber*'s spur appear to be in the correct position relative to the sieve tube elements to be considered companion cells and the orientation of the wall ingrowths also suggests this. However, typically, companion cells have a smaller diameter than sieve tube elements, so it is possible that these are phloem parenchyma cells which have ingrowths not characteristic of phloem parenchyma transfer cells as described by Pate and Gunning (1972).

As the vascular tissue in *C. ruber* appears to be the main source of the sugar components for the floral nectar, the pre-nectar begins its path towards secretion in the spur's abaxial vascular bundle. The wall ingrowths in the phloem's companion cells allow for more efficient movement of solutes into the nectary parenchyma cells or into the small intercellular spaces between them. Modification of the nectar components may occur in the nectary parenchyma cells, the nectary epidermal cells and in the secretory trichomes themselves. Plasmodesmata observed between cells of these three tissue types suggests that components are likely moving via a symplastic route towards the unicellular secretory trichomes. Once arriving at the secretory trichomes, final modifications to the nectar likely occur before the nectar travels across the plasmamembrane which has been vastly increased in surface area due to the labyrinthine layer of wall ingrowths. Nectar accumulates under the cuticle in mid- and late-development and is finally released into the spur's lumen at anthesis (Stage 7), potentially via the microchannels which were observed traversing the cuticle layer.

2.4.1 Conclusions

This study has provided information not currently available in the literature regarding the morphology, anatomy and ultrastructure of *C. ruber*'s floral nectar spur and nectary throughout development. The nectar spur of *C. ruber* contains a trichomatous nectary composed of

unicellular, secretory trichomes, which initiate in early development (Stage 3) and secrete nectar into the lumen of the spur at anthesis (Stage 7). The presence of wall ingrowths, characteristic of transfer cells, in both the companion cells of the nectary's phloem tissue and in the secretory trichomes themselves, increases the efficiency of solute transport between the phloem sap and the nectariferous tissues and between the secretory trichomes and the inner surface of the spur. Mitochondria and plastids were the primary organelles observed within the secretory trichomes, particularly in the mid to late developmental stages. The mitochondria provide energy for the secretion process, while the plastids use their stored starch, which accumulates in mid development (Stages 4-5), to provide sugar components for the nectar or energy for the secretion process.

Despite their importance for the pollination and reproduction of plants, nectaries still remain an understudied aspect of floral biology (Bernardello 2007). Variation in floral nectary anatomy and ultrastructure between different taxa illustrates the importance of investigating numerous species in order to develop a generalized understanding of nectaries, as well as an appreciation of the diversity amongst angiosperms. The study of nectaries provides important information regarding both plant-pollinator interactions, as nectary structure is often related to the behavioural and morphological traits of a plant's pollinators, and plant systematics, as nectary characteristics are often similar in closely related taxa (Pacini and Nicolson 2007).

Chapter 3: SPUR GROWTH IN *CENTRANTHUS RUBER*

3.1 Introduction

3.1.1 Cellular basis for spur growth

The cellular basis for growth and development of floral spurs in angiosperms is still generally uncharacterized. Therefore, one of the goals of this project was to examine this process in the nectar spur of *Centranthus ruber*. It was hypothesized that *C. ruber*'s spur follows the growth pattern outlined by the classical meristem hypothesis, in which cell division contributes to growth until the mature spur length is reached (Tepfer 1953, Puzey *et al*. 2012). It was predicted that there may be one or more distinct points of multiple cell divisions at the spur apex, reminiscent of apical meristems, responsible for the growth of the nectar spur. SEM micrographs of *C. ruber*'s spur tip reveal what appear to be two regions of relatively small, isodiametric and potentially meristematic cells (Fig. 3.1A). This hypothesis was tested by analyzing the patterns of cell divisions on the nectar spurs throughout their development.

3.1.2 Meristematic regions

Broadly defined, a meristematic region refers to a "spatially coherent" group of cells with an increased rate of mitotic activity compared to the surrounding tissues (Veit and Foster 2002). The word meristem originates from "meristos", meaning "to divide" and therefore refers to a region where division occurs, which may have previously been referred to as a "zone of cell division" (Green 1976). Sussex and Kerk (2002) describe meristematic tissues as "input/output" systems because while cells are being continually added by division, older cells are being removed via differentiation. In order to maintain active meristematic regions, precise regulation of the frequency and polarity of cell divisions in 3-dimensional space is necessary (Veit and Foster 2002). This regulation is governed by the developmental organization of the meristem and

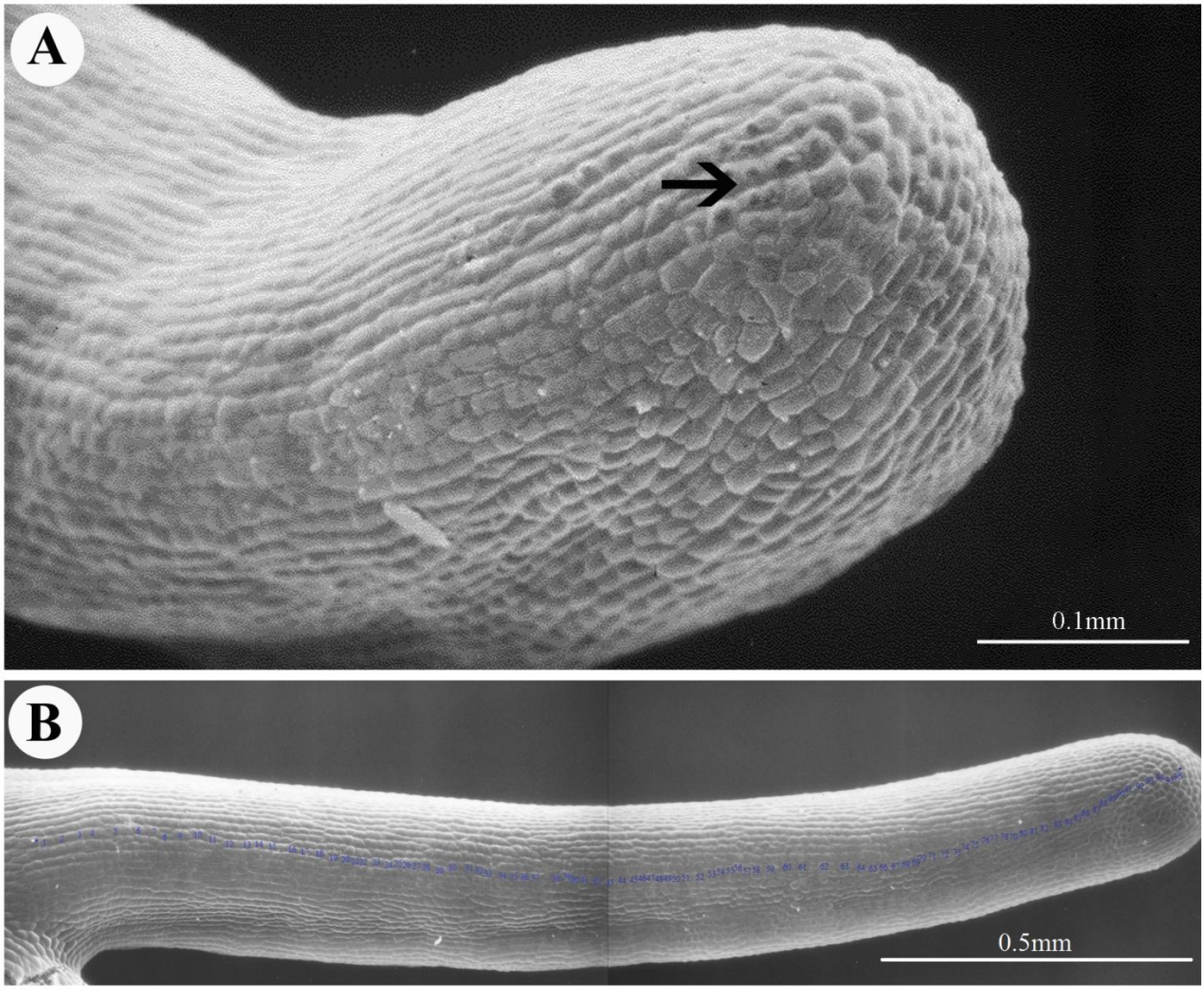

Figure 3.1- Hypothesized meristematic region on lateral tip of spur and lateral view of a Stage 4 spur of *Centranthus ruber* showing an example of the single cell file count along the spur's side. A: Tip of a Stage 4 spur showing a cluster of small, isodiametric cells (arrow) which were hypothesized to be meristematic on each side of the spur's tip. B: Example of a single cell file count along the lateral side of a floral nectar spur. For each developmental stage, cell file counts were taken from six spurs, three each from two different plants. The blue numbers along the spur indicate each cell counted in the cell file.

by interactions between hormonal, genetic and environmental factors which affect the cell cycle machinery (Dewitte and Murray 2002).

Plants exhibit an open form of growth, meaning that they are capable of continually producing new organs and tissues by means of their meristematic regions (Srivastava 2002). Meristematic cells have great developmental potential, however they are not completely unspecialized, as they must be organized for continued cell division (Clowes and Juniper 1968). Additionally, they are not undetermined developmentally because each meristem is destined to produce specific organ and tissue types (Sussex and Kerk 2002).

The most familiar meristems are the root and shoot apical meristems which are responsible for the primary growth of the plant body (Srivastava 2002). Lateral meristems provide a means for secondary growth of the plant body, resulting in increased girth (Srivastava 2002). Apical and lateral meristems are considered indeterminate because they are active throughout the entire life of the plant, whereas determinate meristems, such as those necessary for leaf and flower development, are only active for a short period of time (Srivastava 2002). Under the broad definition of meristem, there may be numerous temporary meristematic regions present on the plant body during development which are responsible for the development and/or initiation of various plant structures.

3.1.2.1 Meristem hypothesis of spur development

Tepfer's classic 1953 study on *Aquilegia formosa* Fisch. Ex DC. var. *truncata* (Fisch. & C.A.Mey.) Baker created a basis for understanding how nectar spurs develop. The highly specialised petals of the pentamerous flowers of *Aquilegia* are each primarily composed of a nectar spur, with only minor development of the petal lamina (Tepfer 1953). Tepfer's investigation of longitudinal sections through the petal spurs, just prior to anthesis, revealed

several active meristematic regions on the developing petals. The first region was located at the base of the developing spur and a second was located at the petal margin (morphological apex). Tepfer (1953) also noted meristematic regions close to the insertion points of the petals, near the bases of the lamina which were comparable to the adaxial meristems of leaf primordia. Tepfer stated that much of the later development of the nectar spur occurs by intercalary growth, which suggests that the meristem at the base of the developing spur is not responsible for continued growth, but rather those flanking the attachment point were responsible for driving continued spur growth. Although Tepfer suggested that the petal spurs develop via these meristematic regions flanking the spur, he also noted that the late stages of petal development, which are rapid and account for much of the increased size of the petals, include cell enlargement in addition to cell division. Since Tepfer's *Aquilegia* study, the meristem hypothesis, which states that spurs develop by adding one cell at a time via meristematic regions, has been largely accepted (Puzey *et al*. 2012).

3.1.3 Tracking patterns of cell division with confocal microscopy

Confocal microscopy has a history of use in mapping patterns of cell division, having been frequently applied to developing tissues, such as apical meristem surfaces (Lemon and Posluszny 1998). It is, therefore, an ideal technique for tracking cell division patterns in the developing nectar spurs of *C. ruber*. To obtain an image with the confocal microscope, a laser is scanned across a sample pre-stained with fluorochrome, which then emits fluorescence or reflected light back through a confocal aperture to a detector, producing an image (Haseloff 2003).

Using a chromosome-specific stain makes it possible to determine which stage of the cell cycle each cell is currently undergoing. The arrangement of the chromosomes at the different

stages of mitosis are clearly visible when stained and viewed with the confocal microscope. DNA specific stains such as DAPI allow these cell division patterns to be assessed. Although following individual spurs throughout their development is not possible using this technique, comparing multiple specimens from each developmental stage can provide important information about which regions have the most mitotically active cells and at what developmental stage the majority of cell division occurs.

3.2 Materials and methods

3.2.1 Designation of floral developmental stages

All seven stages of floral development (Table 2.1), designated in Section 2.2.1, were investigated for the pattern of cell divisions in the spur's outer epidermis.

3.2.2 Scanning electron microscopy

Samples were prepared for SEM following a protocol similar to that described in Wist and Davis (2006). Six representative spurs, from two *C. ruber* plants (three spurs per plant), from each of the seven developmental stages were fixed in 2.5% glutaraldehyde, post fixed in 1% osmium tetroxide in 25mM sodium phosphate buffer and dehydrated with a graded acetone series. Samples were critical point dried, mounted onto SEM stubs, gold coated and viewed with the Philips 505 scanning electron microscope. The film used to capture the images was Fuji FP-100B black and white instant film. Along the entire length of the side of each spur, the number of cells in a single cell file was counted and recorded (Fig. 3.1B). Also, the cell lengths and widths for each of the cells in the cell files were measured from scanned micrographs using the ruler tool in Abode Photoshop CS3® in order to determine if cell elongation is more pronounced in particular spur regions throughout development, or if the elongation is comparable along the

length of the spur.

3.2.3 Confocal/Apotome fluorescence microscopy

Flowers of the seven developmental stages (Table 2.1) were fixed in 2.5% GA between 8:00-15:00 hours and left overnight. The following day, samples were rinsed with 25mM sodium phosphate buffer followed by rinsing with distilled water. Samples were then stained with DAPI in distilled water (2µg DAPI per ml) for 2-3 hours. After staining, samples were rinsed three times with distilled water to remove excess stain, before mounting the flower intact in distilled water in shallow well slides for viewing. Approximately 40-50 flowers per stage were stained with DAPI for observation, but only 20 per stage were used for the assessment of mitotic activity. These 20 sample flowers were selected randomly from those which had stained adequately and for which the number of mitotic figures along the spur could be determined with confidence.

Some samples were observed with a Zeiss LSM 510 Confor2 confocal laser scanning microscope equipped with an Axiovert 200M microscope and 63x water immersion objective lens, using a Diode (405nm) beam. Images were captured using the LSM 5 software program. Other samples were observed with a Zeiss Axio Imager Z1 Apotome fluorescence microscope with a 365 LED module and a DAPI filter set and images were captured with Axiovision 4.8. All spurs were scanned thoroughly for mitotic figures from the spur tip to just above the point of attachment to the corolla tube, near the sepal ring atop the ovary. All mitotic figures observed in each stage were recorded and their position along the spur was noted. All of the mitotic figure data from each developmental stage was compiled to determine regions of mitotic activity on the developing spur epidermis.

3.3 Results

3.3.1 Scanning electron microscopy

3.3.1.1 Epidermal cell file counts throughout spur development

Spurs showed significant increases in their single cell file counts at each of Stages 1-4 (Fig. 3.2). Stage 4 and 5 had similar average cell file count values, showing no statistically significant difference (Fig. 3.2). Stages 6 and 7 also showed no significant difference from each other, but both had average cell file counts significantly higher than Stages 4 and 5 (Fig. 3.2). The rate of increase in numbers of epidermal cells per file count begins to decline at Stage 4 and essentially stops at Stage 6 (Fig. 3.2). However, there continues to be a statistically significant increase in spur length until Stage 6 (Fig. 3.2).

3.3.1.2 Epidermal cell dimensions throughout spur development

Epidermal cells on the spur's outer surface increased both in length and width throughout development; however, the increase in length occurred at a much more rapid rate (Fig. 3.3). This cell elongation led to increasingly anisotropic cells throughout development (Fig. 3.4). Early in development (Stages 1-3), the cells remained primarily isotropic, with cell length: cell width ratios close to 1.0 (Fig. 3.4). For Stages 4-7, each stage had a statistically significant increase in spur cell anisotropy, with the cells reaching a cell length: cell width ratio of approximately 2.0 at anthesis (Stage 7) (Fig. 3.4). The pattern of increasing cell widths and lengths remained comparatively consistent between the different regions of the spur, from spur tip to the point of attachment at the spur base (Fig. 3.3). Overall, the greatest differences in rate of epidermal cell elongation were apparent between Stages 4 and 5, with cell width changing at those same stages most markedly in Q3 and Q4 of the spur (Fig. 3.3).

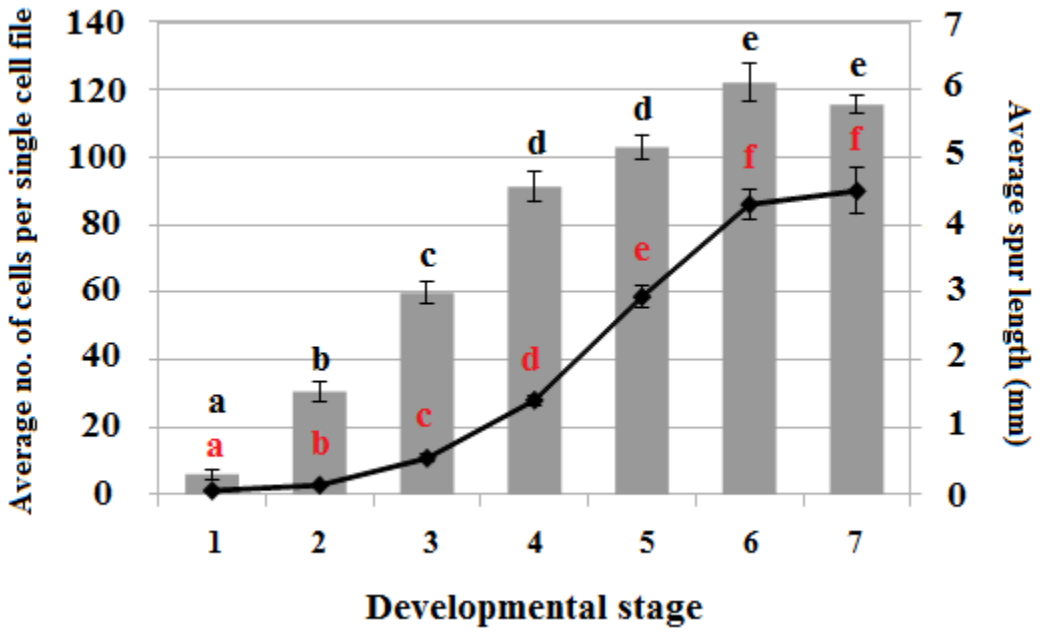

Figure 3.2 – Single cell file counts from the outer epidermis along the side of the spur of *Centranthus ruber* throughout its length during development. This figure shows the average number of cells in a single cell file (bars), along with the average spur length (points), for each of the seven developmental stages of the spur. For the single cell file counts, six spurs (n=6) from two different plants (three per plant) were observed for each developmental stage.

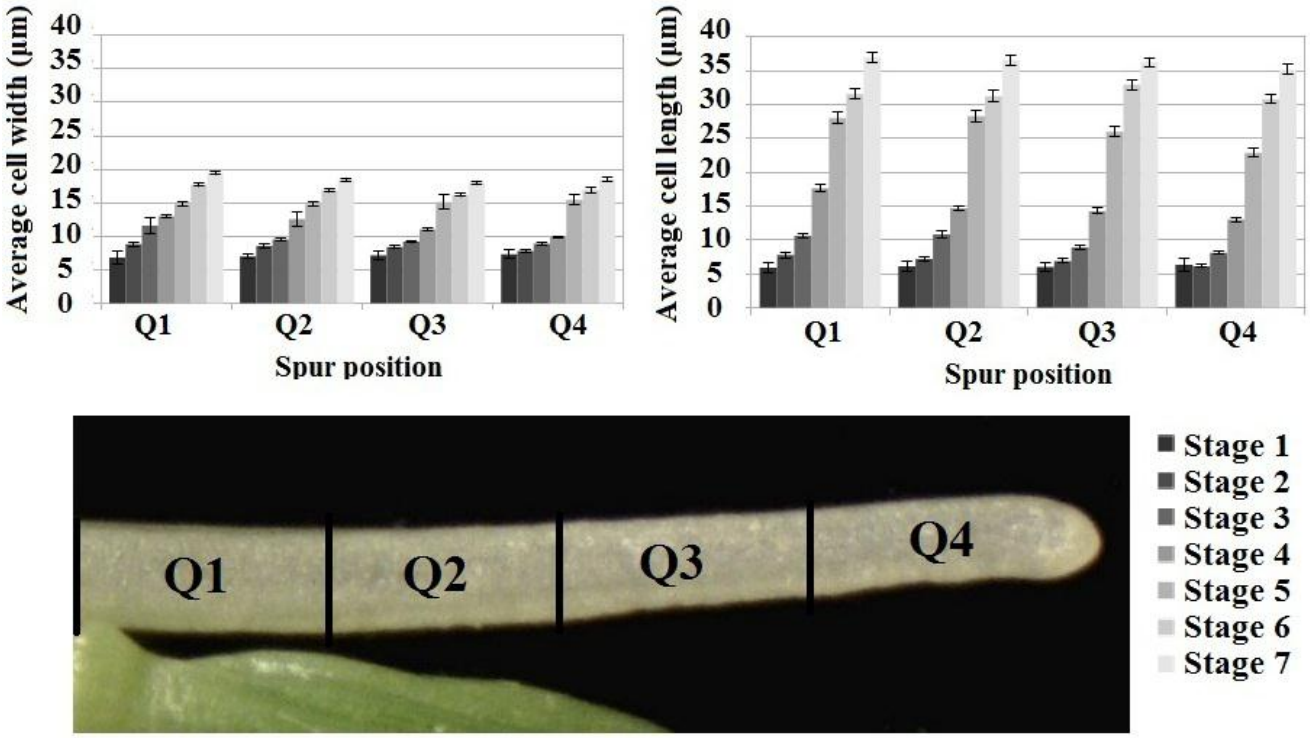

Figure 3.3 - Increases in length and width of outer epidermal cells throughout spur development in *Centranthus ruber.* Lengths and widths of epidermal cells from the single cell files were measured for each of the seven developmental stages (6 spurs per stage). The spurs were broken down into quarters to determine whether or not the cells in different regions followed similar patterns of development. To designate each spur sample into quarters, the distance of each cell from the spur tip (S) was divided by the instantaneous length of the spur (L). L/S of Q1: 1.00 - >0.75, Q2: 0.75- >0.50, Q3: 0.50- >0.25, Q4: 0.25->0.00.

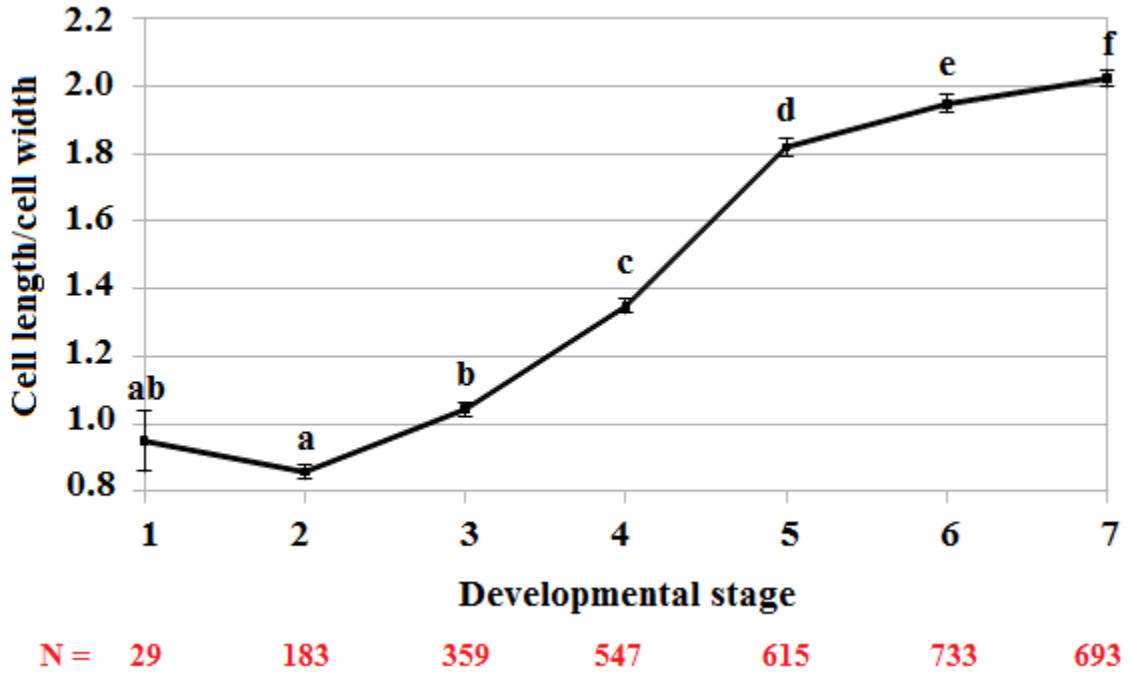

Figure 3.4 - Increased epidermal cell anisotropy throughout development of the floral spur of *Centranthus ruber*. This graph shows the degree of anisotropy of the cells from the single cell file counts in Stages 1-7. They represent the combined data for all 4 quarters of the spur. When cell length and cell width are nearly equal, the cells have a length/width ratio near 1 and the cells are isotropic. As the cells elongate, the length increases more than the width, resulting in cells which are anisotropic, having a length/width ratio near 2. N represents that total number of epidermal cells in the single cell file counts for six spurs (n=3 spurs for each of two plants) per developmental stage. Overall, 3,159 epidermal cells were measured.

3.3.2 Confocal/Apotome fluorescence microscopy

3.3.2.1 Timing and position of mitotic activity in the developing spur

Mitotic figures were observed in epidermal cells of outer surfaces of spurs at Stages 1-3 (Figs. 3.5A, B1, 3.6), but not for Stages 4-7 (Figs. 3.5C, D1, 3.6). The average number of mitotic figures observed per spur was 0.80 ± 0.30 for Stage 1, 2.60 ± 0.79 for Stage 2, 1.85 ± 0.39 for Stage 3 and 0 ± 0 for all Stages 4-7 (n=20 buds for all stages) (Fig. 3.6). This difference in average numbers was statistically significant between Stages 1 and 2 and between Stages 1 and 3, but not between Stages 2 and 3 (Fig. 3.6). The numbers of mitotic figures per spur ranged from 0-5 in Stage 1, 0-11 in Stage 2 and 0-6 in Stage 3. The median number of mitotic figures per spur was 0 for Stage 1, 1.5 for Stage 2 and 2.0 for Stage 3. Mitotic figures observed in Stages 1-3 were observed along the entire length of the spur and were not limited to a single region (Fig. 3.7A-C). Nuclei in early developmental stages (Stages 1-3) were large, easily visible and took up the majority of the cell's volume (Figs. 3.5A, B1). In the later developmental stages (Stage 4-7), cells were larger (Fig. 3.5C) and more irregularly shaped (Fig. 3.5D2) and the nucleus was often pressed up against the plasma membrane by the large central vacuole, resulting in deformation and reduced visibility (Fig. 3.5D1).

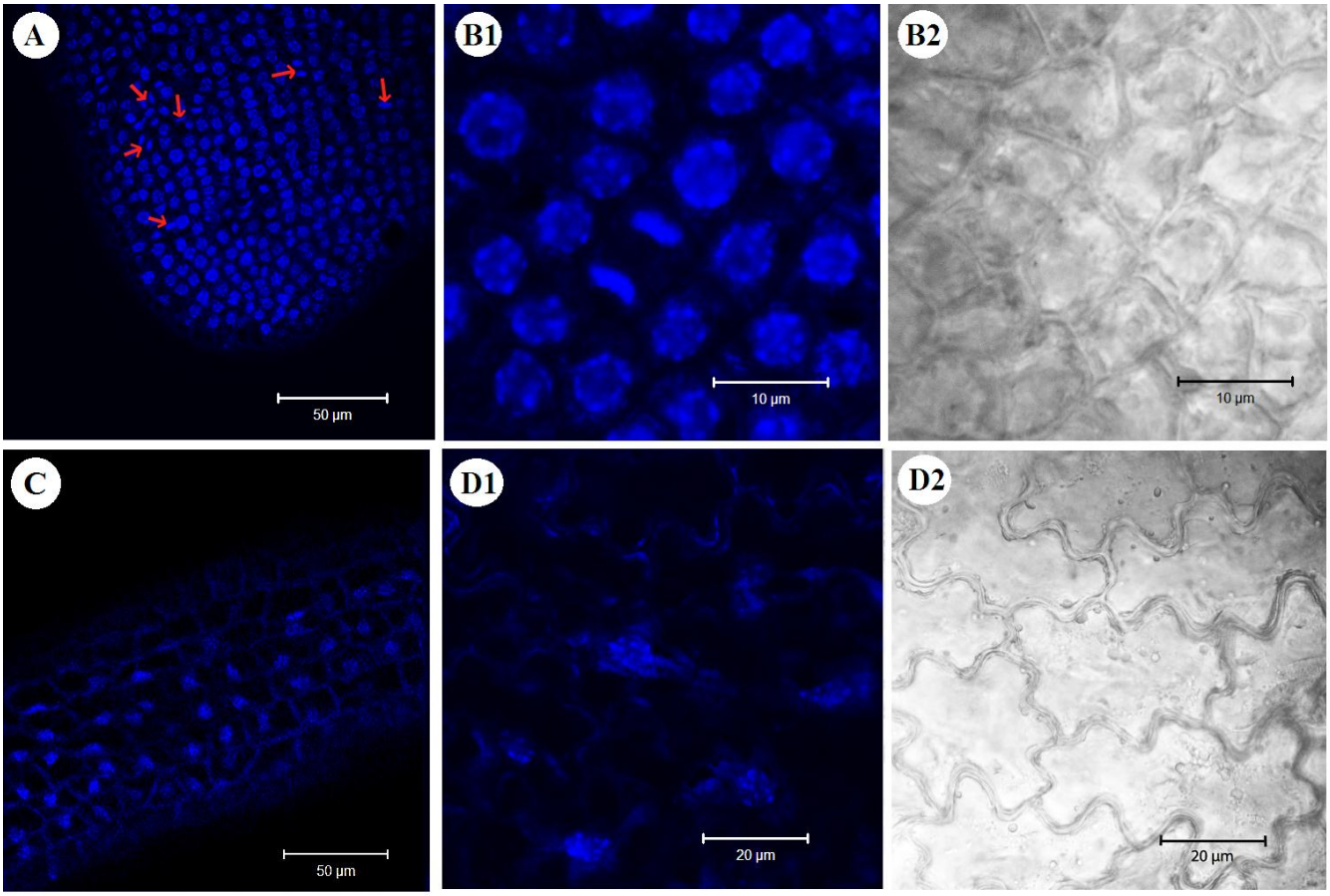

Figure 3.5 - Mitotic figures in outer epidermal cells of DAPI stained spurs of *Centranthus ruber*. These images show examples of DAPI stained spurs and the mitotic figures observed using confocal microscopy. A: Stage 2 spur. Red arrows point to mitotic figures. B1, B2: Cells from a Stage 3 spur. B1: Confocal microscopy, B2: Bright-field microscopy. C: Stage 5 spur. D1, D2: Cells from a Stage 7 spur. D1: Confocal microscopy, D2: Bright-field microscopy. In early developmental stages (1-3), nuclei are large and tend to take up most of the cell, whereas in later stages (4-7), nuclei take up a much smaller portion of the cell volume and are generally pressed against the plasma membrane by the large central vacuole, often distorting nuclear shape.

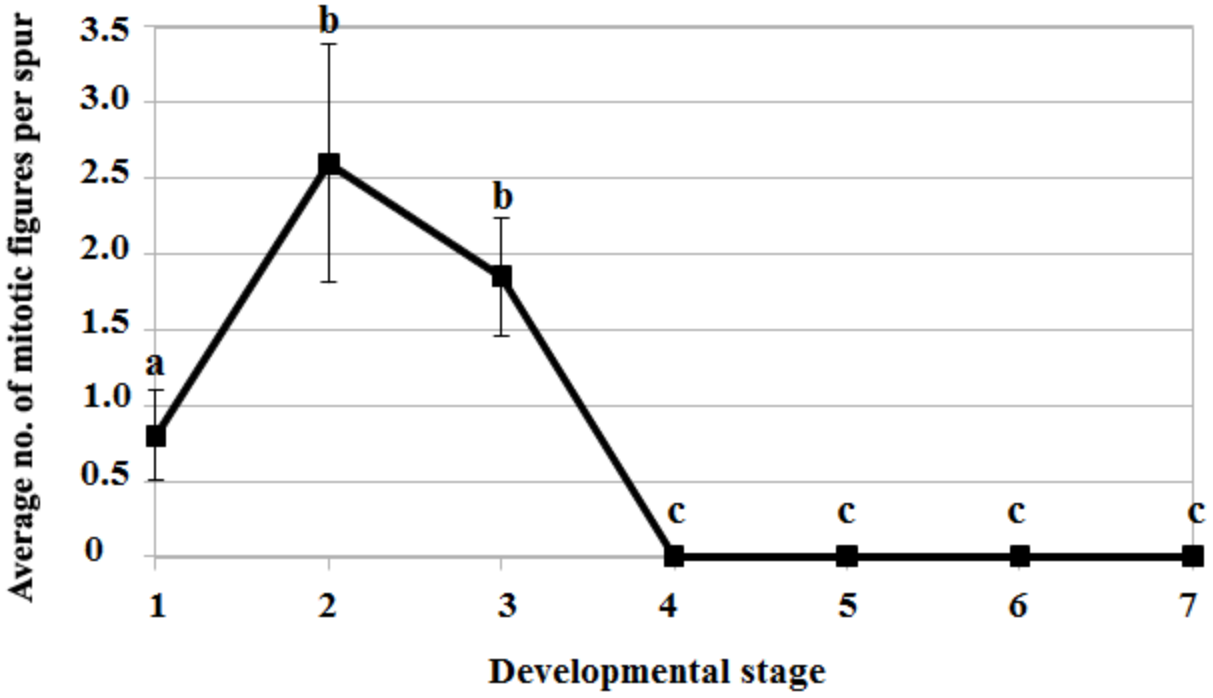

Figure 3.6 - Average number of mitotic figures in outer epidermal cells per spur of *Centranthus ruber* throughout development. This graph shows the average number (± s.e.) of mitotic figures per spur for each of the seven developmental stages. Each data point represents 20 spurs. Different letters above the data points denote statistically significant differences at $\alpha = 0.05$.

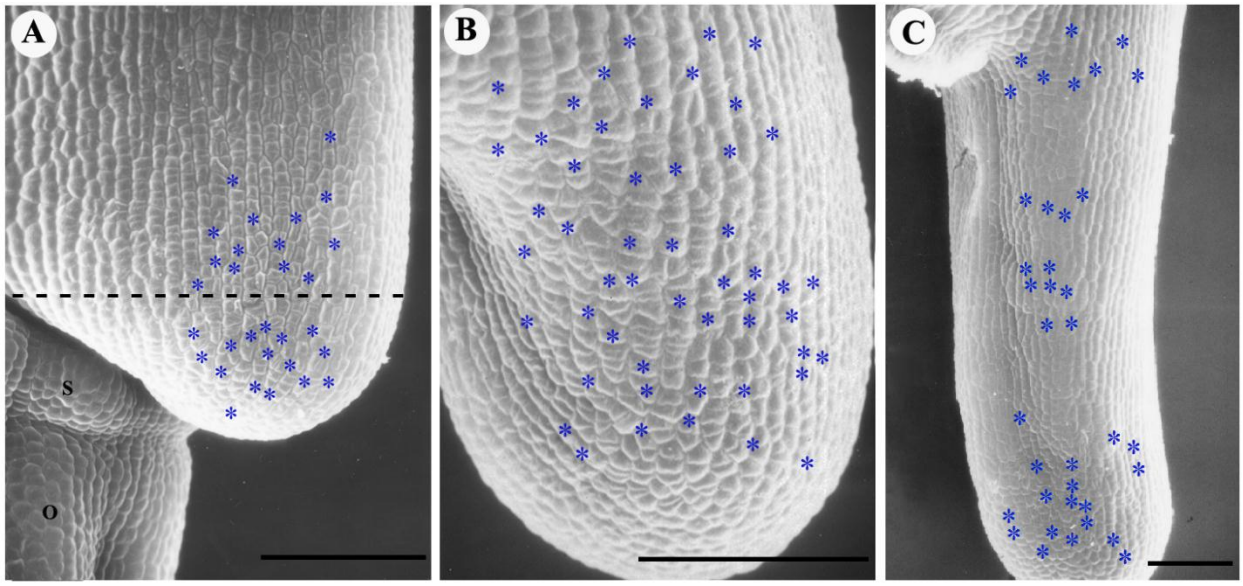

Figure 3.7 - Combined mitotic figure data from developing nectar spurs (Stages 1-3) of *Centranthus ruber*. These images show the combined mitotic figure data for Stages 1(A), 2 (B) and 3 (C). The total number (n) of spurs examined was 20 for Stage 1, 20 for Stage 2 and 21 for Stage 3. These SEM images depict representative spurs from each stage and each blue asterisk represents a single mitotic figure observed in one of the samples. Asterisks were placed on the representative spur based on their approximate position observed via confocal/apotome fluorescence microscopy. The spur is measured from the attachment point at the top of the sepal ring (S) which is situated above the inferior ovary (O). In the Stage 1 spur, the dashed line divides the spur from the base of the corolla tube.Scale bars A-C= 0.1mm.

3.4 Discussion and conclusions

3.4.1 Changes in single cell file count numbers throughout spur development

In *C. ruber*, the single cell file count data indicates that cell division in the outer epidermis of the floral spur continues until approximately Stage 6. However, once the floral bud has reached Stage 4, the increase in cell file count numbers between stages decreases dramatically. A dramatic increase in single cell file count numbers occurs between Stages 1 and 2, with the Stage 2 spurs having on average 5.1 times the number of cells (30.5 ± 3.0) per file as those of Stage 1 (6.0 ± 1.6), indicating that at this early bud stage, cell division activity is particularly important for spur development. Between Stages 2 and 3 there is a 2.0-fold increase in the number of cells (30.5 ± 3.0 to 59.8 ± 3.2) in a single cell file and between Stages 3 and 4 the proportional increase continues to fall, the increase being only 1.5 times (59.8 ± 3.2 to 91.2 ± 4.4). Stages 4 and 5 showed no significant difference in the number of cells (91.2 ± 4.4 and 103.0. ± 3.7). Likewise, there was no significant difference in cell file count numbers between Stages 6 and 7 (122.7 ± 5.7 and 115.5 ± 2.5); however, they were both significantly higher than Stages 4 and 5. On average, Stage 6 and 7 spurs have 1.22 times the number of cells in the outer epidermis as those from Stages 4 and 5. Taken together, all of these data indicate that cell division is prominent in early developmental stages of the floral spur and progressively becomes less important as spur development progresses. Although the numbers of cells per single cell file show this decreasing pattern, the average length of the spur continues to increase significantly between stages until Stage 6. The floral spur in *C. ruber* is 1.3% of its mature length at Stage 1, 3.1% at Stage 2, 12.0% at Stage 3, 31.1% at Stage 4, 65.1% at Stage 5 and 95.6% at Stage 6. These values indicate that the majority of spur length increase actually occurs between Stages 4 and 6 despite the minor changes in single cell file counts at these later developmental stages.

These results are similar to those found by Puzey *et al.* (2012) for *Aquilegia vulgaris* L. in which cell division occurs primarily in early development, until the spur reaches a cup-like shape and a length of approximately 5mm, just 20.8% of the average mature length of 2.4cm. Like in *C. ruber*, the majority of the increase in spur length can be attributed to increased cell elongation.

3.4.2 Changes in cell lengths and widths throughout spur development

The cell length and width measurements demonstrate continued cell growth throughout spur development in *C. ruber*. The widths of the cells along the entire length of the spur do not increase significantly between each stage of development, but overall, they show an increasing trend throughout development, with the cell widths at Stage 7 being 2.7 times greater than those at Stage 1. The cell lengths increase at a much more rapid rate throughout development, showing a significant increase between most stages, particularly the mid and late stages. On average, the cell lengths along the entire length of the spur are 6.0 times greater in Stage 7 than they were at Stage 1. Together, these changes in cell size throughout development lead to increased cell anisotropy. In the early stages of development (Stages 1-3), the ratio of cell length: cell width remains very close to 1.0. As development progresses, and the rate of cell length increase becomes much greater than the rate of cell width increase, such that the cell length: cell width ratio becomes closer to 2.0. Similar patterns have been observed in *Linaria vulgaris,* with small, isodiametric cells making up the spur epidermis early in development but changing to greatly elongated cells later in spur development (Box *et al*. 2011). This pattern suggests a brief period of cell division in early development, followed by a more extensive period of cell elongation which contributes to the majority of the spur's mature length. Similar observations of increasing spur epidermal cell anisotropy throughout development have also been made in *Aquilegia coerulea* E.James, however, unlike *C. ruber,* this species showed a non-uniform increase along

the spur, with cells closer to the nectary (i.e., located at the tip of the spur) being much more anisotropic compared to those at the spur base, near its point of attachment (Puzey *et al.* 2012).

3.4.3 The timing and location of cell division activity in developing spurs

The confocal microscopy data demonstrated cell division activity in the early stages (Stages 1-3) of *C. ruber*'s spur development. Although the cell file count data suggests that there is a small amount of cell division occurring in the mid (Stages 4-5) and late (Stages 6-7) developmental stages, no mitotic figures were observed in samples from any of these stages. The confocal technique for assessing cell division activity allows for a general pattern to emerge and is not able to catch every cell division. Accordingly, multiple samples from each stage are required in order to construct an understanding of the timing and location of mitotic activity in the developing spur. Apart from the apparent infrequency of cell divisions in late spur development, the fact that no cell divisions were observed for Stages 4-7 may have been due to the sample size of 20 spurs per stage. It is possible that if the sample size were increased to 100, several mitotic figures would be observed in these stages. Another possibility is that mitotic figures present in the samples observed were not readily visualized with the confocal microscope. The nuclei of cells in early development stained readily and were viewed with ease, whereas the nuclei of cells in later development tended to stain less readily and were often pressed tightly against the plasma membrane by the large central vacuole, deforming their shape. Although all epidermal cells were scanned carefully along their entire length for mitotic figures, it is possible that some were missed. Despite these potentially contributing factors, the mitotic figure data, along with the SEM work, do demonstrate that cell division is paramount in early development and becomes less so as spur development progresses, and that the outer epidermal cells continue to enlarge, particularly in length, resulting in anisotropic cells.

The location of the cell divisions in Stages 1-3 observed with confocal microscopy was diffuse, with cell divisions observed along the entire length of the spur. These observations were contrary to the hypothesis that there would be distinct points on the spur from which the new cells originated. Puzey *et al.* (2012) showed a similar pattern in *Aquilegia vulgaris* using *in-situ* hybridization, with diffuse cell division activity observed along the entire length of the spur. These results are not in agreement with Tepfer's interpretation of spur growth in *Aquilegia* based on histological work. Tepfer (1953) stated that continued spur growth occurs primarily by cell enlargement and intercalary growth, whereas Puzey *et al*. (2012) propose that cell growth alone is the primary driver of spur development after the initial period of cell division which ceases early in development. Puzey *et al*. also note that in various *Aquilegia* species, variation in the degree of spur epidermal cell anisotropy accounts for 99% of the differences in spur lengths, with species showing less than 30 +/- 21% difference in the number of cells in a single cell file, but having spur length varying up to 600%. Although *Centranthus* and *Aquilegia* are not closely related, they appear to have similar patterns of spur growth, apart from the non-uniform increase in cell anisotropy along the spurs of *Aquilegia*, suggesting convergence in growth pattern. It would be interesting for future work on other *Centranthus* species, those with longer or shorter spurs, to examine whether this trend seen in *Aquilegia* also holds true in other genera.

3.4.4 The meristem hypothesis of spur growth

Since Tepfer's *Aquilegia* study, it has been accepted that nectar spurs develop by meristematic regions, essentially by adding one cell at a time, despite a lack of additional evidence to support the claim (Puzey *et al*. 2012). Although, Tepfer's work on *Aquilegia* has suggested that cell division continues to occur throughout spur development and contributes to spur elongation until the spur reaches its mature length, Tepfer did recognize the important role

of cell enlargement during the later stages of development. Tepfer's work was not focused on spur development and the longitudinal section used to draw the conclusions about continued meristematic activity was a young spur which had not yet developed the mature, elongated morphology. At this early stage, cell division activity likely was still occurring in the spur. It was not until recently that work on *Linaria vulgaris* and *Aquilegia* spp. has demonstrated that cell division actually plays a very minor role, primarily during early spur development, compared to the more extensive role of cell expansion in the later stages of rapid growth (Box *et al*. 2011, Puzey *et al*. 2012). It was concluded that the meristem hypothesis must be rejected in *Aquilegia* because cell division activity is limited to the early stages of spur initiation and development, with cell expansion acting as the main driving force of spur growth for the majority of its growth (Puzey *et al*. 2012). Likewise, the meristem hypothesis must be rejected in *C. ruber* because cell division is not driving spur growth throughout the entire developmental period. However, even though the meristem hypothesis must be rejected, there still appears to be a period of extensive cell division activity which could be considered meristematic, during early development.

3.4.5 Petal growth and development

The development of floral organs is controlled by numerous genetic, environmental and hormonal factors (Meyerowitz 1997, Mizukami and Fischer 2000). The shoot apical meristem produces floral primordia which develop into floral meristems, on which the floral organs are initiated by localized cell division (Meyerowitz 1997). The ABCE quartet model of floral organ identity describes regulation of organ identity by the combinatorial action of numerous *MADS BOX* organ identity genes (Coen and Meyerowitz 1991, Causier et al. 2010, Theissen and Saedler 2001). It is the A, B and E classes of floral organ identity genes which determine the identity of the second whorl as petals (Theissen and Saedler 2001). The relationship between the

determination of floral organ identity and the subsequent growth of the organs remains somewhat unresolved, but it appears that the *MADS BOX* genes continue to play an important role in the later stages of organ development as well (Dornelas *et al.* 2011). In developing petals, their final shape is determined by several factors, including the growth rate, the growth anisotropy and the direction of growth (Das 2011). The final size of the organ is primarily determined by the size and number of the cells which makes it up. Therefore, developing floral organs require controlled cell division and enlargement in order to obtain the correct patterning and shape (Meyerowitz 1997).

The development of petals requires numerous groups of genes, many which work in concert via feedback loops, in order to establish the second whorl domain, to determine the petal identity and to specify the particular cell types required (Irish 2008). The hormones which are most important for the process of petal development are auxin, gibberellins and jasmonic acid (Chandler 2011). Several genes involved specifically in the process of petal growth have been identified, however understanding of the overall genetic control of this process remains limited, particularly in the later stages of development (Manchado-Rojo *et al*. 2012, Abraham *et al.* 2013). The petals usually develop by an initial stage of cell division, after which cell division activity ceases basipetally, followed by a period of cell growth and differentiation (Reale *et al*. 2002, Abraham *et al.* 2013). *ERECTA* is a regulator of petal shape in *Arabidopsis*, which may function by regulating this switch from cell division to cell expansion in the developing petals (Abraham *et al*. 2013). In *Arabidopsis*, the gene *AINTEGUMENATA* (*ANT*) is one of the regulatory genes implicated in the control of cell number during organ development, with loss of function mutations resulting in organs with decreased cell numbers and ectopic expression resulting in enlarged organs due to an increased period of cell division (Mizukami and Fischer

2000). Conversely, the *BIG BROTHER* gene in *Arabidopsis* has an opposite role, limiting the length of the cell division period and thereby restricting organ growth to specific parameters by degrading factors which promote growth (Disch *et al*. 2006). Another gene which is involved in floral organ growth and determination of organ shape in *Arabidopsis* is *JAGGED* (*JAG*), which functions by the activation or the maintenance of cell division activity, in addition to involvement in the regulation of cell expansion (Dinneny *et al.* 2006, Ohno *et al.* 2003). *BIG PETAL* has a role in determining the degree of cell expansion following mitotic activity in *Arabidopsis*, with mutations causing increased cell expansion, resulting in oversized petals (Szecsi *et al*. 2006). It is thought that auxin signaling plays a key role in petal development due to its interaction with the process of cellular expansion (Irish 2010). Understanding how simple petals are formed will provide a framework for understanding the development of more complex petal forms such as those bearing petal spurs.

3.4.6 Conclusions

It has been suggested that the evolution of adaxial-abaxial polarity may account for much of the diversity in form in the lateral organs between different taxa (Bowman *et al*. 2002). In particular, there exists tremendous variation in petal morphology across angiosperm taxa, more than with any other floral organ type (Takeda *et al.* 2013). It is the combined action of numerous genetic and hormonal factors which collectively control the development of the petals, and spurred species clearly require unique patterns of gene expression compared to non-spurred species in order to derive their complex form. In some species, such as *Aquilegia*, the evolution of nectar spurs is considered to be a key innovation which has allowed diversification and rapid speciation (Hodges 1997, Hunter 1998). Despite this importance, information on the cellular and genetic bases for spur development is just beginning to be characterized.

KNOX gene expression was first implicated in nectar spur development by Golz *et a*l. (2002) when investigating outgrowths on the petals of *Antirrhinum majus* L. which could be induced by dominant mutations resulting in ectopic expression of the *KNOX* genes *Hirzina*-d153 (*HIRZ*) and *Invaginata*-d1 (*INA*) in the petals. The closest relatives of *A. majus* possess nectar spurs and it is unknown whether *A. majus* arose from a spurred or non-spurred ancestor (Golz *et al*. 2002). A role for *KNOX* genes in nectar spur development has also been demonstrated in *Linaria vulgaris* and *Dactylorhiza fuchsii*, but despite this role, these genes do not set the fate of the spurs (Box *et al*. 2011, 2012). Other genes expressed in the developing petals and involved in the regulation of cell division and cell expansion, such as *ANT* and *JAG*, could be important for determining spur fate (Box *et al*. 2011). Understanding the timing and position of cell division activity and the extent of cell elongation in developing nectar spurs will help to develop a generalized model of spur development which can be complemented by future genetic work.

Chapter 4: NECTAR SECRETION DYNAMICS OF *CENTRANTHUS RUBER*

4.1 Introduction

Nectar and pollen are the two primary rewards offered by angiosperms to their potential pollinators, with nectar being most widely appealing as it is used by the greatest number of floral visitors (Fahn 1979, Simpson and Neff 1981). Nectar secretion is of great importance because, in a large proportion of angiosperms, it is vital to successful pollination and the subsequent production of fruits and seeds (e.g. Pleasants and Chaplin 1983, Pyke 1981, Vesprini *et al*. 1999). The secretion of floral nectar provides a substantial nutritional reward for floral visitors and encourages them to continue to maintain their relationships with the flowering plants (Hodges 1995, Ren *et al*. 2007).

4.1.1 Nectar composition

Sugars, primarily sucrose, glucose and fructose, are the key nutritive components of the nectar and they are either derived from the phloem sap (Fahn 1979, De la Berrera and Nobel 2004) or from hydrolysis of starch stored in the nectary tissues (Ren *et al*. 2007). Apart from sugars, nectar is composed of a wide range of different compounds including amino acids (Baker 1977, Baker and Baker 1977, 1986), proteins (Nicolson and Thornburg 2007), lipids (Baker and Baker 1975, Baker 1977), volatiles (Ecroyd *et al*. 1995, Raguso and Pichersky 1995, Raguso 2004), potentially toxic substances such as alkaloids, phenols and glycosides (Baker 1977), vitamins (Lüttge 1977), antioxidants (Baker 1977), mineral ions (Lüttge 1977), among others, with composition and concentration often varying significantly between species (Baker and Baker 1983). In some cases, differences in nectar chemistry have been suggested to be related to the phylogeny of the plants (Barnes *et al*. 1995, Van Wyk *et al*. 1993, Galetto and Bernardello 2003). However, pollinators also have various nutritional requirements, preferences and physical

constraints which cause them to consume particular types of nectar (Wykes 1952, del Rio and Karasov 1990, Erhardt 1991). In these situations, it has been frequently demonstrated that the chemical composition of nectar is similar between plants with similar pollination syndromes and thereby visited by similar types of pollinators (Baker and Baker 1975, Freeman *et al*. 1984, Nicolson and Thornburg 2007, Krömer *et al*. 2008). This suggests that pollinator mediated selection is an important causal factor for the variation in nectar chemistry across angiosperm taxa (Hodges 1995).

4.1.1.1 Water

As an aqueous solution, nectar production requires a source of water, which is usually derived from the phloem and/or xylem connection which serves the nectary tissue (Pacini and Nicolson 2007). For many pollinators, especially in arid environments, nectar is a source of water in addition to a caloric resource (Nicolson 1998, Pacini and Nicolson 2007). Optimal water levels in the floral nectar can be maintained by microclimatic floral features or by its physical or chemical attributes (Corbet and Willmer 1981, Corbet *et al*. 1979).

4.1.1.2 Carbohydrates

During nectar production, sucrose is either transported from the phloem sap or generated directly within the nectary tissues. Some of this sucrose is hydrolyzed by invertases into fructose and glucose in many species (Heil 2011). The *CELL WALL INVERTASE 4* gene, which encodes a putative sucrose hydrolysis enzyme, has been demonstrated to be required for the production of floral nectar in *Arabidopsis* (Ruhlmann *et al*. 2010). Although nectar carbohydrate composition usually comprises primarily sucrose, fructose and glucose, a variety of other monosaccharides, disaccharides and oligosaccharides have been identified in floral nectars, usually in

comparatively smaller amounts (Percival 1961, Nicolson and Thornburg 2007). However, there are exceptions to this trend as seen in several species in the Proteaceae which have xylose-rich nectar (Jackson and Nicolson 2002). The final ratio of sucrose:fructose:glucose in the nectar varies based on the species, among other factors (Percival 1961). The carbohydrate composition has been related to the pollinator type, with a demonstrated trend for species with hexose rich nectar to be pollinated by short-tongued bees, bats, flies and perching birds, versus species with sucrose dominant nectar which are often pollinated by hummingbirds and long-tongued insects (Perret *et al*. 2001 and references therein).

In many cases, variation in nectar carbohydrate composition between plants of the same species in a population has been reported to be negligible (Percival 1961, Davis *et al*. 1998), but several studies have detected significant variations between flowers of the same plant and even nectaries of the same flower (Percival 1961, Davis *et al*. 1998, Herrera *et al*. 2006, Canto *et al*. 2007). Herrera *et al.* (2006) suggest that extreme within plant variation could be an adaptive mechanism, causing foragers who are sensitive to variance to visit a reduced number of flowers on a given plant, thereby reducing the chances of geitonogamy (i.e., pollination of other flowers within the same plant). However, Canto *et al*. (2007) demonstrated that the intraspecific variation observed in field specimens of *Aquilegia vulgaris* and *A. pyrenaica* DC. was eliminated when the plants were grown in the greenhouse, suggesting that other factors, such as microorganism contamination, may be the cause of differences in nectar carbohydrate composition in some species.

4.1.1.3 Amino acids

The presence of free amino acids in the floral nectar is considered to be universal amongst nectar secreting angiosperms and members of the same species tend to contain the same

amino acid complement even if inhabiting strikingly different habitats (Baker and Baker 1977, Gardener and Gillman 2001a). This constancy suggests that this aspect of nectar composition is under genetic control and that the phenotypic expression is not affected by environmental conditions (Baker and Baker 1977). Additionally, different coloured varieties of the same species, which may be expected to have different amino acid complements due to the physiological differences associated with the expression of different phenotypes, all contain the same amino acid complement (Baker and Baker 1977). For example, this consistency has been demonstrated in *Centranthus ruber*, with the red, pink and white flowered varieties all containing the same amino acid complement in their nectar (Baker and Baker 1977).

Unlike the apparently stable complement of amino acids, the concentrations can vary significantly between different populations and even between different plants within a population. Work on *Impatiens capensis* demonstrated that concentrations of particular amino acids vary significantly at the plant and at the population level, although no significant variation was observed at the flower level (Lanza *et al*. 1995). Fertilization experiments on *Agrostemma githago* L. demonstrated a positive relationship between the use of soil fertilizer and the total concentration of amino acids in the nectar, with increasing fertilization resulting in significantly increased levels of glutamine and proline, demonstrating the role of the environment in regulation of the levels of some amino acids (Gardener and Gillman 2001b).

The pollinator type can give an indication of the expected levels of amino acids in the floral nectar, with angiosperms pollinated by some species of wasps, butterflies and settling moths tending to have higher levels of amino acids in their nectar compared to other pollinator types (Baker 1977, Alm *et al*. 1990). Additionally, proline-rich nectars are attractive to insects such as honeybees because proline is important for flight (Carter *et al*. 2006). It has also been

demonstrated that some species of butterflies show a preference for nectar with a higher content of proline, along with other amino acids (Alm *et al*. 1990, Watt *et al*. 1974). Map butterflies (*Araschnia levana* L.) fed an amino acid rich diet had greater fecundity than those fed a lower amino acid diet, demonstrating a fitness benefit for butterflies which consume nectar high in amino acids (Mevi-Shütz and Erhardt 2005, Jervis and Boggs 2005).

4.1.1.4 Lipids

The presence of lipids in floral nectar has been described in many different species (Baker 1977, Forcone *et al*. 1997). In most nectars, lipids are present in just small or trace amounts, but the nectar of certain species, such as *Catalpa speciosa* (Warder) Engelm., *Jacaranda acutifolia* Bonpl., and *Trichocereus andalgalensis* (F.A.C.Weber ex K.Shum.) Hosseus, contain more significant levels, giving the nectar a milky white appearance (Baker and Baker 1983). Lipids are high energy compounds, so their presence in floral nectar can significantly enhance a pollinator's diet (Nicolson and Thornburg 2007). An additional suggested role for lipids in the floral nectar is the formation of lipid monolayers on the nectar surface, which can reduce evaporation and maintain more dilute nectar than would be otherwise expected in low humidity environments (Corbet *et al*. 1979).

4.1.1.5 Microorganisms

In addition to chemical compounds, living organisms such as bacteria, yeasts and fungi are commonly associated with floral nectar despite the presence of antimicrobial compounds (Alvarez-Perez *et al*. 2012). Yeasts are the most common microorganisms associated with the nectar and their activities can greatly affect the composition and sugar concentration of the nectar (Herrera *et al*. 2008). Differences in microorganism density in the nectar of flowers within the

same species, and even within nectaries of the same flower, can result in significant intraspecific and intra-plant variation in nectar composition, suspected to be due to the hydrolysis of sucrose by the microorganisms, followed by the metabolism of the resulting fructose and glucose (Canto *et al*. 2007, Herrera *et al*. 2008). It has been demonstrated that visitation and probing by *Bombus terrestris* and *B. pratorum* on the flowers of *Helleborus foetidus* L. results in decreased sucrose levels and increased fructose and glucose levels, changes which are not observed when flowers are visited and probed by other insects such as *Andrena*, *Apis mellifera* and *Lasioglossum* (Canto *et al*. 2008). This discrepancy suggests that some floral visitors may be transferring microorganisms to the nectar via their mouth parts, resulting in the hydrolysis of sucrose into its component monosaccharides (Canto *et al*. 2008). In addition to fungi and yeasts, the presence of unique bacterial communities has been demonstrated in several different species and these organisms may play a role in influencing nectar chemistry, perhaps by producing volatile compounds (Fridman *et al*. 2012).

4.1.1.6 Proteins

Nectar proteins called nectarins appear to serve an important role in nectar protection against microbial infection (Park and Thornburg 2009). Proteins have been demonstrated to occur in the nectar of various species, but have only been thoroughly characterized in the ornamental tobacco model system and in *Petunia* Juss. (Peumans *et al*. 1997, Kram *et al*. 2008, Park and Thornburg 2009, Hillwig *et al*. 2010, 2011). In tobacco, hydrogen peroxide is produced by nectar proteins via the nectar-redox cycle and it is accepted that the hydrogen peroxide plays a role in killing nectar microorganisms, or at least limiting their growth (Carter and Thornburg 2004, Park and Thornburg 2009). In *Petunia*, however, only small amounts of hydrogen peroxide are produced and the nectar proteins, which have been identified as ribonucleases, endochitinases

and peroxidases, protect the nectar against microorganism invasions using alternate strategies, demonstrating that antimicrobial mechanisms may not be as conserved as once predicted (Hillwig *et al*. 2010, 2011). The survey and identification of nectar proteins in additional species will be required in order to make generalizations about their roles and modes of action.

4.1.1.7 Volatile organic compounds (VOCs), phenolics, alkaloids and glycosides

Although produced in small quantities, the presence of volatiles, phenolics, alkaloids and glycosides can have a significant effect on floral visitation. Volatiles can provide odour cues to potential pollinators, signaling the presence of nectar (Raguso and Pickersky 1995, Blight *et al*. 1997, Andrews *et al*. 2007). In general, presence in the nectar of secondary metabolites which plants use for defense, such as phenolics and alkaloids, is regarded as a strategy to deter herbivores and nectar robbers, floral visitors which consume floral nectar without providing a pollination service to the plant (Gonzalez-Teuber and Heil 2009). Iridoid glycosides in the floral nectar of *Catalpa speciosa* intoxicate nectar robbers and deter them from consuming large quantities (Stephenson 1982). Phenolic compounds in the nectar of *Aloe vryheidensis* Groenew. give the nectar a bitter taste which is unpalatable to incompatible floral visitors, while also imparting a dark colouration which serves as a cue for its pollinators (Johnson *et al*. 2006). Clearly, the evolution of nectar chemistry is driven both by interactions with beneficial pollinators and with destructive visitors such as herbivores and nectar robbers. Therefore it is important to consider the selection pressures from both directions when investigating nectar composition (Adler 2000).

4.1.2 Nectar solute concentration

Nectar solute concentration is usually recorded with a refractometer which gives the

concentration as a percent on a "weight to weight" basis (g solute per 100g solution) (Bolten *et al*. 1979). This concentration by weight can then be converted to a nectar concentration by volume (g solute per 1mL solution) using the quadratic equation proposed by Burquez and Corbet (1991), which will then allow the calculation of total nectar solutes given a known nectar volume. Nectar solute concentrations can vary tremendously between different species and also within a species (Nicolson and Thornburg 2007). The solute concentration of the phloem sap of most plants ranges from 18-30% and it is changes which occur during nectar production that influence the final solute concentration (Lüttge 1977). The nectar of bird pollinated species tends to be quite dilute (Bolten and Feinsinger 1978, Johnson and Nicolson 2008), with South African passerine bird pollinated species demonstrating particular dilute (10-15%), hexose rich nectars (Nicolson 2002). Insect pollinated species tend to have more concentrated nectars, such as that of *Carum carvi* L. ranging from 48-76.5% (Langenberger and Davis 2002) and that of *Arceuthobium abietinum* (Engelm.) Engelm. Ex Munz spanning 58-92% (Brewer *et al.* 1974). In several spurred orchid species, it has been demonstrated that the nectar at the tip of the spur has a higher solute concentration than the nectar more easily accessible at the base of the spur near its point of attachment, perhaps functioning to increase successful pollination by forcing the floral visitors to increase physical contact with the reproductive floral organs (Martins and Johnson 2007).

Climatic factors such as low or high humidity can cause differences in the nectar solute concentration if the nectar is exposed (Corbet *et al*. 1979, Corbet and Willmer 1981, Nicolson 1998). In a tropical climate, it has been shown that morphological features such as tubular corollas help protect nectar from becoming too dilute by maintaining a stable microclimate inside the flower (Corbet and Willmer 1981). In arid environments, several different mechanisms

for maintaining nectar sugar concentrations lower than would be expected based on the equilibrium with the ambient humidity have been described. These include microclimatic mechanisms such as the protection of nectar by a tubular corolla, nectar spur or barrier trichomes, chemical mechanisms such as the establishment of concentration gradients, or physical mechanisms such as the development of a water-proof lipid layer atop the secreted nectar (Corbet *et al.* 1979).

4.1.3 Nectar volume

Nectar volume is highly variable between species and can depend on numerous factors such as flower size (Murrell *et al*. 1982), nectary size (Petanidou *et al*. 2000), pollinator type (Symes and Nicolson 2008), the plant's energetic status (Pleasants and Chaplin 1983) and environmental conditions (Wyatt *et al*. 1992, Pacini *et al*. 2003). To some extent, pollinator mediated selection appears to have resulted in nectar volumes being stabilized at particular levels in different species (Barrows 1976, Hodges 1995). It has been demonstrated that increasing or decreasing these levels by artificial manipulation can result in decreased plant fitness (Hodges 1995) or, in other cases, increased plant fitness (Zimmerman and Cook 1985). Increased nectar volumes can lead to decreased fitness by increasing geitonogamy, which can cause reduced fitness in both self-compatible and self-incompatible species (Waser and Price 1991, Broyles and Wyatt 1993, Hodges 1995). However, in plants which are pollinator limited, increased nectar production can have the opposite effect on fitness, resulting in increased fruit set (Real and Rathcke 1991). In other cases, decreasing nectar volumes have been demonstrated to increase the distance of pollen transport, resulting in greater chances of outcrossing and therefore enhanced fitness (Zimmerman and Cook 1985, Maloof and Inouye 2000). Because of these opposite relationships between increased or decreased nectar in different species and situations, it is

difficult to reach any generalized conclusions about the role of nectar volume in pollination and reproductive success, other than that it is highly dependent on the circumstances.

At the within plant level, variation in the volume of nectar secreted per flower is variable between species, with some plants secreting similar amounts of nectar per flower and others producing widely different volumes between flowers (Feinsinger 1983, Biernaskie and Cartar 2004). “Bonanza-blank” nectar secretion patterns, in which flowers can produce either a miniscule or a large quantity of nectar, have been implicated as an adaptation to ensure reproductive success by forcing pollinators to visit many flowers in search of the bonanza flowers (Feinsinger 1978, 1983).

4.1.4 Nectar reabsorption

The production and secretion of nectar is an extremely costly process for flowering plants, requiring the allocation of a significant portion of their energy (Southwick 1984). Nectar reabsorption is a complementary process whereby solutes are transferred from the nectar back to the nectary following secretion (Burquez and Corbet 1991). This process, which serves to recover energy and maintain low sugar concentrations during evaporation, has been demonstrated in numerous species (Burquez and Corbet 1991, Nicolson 1995, Davis 1997, Nepi *et al*. 2001, 2003). In some species, such as *Mystacidium venosum* Harv. Ex Rolfe, pollination can induce nectar reabsorption and the reabsorption of unused sugars results in better quality fruits (Luyt and Johnson 2002). Experimental removal of nectar has been shown to increase nectar accumulation in some species, either by increased production in visited flowers or reabsorption in unvisited flowers (Nicolson 1995, Gill 1988). Nectar reabsorption can be confirmed by radiolabelling experiments which allow detection of reabsorbed labelled sugars using microautoradiographical techniques (Stpiczynska 2003a).

4.2 Materials and methods

4.2.1 Determination of nectar volumes and solute concentrations in the floral spurs of *C. ruber* throughout anthesis

Closed buds from two different *C. ruber* plants, which were tagged with string and small numbered labels, were observed at least once every two hours until anthesis. This frequent observation ensured that the time of anthesis was known within two hours. Nectar samples were collected for 6-hour intervals ranging from 0-6 hours to >114-120 hours after anthesis. Any samples collected at >120 hours were included in a single 120+ hours after anthesis category. Flowers were destructively sampled and nectar was collected in 1.0 µL Drummond microcapillary tubes (Microcaps®) to determine the total nectar volume. Nectar solute concentration by weight (g sucrose per 100g solution) was determined by expelling a drop of the nectar onto a portable, hand-held refractometer (Bellingham and Stanley). The nectar concentration by volume (g sucrose per mL of solution) (NCV) was calculated from the nectar concentration by weight (NCW) using the equation from Burquez and Corbet (1991):

$$NCV = NCW^2 (59.6 \times 10^{-6}) + NCW (9.224 \times 10^{-3}) + 7.08 \times 10^{-3}$$

This equation results in less than 1% error for nectars with solute concentrations ranging from 10-80% (Burquez and Corbet 1991). The total amount of nectar solutes (µg) in each sample was calculated by multiplying the calculated NCV by the nectar volume collected.

4.2.2 HPLC analysis of nectar carbohydrate composition

Nectar samples from two plants were collected at known periods after anthesis and nectar was dispelled from the Microcap® onto small pieces of filter paper. At least 4 nectar samples per plant were analysed for each of the following time categories following anthesis: 0-6h, >6-12h, >12-18h, >18-24h, >30-36h, >42-48h, >54-60h, >66-72h, >84-90h, >102-108 and >108-114.

The filter paper wicks were placed into microfuge tubes, diluted with a known amount of double distilled water and agitated for at least 30 minutes to ensure all of the nectar solutes were transferred from the filter paper to the water. Samples were filtered using sterile syringes with a 0.2µm pore size and their carbohydrate composition was analysed using a Dionex ICS 5000 high performance liquid chromatography system (Thermo Fischer Scientific). The system included a Dionex AS autosampler, an ICS 5000 electrochemical cell with a gold electrode, a 25µL injection loop and a Dionex CarboPac PA1 column. An isocratic mobile phase of 80mM NaOH was run through the system at a flow rate of 1.0mL/min to separate the sugars. Data was collected using Dionex Chromeleon 7.0 software. All samples were run in duplicate, apart from several which had too little nectar available for dilution. Linear standard curves for glucose (1-100ppm, $r^2 = 0.998$), fructose (1-100ppm, $r^2 = 0.999$) and sucrose (40-175ppm, $r^2 = 0.991$) were created to determine the sugar concentrations for the samples. These concentrations were used in combination with the original volume and percent solute data to calculate the amounts (µg) of glucose, fructose and sucrose which were present in the nectar of each original sample. Glucose, fructose and sucrose quantities were converted to percentages to allow comparisons to be made between the carbohydrate profiles of the nectar collected at different time periods throughout anthesis.

4.3 Results

4.3.1 Nectar volumes and solute concentrations following anthesis

Total nectar volume per spur throughout anthesis ranged from 0-1.45µL (mean ± s.e.: 0.39 +/- 0.016µL, median: 0.34µL, n=376) for the first plant and 0-1.20µL for the second plant (mean: 0.28 +/- 0.017µL, median: 0.19µL, n=251). When compared with a t-test, the differences between Plant 1 and Plant 2 were found to be highly significant ($p < 0.001$). In the first plant,

flowers with no nectar occurred in 8.8% (33/376) of samples and these occurred at an average time of 90.5 +/- 5.3 hours (median: 88.0, range: 22.5-142.0) following anthesis. In the second plant, flowers with no nectar represented 7.1% (18/251) of the samples and these occurred at an average time of 75.3 +/- 7.7 hours (median 73.0, range: 4.5-144.25) following anthesis. On the other hand, flowers with abundant nectar (>0.75µL) represented 14.4% (54/376) of samples in the first plant and these occurred at an average time of 45.5 +/- 4.0 hours after anthesis. In the second plant, only 7.2% (18/251) of sampled flowers had abundant nectar, occurring at an average time of 62.1 +/- 7.5 hours following anthesis. When flowers yielded 1µL or more, the nectar often completely filled the spur and a small portion of the base of the corolla tube. For the first plant, 63.8% of the samples were collected between the months of May and August and the remaining 36.2% were collected between November and April. For the second plant, 51.8% of the samples were collected between May and August and 48.2% were collected between November and April.

The nectar solute concentrations ranged from 11-50% in one plant and 15-50% in the second plant. It is possible that some nectar solute concentrations exceeded 50%, but low nectar volumes often only allowed one attempt for a refractometer reading and because most nectar samples were under 50%, a 0-50% refractometer routinely was used. The average and median nectar solute concentrations were 34.4 +/- 0.53% and 33% (n=287), respectively, for the first plant and 39.3 +/- 0.66% and 42% (n=174) for the second. When compared with a t-test, the results from the two plants were found to be very statistically significant ($p < 0.0001$). The average and median total solute amounts per flower were 165.5 +/- 7.0µg and 155.0µg (n=322) for the first plant and 156.2 +/- 9.1 µg and 131.7µg (n=187) for the second plant. When compared with a t-test, the differences in average solute amounts per flower (µg) were not found

to be statistically significant ($p = 0.4178$).

From anthesis to senescence, the first plant appears to show an initial increase in nectar volume (μL) and total nectar solutes (μg), peaking 36-60 (midpoint 48) hours after anthesis (n=31, average volume: 0.58 +/- s.e. 0.044μL; n=29, average amount of solute: 293.39 +/- s.e. 22.07μg), followed by a decrease in both (Figs. 4.1, 4.2). The second plant shows a similar trend of increase in volume and solutes followed by a subsequent decline, but the peaks are lower and later than those in the first plant Figs. 4.1, 4.2). The volume and solute amounts in the second plant peak at 84-108 (midpoint 96) hours following anthesis (n=55, average volume: 0.33 +/- s.e. 0.038 μL; n=42, average amount of solute: 209.67 +/- s.e. 21.38 μL) (Figs. 4.1, 4.2).

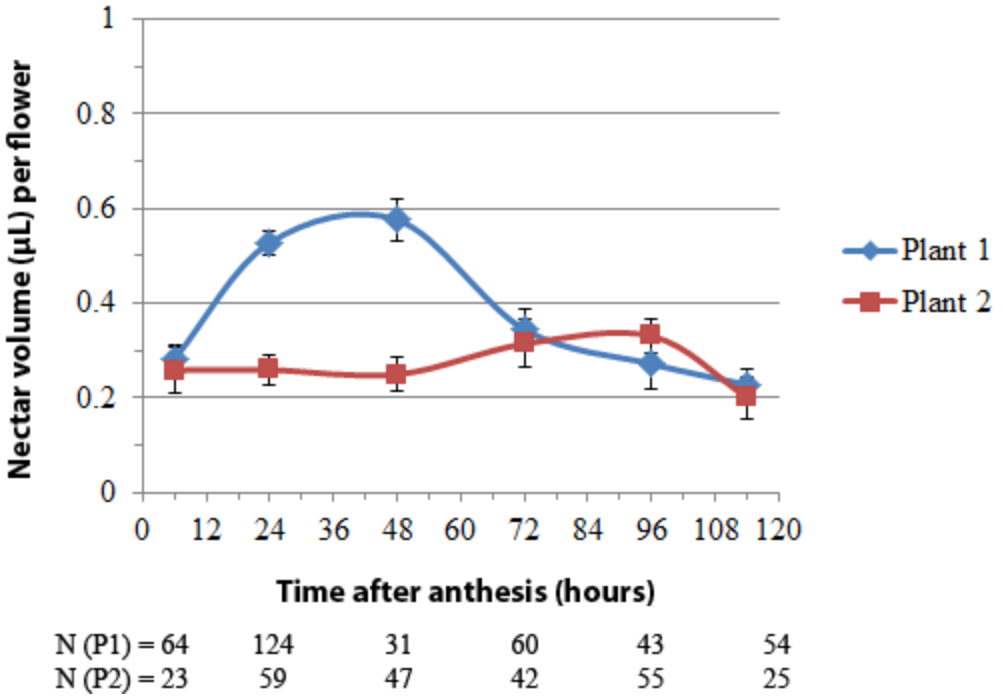

Figure 4.1 - Average total nectar volumes (± s.e.) per spur throughout flowering phenology in two greenhouse plants of *Centranthus ruber*. This graph shows the average volumes of nectar collected from flowers at different times after anthesis. The total number of flowers (N) sampled per interval are shown for Plant 1 (P1) and Plant 2 (P2).

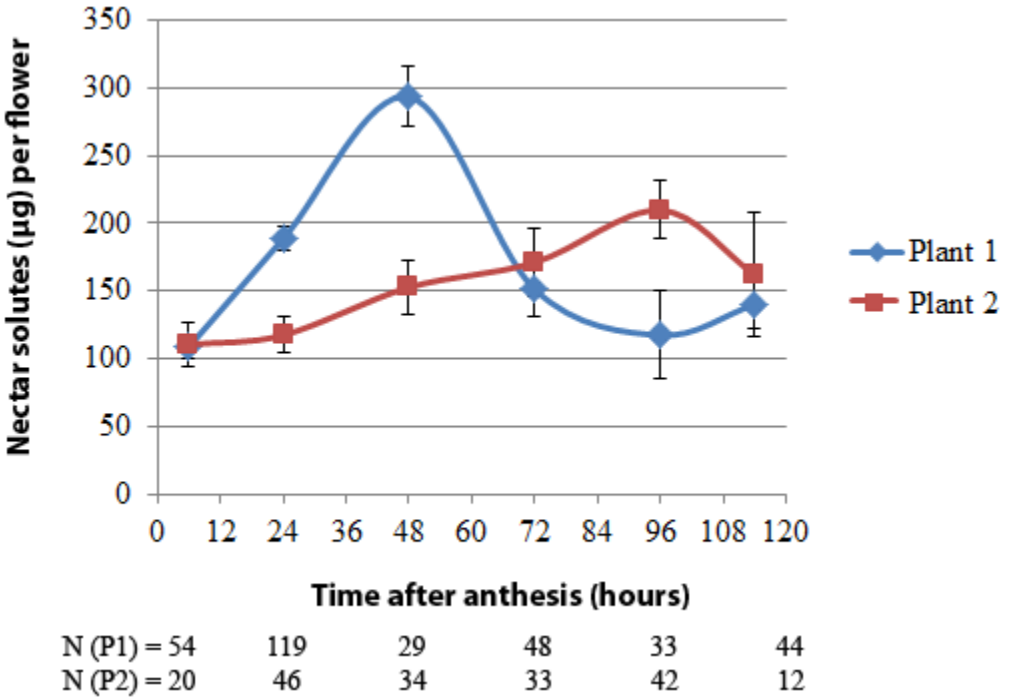

Figure 4.2 - Average total nectar solutes (± s.e.) per spur throughout flowering phenology in two greenhouse plants of *Centranthus ruber*. This graph shows the average total solutes present in the nectar collected from flowers at different times after anthesis. The total number of flowers (N) sampled per interval are shown for Plant 1 (P1) and Plant 2 (P2).

4.3.2 Nectar carbohydrate composition

There were no significant differences between the results of the HPLC analysis between the two replicates for any of the nectar samples analysed. Nectar carbohydrate composition within the floral spurs in both plants tested was highly consistent (Fig. 4.3). There was no significant variation in carbohydrate composition throughout flowering phenology at the 11 intervals tested ranging from 0-6 to >108-114 hours following anthesis, showing a constancy throughout the flowering period (Figure 4.3). Glucose amounts in floral nectar ranged from 2.2-34.4 % in the first plant and 8.8-26.4% in the second plant. Fructose quantities ranged from 2.2-32.8% in the first plant and 8.4-29.5% in the second plant, whereas sucrose amounts spanned 32.8-95.5% in the first plant and 45.3-82.8% in the second plant. Miniscule peaks on the HPLC graphs, representing compounds eluting before the larger glucose, fructose and sucrose peaks, could indicate the presence of additional sugar components in the floral nectar of *C. ruber*; however, this possibility was not investigated in this study.

Owing to the lack of significant differences in nectar carbohydrate composition among the two plants (Fig. 4.3), these results have been combined for the species (Fig. 4.4). Overall, the floral nectar of *C. ruber* is sucrose dominant (average: 68.6 ± 8.1%, n=102), with roughly equal amounts of glucose (average: 16.2 ± 4.0%, n=102) and fructose (average: 15.2 ± 4.2%, n=102) (Fig. 4.4).

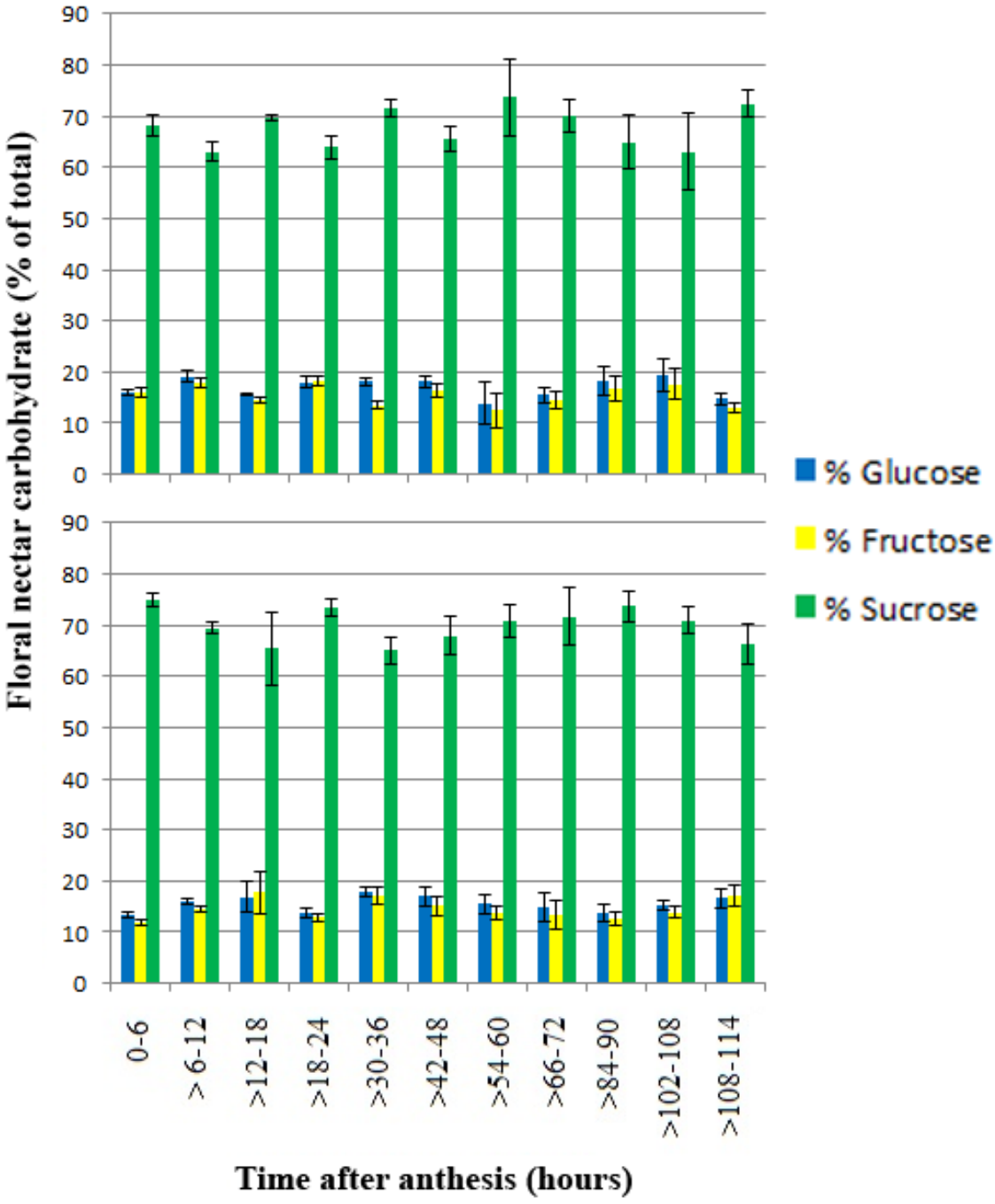

Figure 4.3 – Nectar carbohydrate composition at various time intervals after anthesis in two greenhouse plants of *Centranthus ruber*. These graphs show the carbohydrate composition (glucose, fructose and sucrose) of the spur's floral nectar throughout flowering phenology. Data (mean ± s.e.) at each interval represent a minimum of four nectar samples collected and analysed per plant.

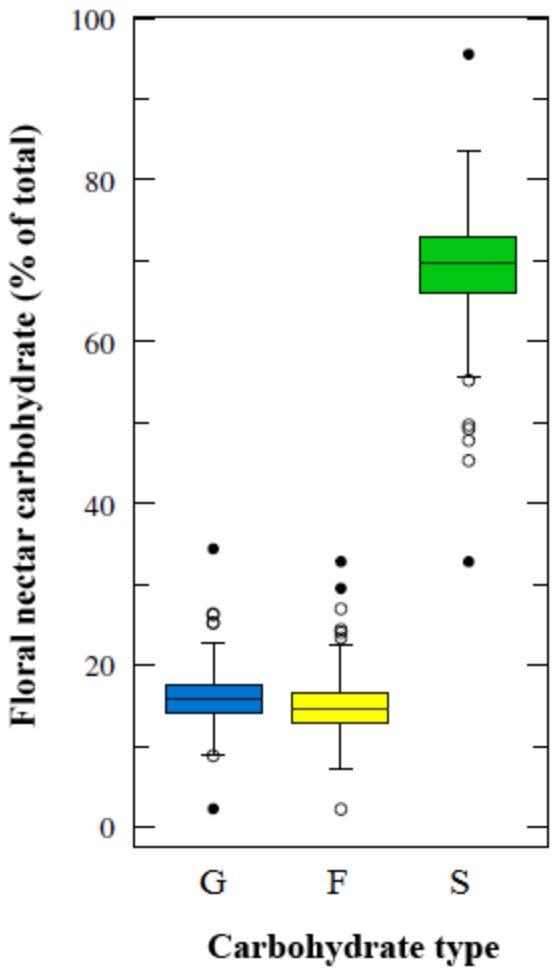

Figure 4.4 - Overall nectar carbohydrate composition in *Centranthus ruber*. This box plot shows the results of the HPLC analysis of *C. ruber*'s nectar. The data was pooled for two separate plants which showed no significant differences from one another (n=102). The nectar is sucrose (S) dominant (overall average: 68.6 ± 8.1%), with roughly equal amounts of glucose (G, overall average: 16.2 ± 4.0%) and fructose (F, overall average: 15.2 ± 4.2%). Whiskers show the minimum and the maximum values, apart from outliers and suspected outliers. Boxes show the intraquartile range (IQR) from quartile 1(Q1) to quartile 3 (Q3), with the line showing the median (Q2). Open circles represent suspected outliers (1.5 times the IQR or greater below Q1 or above Q3). Closed circles represent outliers (3 times the IQR or greater below Q1 or above Q3).

4.4 Discussion and conclusions

4.4.1 Nectar volume and solute concentrations in *C. ruber*

The nectar volumes for both plants (0.2-0.6µL per floral spur throughout anthesis) were quite low when compared to other spurred species such as *Impatiens capensis* (up to 2.03µL secreted per hour; Marden 1984), *Linaria vulgaris* (2-3µL secreted per flower in one population, 6-8µL in a second population; Nepi *et al*. 2003) and *Platanthera chlorantha* (over 10µL secreted per flower; Stpiczynska 2003a). However, *C. ruber*'s flowers are rather small in comparison to the flowers of these species, which could explain the lower nectar volumes. Flower and nectary size have been described as floral characteristics which can influence the volume of nectar secreted (Murrell *et al*. 1982, Petanidou *et al*. 2000). *C. ruber*'s flowers are also arranged into large, showy inflorescences, so the total amount of nectar available to pollinators may be comparable to plants with fewer flowers, containing higher nectar volumes. Interestingly, the differences in average nectar volumes found between Plant 1 (0.39 +/- 0.016µL) and Plant 2 (0.28 +/- 0.017µL), when averaged throughout anthesis, were found to be statistically significant. These differences were most likely due to genetic differences between the two plants, or because the time of year that the majority of samples were collected for each plant varied.

Plants which have inflorescences composed of numerous flowers, such as *C. ruber*, often produce more nectar in some flowers than others in order to encourage pollinators to leave the plant before visiting too many flowers, thereby increasing the chances of out-breeding (Biernaskie *et al*. 2002). Both plants in this study had some flowers which did not contain any nectar, however they tended to be flowers which were sampled, on average, over halfway through their lifespan which could indicate the evaporation or reabsorption of some of the nectar. Only a few flowers sampled early had no nectar, suggesting that this strategy is not likely at

work in *C. ruber*.

All nectar solute concentrations, from which total nectar solute amounts were calculated, were determined using a portable, hand-held refractometer which indicates the amount of nectar solutes in their sucrose equivalents. Although refractometers are commonly used to estimate sugar concentrations, other non-sugar molecules in the nectar, such as amino acids known to occur in the floral nectar of *C. ruber* (Baker and Baker 1977), also contribute to the refractive index and therefore a small amount of error is inherent in using this method to calculate the energy content of the nectar (Inouye *et al*. 1980, Corbet 2003). As with the nectar volumes, the differences in average nectar solute concentrations between Plant 1 (34.4 +/- 0.53%) and Plant 2 (39.3 +/- 0.66%) were found to be very statistically significant. Once again, these differences were most likely due to genetic differences or differences in the time of sampling. Solute concentrations of this level are considered to be mid-range and, compared to other species which have been tested, are neither particularly high nor low. The solute concentrations in both plants had a fairly wide range (Plant 1: 11-50+%, Plant 2: 15-50+%), which is similar to reports in some other species, although not all species display such variance in nectar sugar concentrations (Nicolson and Thornburg 2007).

When studying nectar secretion dynamics, it is important to examine and report both the nectar volumes and solute concentrations, as each provides different information and together they can be used to determine the total solute amount (Bolten *et al*. 1979). Throughout anthesis, it was found that *C. ruber* flowers from the two study plants had nectar volumes and solute concentrations which were both significantly different from one another. Plant 1 had higher volumes, but lower solute concentrations, whereas Plant 2 had lower volumes and higher solute concentrations. Despite these differences, the differences in average solute amounts per flower

were not statistically significant. It appears, therefore, that Plant 1 was able to use a greater amount of water for the production of nectar, resulting in greater amounts of more dilute nectar on average. Both plants were housed in the same greenhouse under identical conditions, so these differences in nectar characteristics were not likely due to differences in amounts of light, water or nutrients received. It is possible that genetic differences between the plants were responsible for the differences in the average volume and solute concentrations. Another possibility is that the time of year influences the volume and solute concentration of nectar secreted, as Plant 1 had a larger proportion of samples collected in the May-August time period compared to the November-April time period, whereas Plant 2 had a similar proportion of samples collected in each time period.

4.4.2 Nectar carbohydrate composition

In this study, *C. ruber*'s floral nectar was found to be sucrose dominant (68.6 ± 8.1%), with roughly equal amounts of glucose (16.2 ± 4.0%) and fructose (15.2 ± 4.2%). Although it was assumed that all of the solutes present in the nectar comprised these three carbohydrates, it is highly likely that trace amounts of various other sugars were present as well. Percival's extensive 1961 survey on the carbohydrate composition of 889 different angiosperm species reported *C. ruber* (listed as *Kentranthus ruber*) to have SFG balanced nectar, indicating that sucrose, fructose and glucose were detected in equal amounts. However, Percival also reports that sucrose dominant nectar is a general trend for flowers which have protected nectar, such as those with long corolla tubes or floral nectar spurs. Flowers with this type of morphology are also often pollinated by butterflies and moths, another indicator of a tendency for sucrose dominated nectar profiles (Percival 1961). These two characteristics are traits of *C. ruber*, indicating that sucrose-rich nectar would be expected in this species. In this study, the nectar of two *C. ruber* plants both

demonstrated sucrose dominance, differing from Percival's findings, but in line with the general trends she presented.

There are several different possible explanations for the discrepancy between Percival's carbohydrate composition results and those determined in this study. Firstly, Percival's early study used paper partition chromatography which is less reliable than the precise HPLC method used in this study. Percival states that the categorization used in her paper is highly subjective because it is determined based on the size and darkness of spots on the chromatography paper. However, it is unlikely that this difference of method could account for the significant difference between the results of the two studies and it is probable that there was an actual difference between the nectars examined. There are several possible explanations for why plants of the same species would have nectar with different carbohydrate compositions. Firstly, differing carbohydrate compositions can be attributed to genetic or environmental population differences, as has been demonstrated between three populations of *Impatiens capensis* which vary in their sucrose concentrations (Lanza *et al.*1995*)*. Additionally, if Percival's samples were collected in the field, rather than from protected greenhouse plants like in this study, it is possible that microorganism contamination could have resulted in the breakdown of much of the disaccharide sucrose into its component monosaccharides (Canto *et al*. 2007, Herrera *et al*. 2008). Plants grown in greenhouse conditions are generally protected from insect vectors which can spread microorganism contamination between plants (Canto *et al*. 2008).

Nectar carbohydrate composition has been investigated in several other spurred species such as *Habenaria gourlieana* Gillies ex Lindl. and *H. hieronymi* Kraenzl. (Galetto *et al*. 1997), *Linaria vulgaris* (Nepi *et al*. 2003), and *Aquilegia vulgaris* and *A. pyrenaica* (Canto *et al*. 2007). *Habenaria gourlieana* was found to have sucrose dominant nectar, whereas the nectar of *H.*

hieronymi was found to be hexose dominant (Galetto *et al*. 1997). Nepi *et al*. (2003) investigated the nectar carbohydrate composition of *L. vulgaris* at three developmental stages (young flowers 1 day before anthesis, mature flowers at anthesis and wilted flowers at senescence) in two different populations. They found that the young flowers in both populations had sucrose dominant nectar (mean S/(F+G) ratio of 10.25), with only trace amounts of glucose, fructose and raffinose (Nepi *et al*. 2003). In the mature and senescent stages, they found significant differences between the two populations, with the S/(F+G) ratios for the two populations in the mature stage being 1.41 and 3.92 and those in the senescent stage being 0.74 and 3.83 (Nepi *et al*. 2003). *C. ruber*'s nectar, in contrast, retained similar sucrose levels throughout the entire flowering period, perhaps due to the protection provided by the greenhouse environment. *A. vulgaris* and *A. pyrenaica* also produced sucrose dominant nectars, with greenhouse grown plants displaying greater sucrose content than field plants and showing less intraspecific variation (Canto *et al.* 2007).

4.4.3 Nectar reabsorption in *C. ruber*

In general, species in which nectar collects in a region that is not the site of the nectary itself and species in which the petals are shed, tend not to reabsorb nectar (Burquez and Corbet 1991). Neither of these are floral characteristics of *C. ruber*, suggesting that *C. ruber* may be a nectar reabsorbing species. In some spurred species such as *Platanthera chlorantha* (Stpiczynska 2003a) and *Linaria vulgaris* (Nepi *et al.* 2003), it has been demonstrated that nectar is reabsorbed following pollination. Interestingly, nectar reabsorption via the nectar spur in *L. vulgaris* flowers occurs in spite of the fact that the spur is not the site of the nectary itself (Nepi *et al*. 2003). One advantage to spurred species is that the nectar can be reabsorbed over a long period of time because the spur protects that nectar from excessive water loss via evaporation

(Pacini *et al*. 2003).

The nectar volume and solute data collected from the two *C. ruber* plants differed in their dynamics throughout flowering phenology. However, the overall changes in nectar volume and solute amounts suggest that nectar reabsorption may be taking place in both plants, but earlier in Plant 1. It is noteworthy that the floral nectar carbohydrate composition in *C. ruber* remained constant, even during the period of net nectar reabsorption. Thus, the floral nectar of *C. ruber* appears to be reabsorbed as a solution, rather than selectively for individual sugars, in agreement with an earlier study (Davis 1997). The actual route of nectar entry into the spur during nectar's reclamation remains to be investigated.

4.4.4 Conclusions

The nectary of *C. ruber* produces nectar which accumulates in the lumen of the spur at anthesis. The sucrose rich nectar of *C. ruber* is suggestive of butterfly pollination (Percival 1961, Perret *et al*. 2001), which matches observations of its floral visitors (Rehnberg 1987). Both plants observed demonstrated an initial increase in both nectar volume and solute amounts per flower as development proceeded, followed by a decline in both, suggesting nectar reabsorption. Nectar carbohydrate composition remained stable throughout anthesis and was equilvalent for both plants analyzed, suggestive of nectar reabsorption as a solution.

Nectar plays a vital role in the interaction between a plant and its pollinators. Particular pollinators demonstrate preferences for nectars which suit their physiological needs, including preferences for specific solute concentations and chemical compositions. Investigation into nectar secretion dynamics, carbohydrate composition and the presence of various additional compounds in the nectar, such as proteins, lipids and protective molecules, is an important aspect of pollination biology which merits further investigation across angiosperm taxa.

Chapter 5: GENERAL DISCUSSION AND CONCLUSIONS

5.1 Relevance of research

Nectar spurs are important structures from the point of view of numerous disciplines within plant biology, including floral development, co-evolution between plants and their pollinators, and species diversification. A solid foundation regarding these interesting floral structures, based on ecological, developmental and genetic approaches is currently being developed. However, because spurs arose multiple times throughout the evolution of angiosperms (Hodges 1997), and therefore likely possess different developmental origins which may correspond to different mechanisms of formation (Golz *et al.* 2002), it is important to investigate as many different species as possible to approach a full understanding of their structure, growth, and ecological and evolutionary significance.

Although *C. ruber*'s flowers are small, often making them tedious to study, the species is an interesting candidate for spur research. Along with *Plectritis*, *Centranthus* is one of just two genera with spurred members within the family Valerianaceae and one of just five genera with spurred members within the entire order Dipsacales. In the literature, there is no indication that the nectar spurs of *Centranthus* or *Plectritis* have been investigated in any detail. This study was, therefore, able to produce some novel findings, contributing to a fuller understanding of spur biology.

This study provided morphological, anatomical and ultrastructural information about the spur and nectary of *C. ruber* throughout its entire developmental period. Information regarding the nectar spur of *C. ruber* is scarce within the literature and ultrastructural studies on nectaries are limited, especially those investigating spur nectaries and those providing information about ultrastructural changes throughout development. Within the Dipsacales, floral nectary

ultrastructure has only been reported in *Lonicera japonica* and *L. kamtschatica* and there have been no reports within the Valerianaceae. The spur of *C. ruber* was found to house a trichomatous nectary, composed of unicellular secretory trichomes which run along the entire inner, abaxial surface of the spur. The secretory trichomes were found to be characteristic transfer cells with a thick and complex layer of wall ingrowths at their tips to aid in efficient secretion of nectar. The nectary was found to be vascularized by a single vascular bundle composed of both xylem and phloem, and nectar release into the lumen of the spur was determined to occur in conjunction with anthesis. Mature companion cells of the phloem were also transfer cells, but their wall ingrowths were fewer and unbranched compared to those of the secretory trichomes. This possession of two different transfer cells within the same floral nectary has rarely been reported. Within the nectary, the hydrolysis of starch stored in plastids was determined to be a potential contributor to nectar production, although it is likely that most of the nectar sugars are obtained from the phloem tissue in the subnectary parenchyma.

The cellular basis for spur growth was, until recently, generally accepted to follow the meristem hypothesis proposed by Tepfer (1953) based on histological work on *Aquilegia*. This hypothesis proposes that active meristematic regions on the developing spur continually produce new cells throughout spur development, driving spur elongation. Recent morphological and genetic work on several *Aquilegia* species (Puzey *et al*. 2012) and *Linaria vulgaris* (Box *et al*. 2011) has demonstrated that the proposed hypothesis is not completely accurate, at least in these species. Instead of continued meristematic activity, there appears to be a brief period of cell division early in development, followed by a more extended period of cell elongation which contributes more significantly to the increase in spur length. It was also shown that different *Aquilegia* species with significantly different spur lengths did not have spurs with significantly

more cells (Puzey *et al*. 2012), indicating perhaps that the degree of cell elongation is quite labile, which could account for the rapid adaptation and speciation often observed in spurred lineages. This study on *C. ruber* complements these recent studies and demonstrates a similar trend of cell division and elongation during *C. ruber*'s spur development, suggesting that this pattern may be the way that many spurs develop, even though they have different developmental origins. Early in *C. ruber'*s spur development, the outer epidermal cells are isotropic and cell division is required in order to eventually contribute to spur elongation. However, in the mid and late developmental stages, cell division activity slows down and then stops just before anthesis, with the remaining spur length increase greatly attributable to cell elongation, resulting in increasingly anistropic cells as development progresses.

Nectar secretion in *C. ruber* was investigated throughout anthesis at six hour intervals until senescence in two different greenhouse grown plants. Using a short time interval between sampling times may allow detection of subtle changes between stages of development which could be missed otherwise. Determining the volume and solute concentration of the floral nectar provides valuable information about the reward offered to potential pollinators. The determination of carbohydrate composition throughout development provides important information about the nectar's production and could indicate any post-secretion changes which occur to it up until senescence. It is known that *C. ruber* flowers are visited by butterflies (Rehnberg 1987) which generally prefer sucrose dominant nectars. The HPLC analysis confirmed that *C. ruber* has sucrose-rich nectar as would be expected based on its common visitors and potential pollinators. This type of nectar also indicates that invertase activity is not pronounced in this species, as most of the sugars are exuded as sucrose and not broken down into their component glucose and fructose monosaccharides. It also suggests that the nectar may not

be easily contaminated by microorganisms such as yeasts as they can often break down the sucrose molecules, but perhaps this is due to the protected greenhouse growth conditions under which these plants were housed, and/or the relatively protected nature of nectar accumulated within a spur.

5.2 Future research

In order to appreciate the significant differences which exist in spur morphology, anatomy, ultrastructure and development, it will be useful to expand the investigation and attempt to represent spur diversity by studying spurred species from all 13 orders and 21 families with spurred members. Apart from work on some key species, including *Aquilegia*, *Linaria* and *Impatiens*, most of the currently available literature on floral nectar spurs focuses on members of the Orchidaceae, perhaps due to their large, showy spurs or simply because they are so diverse and interesting. Increasing the taxonomic diversity represented in these studies will be beneficial in developing a full understanding of these intriguing floral structures and will ensure that generalized conclusions drawn are not unbalanced by excessive sampling of closely related species.

There is no doubt that the increasing ease with which developmental information can be gathered using genetic techniques will help to clarify the basis of spur growth. The recent work on *Aquilegia* species (Puzey *et al.* 2012), *Linaria vulgaris* (Box *et al.* 2011) and *Dactylorhiza fuchsii* (Box *et al.* 2012) has already started to unravel pieces of the developmental story, but additional work remains for a complete understanding of spur growth and development. *KNOX* genes have been implicated in spur development in *L. vulgaris* (Box *et al.* 2011) and *D. fuchsii* (Box *et al.* 2012), which is not surprising when considering the essential function these genes have in maintaining indeterminacy in meristematic regions. However, although *KNOX* genes

have been shown to play a role in spur development in these species, they do not set spur fate, so continued investigation into other genes, specifically those which regulate cell division and cell expansion, will be beneficial (Box *et al*. 2011, 2012). Additionally, recent work on several Papaveraceae species demonstrated that the expression pattern of *PAPSTL*, the homolog of the Arabidopsis *KNOX* gene *SHOOT MERISTEMLESS* (*STM*), could not be clearly linked to spur development in this group (Damerval *et al.* 2013). These types of investigations into the genetic control of nectar spur development are just beginning and continued research using various genetic approaches has the potential to add considerable insight into the field of spur research.

This study has demonstrated that the nectar of *C. ruber* is sucrose dominant, with approximately equal amounts of fructose and glucose. A previous study (Gardener and Gillman 2001a) has already studied the amino acid complement of *C. ruber'*s nectar and determined it to be high in glycine, serine and valine. The primary carbohydrate composition work presented here will benefit from future work analysing additional sugars present in smaller concentrations, as well as identification of additional nectar components such as nectar proteins, lipids and volatiles, among others. It would also be interesting to compare the carbohydrate composition between the greenhouse grown plants in this study to field grown plants from the same seed stock using HPLC. This approach could help to determine if the different carbohydrate composition reported by Percival (1961) for *C. ruber* (sucrose, fructose and glucose balanced) could have been due to microorganism contamination which often occurs due to insect visitation in wild or field-grown plants.

This study also determined that *C. ruber*'s nectar may be reabsorbed following secretion in order to recover some of the unused energy stored in the nectar as sugars. However, despite a fairly large sample size, the trends were not exceedingly strong and the assertion of nectar

reabsorption in this species could benefit from a more definitive approach. *C. ruber*'s flowers have the morphological characteristics shared with most nectar reabsorbing species, such as a long corolla tube, petals which do not shed and the collection of nectar at the site of the nectary, but by using radiolabelled sugars which could be tracked from the nectar back into the nectary tissue, nectar reabsorption could be confirmed.

The work on *C. ruber* could be complemented with similar research into the other 8-11 species (Larsen 1958, Richardson 1975) of *Centranthus*. With regards to spur morphology, anatomy and ultrastructure it would be worthwhile to investigate and compare the spur and nectary tissues between *C. ruber* and other closely related species to identify any significant differences. With respect to spur growth, it would be interesting to determine whether *Centranthus* species with longer and shorter spurs than *C. ruber* (Richardson 1975) have different single cell file count numbers, or if they are similar as was demonstrated in several *Aquilegia* species (Puzey *et al*. 2012). Additionally, identifying *C. ruber*'s *STM* homolog(s) and using in-situ hybridization to determine the location and timing of their expression in the developing flowers could indicate whether *KNOX* genes are involved in spur development in this species. Hormones also play an important role in the regulation of all aspects of floral development, both by crosstalk between one another and interaction with the genetic machinery (Chandler 2011). Gibberellins and auxins in particular have been implicated in promoting elongation of the epidermal cells, as shown in the developing *Arabidopsis* stamen filaments (Fei and Sawhney 1999). These hormones could potentially have a similar role in the elongation of the epidermal cells in developing spurs. Therefore, in addition to genetic approaches and continued morphological work, the role of various hormones in the process spur elongation should be explored.

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