Effects of Female Postmating Odour on Male Sexual Behaviour, in *Heliconius* Butterflies.

by

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Abstract:

The effects of the female postmating odour on male sexual behaviour were examined in *Heliconius erato* and *H. charithonia* (Lepidoptera: Nymphalidae). Predictions from the antiaphrodisiac hypothesis were tested using the two reproductive strategies of these species. Within the pupal mating strategy, results from behavioural experiments quantified and statistically tested dispersal rates of pupal-perched males to the presence of stimuli with and without the postmating odour. Results do not support an antiaphrodisiac function to the postmating odour. Similarly, within the adult courtship strategy, behavioural test results indicate that males do not alter their expenditure of energy in terms of either the duration or frequency of courtship behaviours elicited by females with and without the postmating odour. The data from both experiments did not support the antiaphrodisiac hypothesis for the function of the female postmating odour. A novel hypothesis predicting that the postmating female odour acts as an oviposition-deterring pheromone is presented.
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Literature Review

I. Taxonomy:
The taxonomy of Heliconius butterflies has been of substantial interest for many years. Both morphological (Emsley, 1963, 1965; Brown, 1972, 1981) and modern molecular studies (Lee, et al., 1992; Martin and Pashley, 1992; and Brower, 1994b) have contributed to our present understanding and classification of these butterflies.

Members of the genus Heliconius are part of the lepidopteran family Nymphalidae and subfamily Heliconiinae. Within this subfamily Emsley (1963) recognized 7 genera, and Brown (1981) revised this to 10. Currently there are 70 heliconiine species all characterized by their elongate forewings, large eyes, and long antennae (DeVries, 1987). The largest heliconiine genus Heliconius contains 38 recognized species (Brown, 1981). Two of these are the focus of this thesis, namely, H. erato (L.), and H. charithonia (L.). In this thesis, members of this genus will subsequently be referred to as heliconiids.

There have been widespread discrepancies in the spelling of H. charithonia (i.e., H. charatonia, H. charatonius, and H. charathonius). I have chosen to adopt the spelling recommended by A. Z. Brower (1994a). Contrary to the commonly used spelling of H. charitonia (Comstock and Brown, 1950), I will be using the more historically accurate spelling of H. charithonia. This spelling was verified, by a review of Linnaeus’ personal copy of the 12th edition of Systema Naturae (1767) by Brower (1994a), to be the first used to describe this species. Furthermore, it has now become the spelling used in the Official List of Specific Names in Zoology (Melville and Smith, 1987 in Brower, 1994a).

II. Geographic location and range:
The heliconiines are considered widespread geographically, distributed from the southern United States throughout Central and South America, with the greatest species diversity being found in the Amazon basin of Peru and Brazil (DeVries, 1987).

Populations of H. charithonia have been found in the southern United States (Florida, Texas, Georgia, Louisiana, California and South Carolina), throughout Central America, South America (northern Peru, Ecuador, Colombia and Western Venezuela) and the West Indies (Comstock and Brown, 1950; Young, 1976). Unlike other Heliconius species, H. charithonia is not involved in any
Müllerian mimicry rings, and displays little racial variation in wing colour pattern across its range from Texas through Central America (Comstock and Brown, 1950).

Closely related to *H. charithonia* and often found sympatrically, *H. erato* populations have been found in Panama, Trinidad, Guianas, Colombia, Brazil, Peru, Venezuela, Ecuador and the Amazon Basin (Emsley, 1964; Jiggens et al., 1997). *H. erato* displays diverse geographical variation in its wing patterns. More than twenty distinct geographical races have been described, each of which is involved in Müllerian mimicry rings with at least one other *Heliconius* species (Brower, 1994b).

III. Habitat:

*H. charithonia* has one of the most widespread geographical ranges of all the heliconiines (Comstock and Brown, 1950). In Costa Rica, adults of *H. charithonia* occur primarily in young secondary tropical forest habitat where adults visit various inflorescences throughout the sunny parts of the day (Young, 1976). This species is unable to compete for adult nutritional requirements with forest dwelling *Heliconius* species, and is therefore relegated to forest edges and disturbed areas (Gilbert, 1984; in DeVries, 1987).

An extensive study conducted by Crane (1955) details the preferred habitat conditions of *H. erato* adults. Adults are common in the various types of rain, montane rain, seasonal and swamp forests. Individuals are rarely found in the depths of the forest itself, instead preferring its edges, clearings, trails and roadsides, as at least partial sunlight is needed for flight. They have also been commonly observed in well-grown, open secondary growth, as well as overgrown citrus and cocoa plantations.

IV. Life Cycle and History:

The general characteristics of heliconiine life cycles are very similar and can be summarized according to the work done by Beebe et al. (1960). The eggs can be characterized by their external network of strong horizontal and vertical ridges. In most cases, the egg stage lasts 4 days or less. For most species of *Heliconius*, the preferred oviposition site is on new growth of juvenile *Passiflora* plants (Mallet and Gilbert, 1995). *Passifloraceae* are also known to produce structures that act as egg mimics. These round yellow plant structures have evolved to reduce herbivory by discouraging the placement of 'more' eggs by ovipositing females (Brown, 1981).
*H. erato* eggs are laid singly, not in clusters, and females will actively seek out a different vine instead of laying an egg on a set of leaflets where one has already been glued (Alexander, 1961a). *H. charithonia* females deposit eggs singly, rather than in clusters, on hostplant meristems (Young, 1976). However, in captivity several females have been observed to oviposit as many as 30 eggs on a single meristem of *Passiflora biflora* (pers. observ.).

All heliconiine larvae are spiny, having two spines on the head capsule and three pairs on most body segments. In some species, these spines have an irritant quality that acts to deter predators, and some people have been known to break out in a rash from handling heliconiine larvae (DeVries, 1987). The larval body, aside from the spines, is otherwise almost smooth. Larval colouration is often cryptic in immature early instars, and brightly coloured in mature instars (Brown, 1981). All larvae undergo 5 instars requiring anywhere from 13 to 17 days for completion (Beebe et al., 1960) depending on temperature and abundance of hostplant material.

*H. erato* larvae are cannibalistic. Newly emerged first instar larvae will feed on other larvae or eggs, and older larvae never share a leaf with another caterpillar without fighting (Alexander, 1961a). *H. charithonia* larvae are not cannibalistic, but are not known to feed gregariously. However, they are occasionally found feeding on the same leaf with another larva (Boggs, 1981a).

Pupal characteristics of most members of the subfamily *Heliconiinae* include a pair of long cephalic appendages, a rough textured surface with tubercles and flanges and gold spots present on the dorsal part of the thorax and abdomen. Pupation sites are varied. They can include the leaf tip, midrib of leaf, leaf margin, living stem material, or a new tendril (Alexander, 1961b). Depending on the species, the pupa can be found hanging, either with the body held horizontal to the pupation substrate, or with the body vertical (DeVries, 1987). The spiny abdominal projections of the pupae are clung to by the newly emerged imago with its first pair of functional legs, and onto the margin of the head case with the other legs (Alexander, 1961b). The duration of the pupal stage is 9 or 10 days (Beebe et al., 1960).

Another interesting fact about heliconiid pupae is that most species exhibit some form of pupal movement and can produce a sudden clicking when disturbed. Depending on the species, the movement exhibited by the pupae is either subtle or vigorous and can range from wiggling to turning (Alexander, 1961b).

Once eclosed, the imago must hang from the pupal case until wing flattening and hardening have taken place. These two processes occur concurrently. The wings of the freshly emerged butterfly
are about one third of their final length, and over the next 2-5 minutes they will expand to their full length (Alexander, 1961b). If forced off the pupal case, the imago has a short period (ca. 8 minutes) in which to locate an alternative hanging site or its wings will not flatten sufficiently to allow flight (Alexander, 1961b). Anything beyond this will result in premature death of the imago.

Generally, the entire life cycle from egg to adult takes between 26 and 30 days (Beebe et al., 1960). In a natural setting, adult nectar feeding will not normally begin until 20-24 hours post emergence (Crane, 1955).

V. Feeding needs and preferences:

i) Larvae

The larvae of the Heliconiinae feed only on various species of passion vines. This life-history fact has garnered them the name the Passion Vine Butterflies. Heliconiid larvae predominantly feed upon leaves, but have been observed feeding upon the tendrils, stalks, flowers and hairs (Alexander, 1961a).

a) Hostplants

Both H. erato and H. charithonia are considered to be generalists, compared to other Heliconius species, in relation to their hostplants (Young, 1976; Benson, et al., 1975; Brown, 1981).

For H. erato there are several Passion Vines that are utilized as hostplants depending on the subspecies and location. DeVries (1987) lists Passiflora vesperillio, P. tuberosa, P. coreacea, P. biflora and P. talamancensis as hostplants for H. erato petiverana. However, Smiley (1978) reported increased larval growth rates for H. erato petiverana on P. biflora suggesting digestive specialization or perhaps a partially evolved monophagy for P. biflora.

For H. charithonia the primary hostplant is P. lobata (Passifloraceae) (DeVries, 1987), but other passifloraceous hostplants are utilized (Benson et al., 1975). H. charithonia is the only Heliconius species to have evolved defences against the usually lethal trichomes on Tetrastylis lobata and Passiflora adenopoda (Jiggins et al., 1998).

b) How specific is hostplant choice?

Several ecological factors determine host specificity. Plant chemistry, searching ability and competitive interactions all combine to determine the optimal hostplant (Smiley, 1978). For heliconiids, eggs are almost invariably laid on the optimal food-plant of the species (Alexander, 1961a). In the case where a female oviposits an egg on a sub-optimal species, there are three possible
outcomes. First, the newly hatched larvae will refuse to eat and will die. Second, they will be forced to leave the sub-optimal species and attempt to locate the preferred hostplant. Third, the sub-optimal food plant is accepted but larval growth may be retarded or reduced (Alexander, 1961a).

It was decided that due to ease of propagation and pest control, only one *Passiflora* species would be reared to serve as hostplant for the heliconiids in the breeding program at the Butterfly Conservatory. *P. biflora* was chosen as this hostplant species. Both *H. erato* and *H. charithonia* readily oviposit on this plant. The effect of utilizing this hostplant in captivity was addressed by Brgan (in preparation). In comparison with the literature, he found neither retarded growth rates of larval instars, nor anomalies in the duration of any life stage of either *H. erato* or *H. charithonia* (Brgan, in preparation).

ii) Adults:

a) Nectar feeding:

Nectar plants for wild heliconiids include the flowers, but not fruit of: *Lantana camera*, *Hamelia* spp., and *Stachytarpheta* spp. (DeVries, 1987). Nectar plants are visited throughout the day, with the most active feeding occurring midmorning.

b) Pollen feeding behaviour:

It is known that adult lepidopterans feed on nectar, but more interestingly several species of *Heliconius* (including *H. erato* and *H. charithonia*) are also able to feed on pollen from the male flowers of vines in the family *Cucurbitaceae* (Gilbert, 1972). Pollen is collected daily soon after anther dehiscence. It is then mixed with nectar on the proboscis, causing the pollen to dissolve. Dissolved pollen mixed with nectar releases free amino acids and peptides. Once released, these nutrients are sucked up, and the spent pollen mass is discarded (Gilbert, 1972).

This extra source of nitrogenous compounds, which supplements the usually finite larval reserves, is responsible for the increased ability of the males to transfer limiting nutrients to the female in the spermatophore (nuptial gift) (Boggs and Gilbert, 1979), the unusually long (up to 6 months) life expectancies (Gilbert, 1972) and increased adult oogenesis (Dunlap-Pianka *et al.*, 1977) compared to non-pollen feeding lepidopteran species. Additionally pollen feeding has been hypothesized to increase the adults’ ability to produce cyogenic glycosides (Nahrstedt and Davis, 1983), as pollen feeding is strongly correlated with unpalatability to a natural avian predator, the jacamar (Chai, 1990).

Adult nitrogen supply, and subsequently reproduction and longevity of adult lepidopterans, is usually limited to reserves accumulated during larval feeding (Schoonhoven, *et al.*, 1998).
overcome this limitation some lepidopteran species have evolved methods for adding to the nitrogenous compound supply of the adults by feeding on fresh bird droppings (ithomiine), rotting fruit, fermenting sap, urine and dung (charixine and nyphaline species) (Gilbert, 1969, in Gilbert, 1972). Although supplementary adult nitrogen collection is practiced by other lepidopterans, the pollen collection strategy is almost unique to the genus Heliconius, the lone exception being the closely related heliconiine Laparus doris (Gilbert, 1991).

VI. Mating practices and reproductive biology:

i) Sperm storage; how and where:

The great majority of lepidopterans, including the heliconiids, belong to the suborder Ditrysia. Figure 1.1 is a general overview of the reproductive morphology of ditrysian members as described by Klots (1970). All anatomical abbreviations used refer to structures in this figure.

Members of this suborder possess two widely separated genital openings. The one for oviposition is still closely associated with the anus, but the one for insemination has moved ventrocephalad to a position between the 7th and 8th sternites (Klots, 1970). The principle reproductive organs of this suborder are the paired ovaria (ovar.), an oviductus lateralis (ovl.) that drain each of these, and the oviductus communis (ovc.) formed by the convergence of the oviductus laterales.

In Ditrysia there is an invaginated genital chamber called the sinus vaginalis. In its wall is the ostium bursae (o.b.), which is the receptive copulatory opening leading into the bursa copulatrix (bu.c.). Sperm travel to the bursa copulatrix via the ductus bursae (du.bu.) and enter at the enlarged sac-like corpus bursae (crp. bu.).

The bursa copulatrix is where the sperm are first received as a spermatophore. From here, the sperm travel to the spermatheca (sp.) which is the main storage organ. From here they reach the vagina via the ductus seminalis (du. sml.). Into the vagina also opens the duct of the glandulae sebaceae (gl.s). This gland secretes an adhesive substance used to attach the egg to the oviposition substrate.

ii) Minimum age of mating:

Generally speaking, lepidopteran females can mate at a younger age than males. Females can mate on the first day of eclosion in most species, whereas males usually mate only after several days. Two reasons have been attributed to the difference in age of first mating between the sexes (Scott, 1973). First, since males usually take the active role in mate-location, it is the male who must be stronger in flight ability, which usually takes a few days to develop. Second, in order to maximize
Figure 1.1 Reproductive morphology of lepidopteran females in the suborder *Ditrysia*. Figure originally published by Klots (1970).
oviposition time for the female, it is beneficial that she is mated as soon as possible. Additionally, the general trend for lepidopterous species to be protandrous (i.e., males emerging before females) (Stern and Smith, 1960; Oliver, 1972; Scott 1973) means that locally, there will be older males present when virgin females emerge.

iii) Mating frequency:

a) Males:

Regardless of the specific reproductive strategy (i.e. courtship, pupal mating or both) male butterflies employ the typical male evolutionary strategy of attempting to maximize their number of copulations. *H. erato* males have been observed to actively court and repeatedly mate well into advanced age (3-3.5 months) (Crane, 1957). *H. charithonia* males have also been observed to mate several times in their life (Boggs, 1990). Larry Gilbert (oral communication with Scott in Scott, 1973) asserted that males of *Heliconius* spp. could mate at least 10 times.

b) Females:

Crane (1955) stated that for *H. erato* in captivity, females can mate at least twice. She did not note the usual frequency of matings in the wild. However, other researchers have concluded that the average lifetime female mating frequency of both *H. erato* and *H. charithonia* is close to one (Boggs and Gilbert, 1979; Boggs, 1981b; Boggs, 1997a). But both *H. erato* and *H. charithonia* females will mate more than once if their first (or second) mating did not supply a sufficient quantity or quality of sperm (Boggs, 1990).

iv) Fertilization from different matings:

When a female mates with more than one male, she can have sperm from several different males stored in her spermatheca simultaneously. Thus, there is the opportunity for sperm precedence and competition to occur. Parker (1970) formulated the general rule of sperm precedence for insects, that of “last in first out.” Sperm from the most recent mating almost exclusively fertilized eggs laid by a lepidopteran female until the next mating e.g., in *Papilionidae: Papilio zelicaon* (Sims, 1979); *Papilio bianor* and *P. maaackii* (Ae, 1962 in Lederhouse, 1981); *Nymphalidae: Euphydryas editha* (Labine, 1966); and *Pieridae: Pieris rapae* (Shapiro, 1970).

The benefit of sperm precedence for males is increased fitness through increased fertilization. However, for females the benefits of sperm precedence are not so obvious. It has been calculated that there is enough sperm in a normal first copulation to provide sufficient sperm to fertilize all the eggs a female is likely to lay (Labine, 1966; Sims, 1979). However, matings with males that have frequently
copulated, or with very young males, result in spermatophores low in sperm concentration (Sims, 1979). It has been hypothesized by Lederhouse (1981) that sperm precedence would be a way to restore fertility levels in females with reduced sperm concentration, or in older females in which the sperm have ceased to be viable.

v) Adult nutrient allocation, investment, and usage:

It is known that males of many insect orders (Thornhill and Alcock, 1983) and certain Lepidoptera species (Boggs and Gilbert, 1979 and Boggs and Watt, 1981) contribute nutrients to the female by transferring resources at the time of mating. By analyzing various sources of nutrients, Boggs and Gilbert (1979) found radiolabelled male donated products, as well as nutrients derived from female larval resources and adult pollen feeding, in the eggs and somatic tissues of H. erato females. They concluded that these resources play an important role in the nutrient and energy budgets of short-lived species or those with little or no adult feeding. The fact that radiolabelled nutrients were found in egg and somatic tissues of H. erato, a long-lived species with significant adult feeding, indicated that the female does indeed use the male donated nutrients for egg production and possibly for somatic maintenance. For both H. erato and H. charithonia, a reduction in adult foraging activity, resulting from male donated nutrients, may provide the female more time for locating oviposition sites, help to decrease the risk of predation, or especially for H. charithonia, afford females the opportunity to left across nutritionally poor environments early in life (Boggs, 1990).

The capacity of female H. erato and H. charithonia to supplement larval nitrogenous reserves by pollen feeding (Gilbert, 1972), suggests that would-be nutrients absorbed from the male spermatophore and accessory fluids are not as critical to survival and fertility as in other lepidopterans. Without the nutritional need for male donated nutrients, the sperm gained from one mating would suffice to fertilize most, if not all female eggs. This coincides with the life history observations that most female H. erato and H. charithonia mate only once in their lifetime.

Boggs (1990) found that females incorporated male derived nutrients up to 15-20 days post-copulation. This fits with a previous observation (Boggs et al., 1981) that after 2 weeks, in dissected H. charithonia females, spermatophores were nearly completely absorbed, with only an “orange oily substance” remaining. Pollen collecting behaviour was also observed to increase sharply after about 15-20 days (Boggs, 1990). Therefore, the results indicate that male derived nutrients are used in H. charithonia in place of pollen-derived resources early in the adult female’s life.

In lepidopterans, male derived resources play other roles beyond providing the female with
sperm and nutrients. These may include inducing oogenesis through hormonal effects (Herman and Barker, 1977, in Boggs 1990), or the prevention of further matings by the female (Labine, 1964, sperm plug; Sugawara, 1979, stretch receptor).

vi) **Pupal mating:**

Pupal mating was first observed and described for *H. charithonia* (Edwards, 1881). Pupal mating *Heliconius* females visit hostplants to oviposit. Pupal mating *Heliconius* males also visit hostplants, but to investigate larvae and pupae. The inspection of the pupae by the males is an attempt to locate and assess suitable female pupae. Once a male has located a suitable pupa, he will defend his position on the pupa from other males with increasing intensity as the female nears eclosion. During the evening prior to eclosion, one or more males’ abdomens will penetrate the pupal case near the wingtips, and align parallel with the female’s abdomen under the pupal skin. It is only upon the female actually beginning to eclose, that the successful male can mate with the female as she descends from the pupal exuvium (Gilbert, 1975; Deinert et al., 1994). Although pupal mating is the primary reproductive strategy, pupal mating species also have an equally well-developed courtship strategy (Crane, 1957; Hernandez and Benson, 1997). This likely represents the maintenance of the ancestral strategy i.e., courtship, in addition to the derived strategy of pupal mating (Lee et al., 1992).

The phylogenetic clade of species referred to as the erato-group (including *H. charithonia, H. erato, H. sara, H. hewitsoni*) are known to pupal mate on their hostplants (Gilbert, 1975; Deinert, et al., 1994). This clade is referred to as the evolutionarily advanced group, and its members exhibit pupal spines. The non-pupal mating group, referred to as the primitive group, do not possess these spines (DeVries, 1987). It has been hypothesized that the presence of pupal spines and head appendages may constitute an adaptation affording a better foothold to pupal mating males (Brower, 1997). Gilbert (1976) observed that *H. erato* males perched on both male and female pupae of *H. melpomene*, a non-pupal mating species. This generally resulted in the dislodging and killing of the newly emerged *H. melpomene* imago. The absence of pupal protuberances in non-pupal mating species may be a way of potentially reducing or deterring lethal harassment of newly emerged adults by pupal mating males (Brower, 1997).

a) **Other organisms that practice this strategy:**

While 42% of *Heliconius* species practice pupal mating (Gilbert, 1991) it has only been reported in one species outside this genus, namely, *Jalmenus evagoras* (*Lycaenidae*) (Elgar and Pierce, 1988). Aside from the Lepidoptera, the strategy of pupal mating is practiced by representatives from
the insect orders: Diptera, Coleoptera and Hymenoptera (see Table 7.5, Thornhill and Alcock, 1983).

b) How males locate and differentiate between male and female pupae:

*Heliconius* individuals search, in learned home ranges, for oviposition sites on larval food plants (*Passiflora* spp.), for nectar and pollen sources (*Psiguria* spp. and *Gurania* spp.), for mates (pupae), and for roosting sites (Gilbert, 1979; Turner, 1981; Mallet, 1986; and Murowski and Gilbert, 1986).

It has been reported that the female pupae of *H. charithonia* release a pheromone that attracts males (Edwards, 1881, Bellinger, 1954). It was noted in these studies that males were able to distinguish male from female pupae, and are increasingly attracted to the female pupae as they near eclosion. Females showed no such attraction to male pupae, and the male pupae did not attract other males.

In another study (Alexander, 1961b) it was noted that pupae of *H. erato* are capable of vigorous movement, the production of a sudden clicking when disturbed and also emit a detectable odour. It was not tested if the gender of the pupa had any effect on the gender of adults that inspected it. However, it was hypothesized that long-range identification is accomplished by olfactory cues, and that olfactory plus auditory cues aid in close range identification.

c) Benefits of pupal mating:

i) Males

In this strategy, potential mates (female pupae) are stationary and non-randomly grouped. Thus, males do not have to expend energy searching out single, cautious and perhaps coy adult females. Instead, they need only locate the larval hostplant to expose themselves to a higher percentage of available females. Wickman (1985) while working with the small heath butterfly *Coenonympha pamphilus* (L.)(Lepidoptera: Satyridae), has shown that site-tenacious males experienced an increased mating success compared to males that roamed widely.

The potential for increased male fitness is another benefit of this strategy. Since perspective mates - female pupae - are guaranteed to be virgins, this represents a potentially high gain in fitness for males that successfully mate a virgin. Rutowski (1991b) states that in many species of butterflies males that achieve matings with virgin females experience the highest fitness gains. Most females encountered by males in the wild are usually mated and, even if receptive, are of advanced age and, thus, of lower potential reproductive value than a newly emerged virgin female. This conclusion would be especially applicable to species in which the females usually mate only once or have mechanisms to
prevent further matings (proteinaceous sperm plugs, or female rejection behaviours) (Rutowski, 1991b).

Increased male fitness can also result from male mate selection. Once a larval hostplant is located, a male has potential access to many females and can, therefore, exercise selectivity. Deinert (1997) experimentally illustrated that males of another pupal mating heliconiid (*H. hewitsoni*) assessed female pupae for size and age. This study concluded that captive males found larger and older pupae more attractive, thus reflecting a component of male mate choice. Due to the male's energy investment in copulation, plus the added refractory period before he can mate again, it would increase the male's fitness to exercise selectivity. The total quantity of material passed by the male during copulation may be as much as 10% of the male's body weight (equivalent to a 90kg human male passing 9kg or about 9 litres of ejaculate in a single copulation!) (Rutowski, 1982).

ii) Females

Males compete with each other for purchase on the pupae, and then for the actual mating of the female. Therefore, while there is no direct female choice, she benefits from the selective pressures of intrasexual competition and passive selection (Lloyd, 1979).

Pupal mating also provides protection for the pre-emergent female. Several species of ants and, in captivity, cockroaches will prey upon *Heliconius* pupae (Edwards, 1881). By the males perching on the pupa, they might protect the captive female by thwarting the ants' attempts to investigate a potential prey item (Young, 1976). This hypothesized protection by the males, coupled with the ability of heliconiid pupae to wiggle and produce clicking sounds provides the only immobile life stage, aside from the egg, with an extra degree of security.

A single copulation can take up a substantial portion of the daylight hours during which temperatures are appropriate for flight. Therefore, copulation may reduce the time a female can allocate to feeding and ovipositing. Oviposition sites, nectar and pollen plants can be widely separated; thus, time constraints on the female can translate into a fitness compromise (Rutowski, 1982). By eclosing already *in copula*, pupal mating eliminates the time the female would spend to find a mate, while concomitantly maximizing the time she has available for oviposition and locating nectar and pollen sources.
VII. Pheromones

Insect sex pheromones have been defined as "odorant molecules transmitted in a gaseous state, which are perceived by olfactory receptors located primarily on the insect antenna" (Seabrook, 1978). These chemical compounds, secreted by an individual, usually influence the behaviour of conspecifics.

It is important to differentiate the pathways in which pheromones operate. Wilson and Bossert (1963) proposed two separate categories of pheromone operation, depending on which biological system they affect. The first affects the endocrine or reproductive systems, and these pheromones achieve their response by altering internal physiology. This type of reaction is termed the 'primer effect'. The second class achieves its effect by the classical stimulus-response process mediated by the central nervous system. This type of reaction is termed the 'releaser effect'. With the primer effect, behaviour is usually not induced by the pheromone, but by subsequent external stimuli. With the releaser effect the pheromone is the primary stimulus and causes a quick behavioural response.

i) Male pheromones:

It has been estimated that up to 50% of all butterfly species have male structures that are in some way associated with pheromones (Scott, 1973). Females of most insect species are the choosy sex and, as a result, male produced pheromones are usually used at close range and are associated with mate choice (McNeil et al., 1999). The pheromone emitting organs of males can be setae modified into long hairs gathered into tufts or setae modified into scales of various types, including long tapered scales with terminal tufts called "androconia". All modified setae are associated with glands. Their location in males is generally on the wings (usually the upper side of the forewings), but occasionally they are found on the legs or abdomen (Scott, 1973).

*Heliconius* males possess specialized scent scales on the friction surface of the anterior portion of the upper hindwing (Müller, 1877). These surfaces are covered by androconial scales. Although they play a definite role in courtship, their odour was only rarely detected by the human sense of smell in courting *H. erato* males (Crane, 1955). During courtship, *Heliconius* males flutter their wings, which expose these scent surfaces to the female. Scott (1973) asserts that this aids in courtship success visually as well as olfactorily by disseminating androconial scales.

Another source of male scent is the region of the abdominal tip. The 7th and 8th segments and the 8th-9th intersegmental region of the abdomen are regularly involved with forming display, scent-producing and distributing structures. These structures consist of pouches and erectile or eversible groups or clumps of modified hairs or scales (Klots, 1970) (Figure 1.2).
Figure 1.2 Scanning electron micrograph of a midsaggital section of a *H. charithonia* male abdomen showing the inside of the male claspers and genitalia (Photo by M. Cornish, 1998).
Eltringham (1925), reiterating Fritz Müller's (1877) observations on heliconiid butterflies, described a male behaviour in relation to its scent organs. “The males, when seized, open wide the anal valvulae, from the inner side of which there appear two glands yielding a strong and nauseous smell” (pg. 270). Eltringham further states, of the male glands, that upon dissecting the abdomen of some males, he found near the base of the lateral valves a “yellowish-white amorphous mass” on each side. Upon dissection of this mass from the tissue, he found that it smelled like carbylamine.

ii) **Female pheromones:**

Generally, female pheromones are used over long distances to attract males (McNeil *et al.*, 1999), and are produced by specifically developed scent-producing glands (Scott, 1973). These may be associated with hair-like setae, or structures similar to those of males, i.e., hair-pencils of male danaines. These scent producing glands are nearly always located near the tip of the abdomen. Evolution would not have favoured the development of scent producing glands on the female’s wings, as in most lepidopteran species the females do not move their wings during successful courtship. Additionally, the male is usually behind the female with his head near the tip of her abdomen prior to joining (Scott, 1973).

a) **Female postmating odour of heliconiines:**

It has long been known that mated *H. erato* females, produce a strong odour which has been described as resembling phenylcarbylamine, or witch hazel. Müller (1878) was the first person to document the “witch hazel-like” odour produced by captured heliconiines. Further observations regarding the postmating odour were collected from both captive and wild caught heliconiine specimens (Longstaff, 1912; Eltringham, 1925; Poulton, 1925, Longfield, 1926; Crane, 1955, 1957; Emsley, 1963; and Parsons, 1989).

b) **Scent glands and stink clubs of female heliconiines:**

In *Heliconius* spp. the female scent-producing glands are located dorsally between the distal and penultimate segments of the abdomen (Crane, 1955). When extruded, two “bulbous excrescences” swell out from on either side of the dorsal midline (Figures 1.3 and 1.4). Below and behind them is the pair of tiny “stink clubs”, first described, along with the scent gland in heliconiines by Müller (1878), and later expanded upon by Eltringham (1925) and Emsley (1963).

Müller (1878) found that the presence of the stink clubs indicated close relationships between heliconiine genera, and that they exhibited clear morphological differences between species. He described the stink clubs as being located “one on each side, on the posterior margin of the penultimate
Figure 1.3 Scanning electron micrograph of a lateral view of the posterior abdominal segments, scent glands (SG) and stink clubs (SC) of a *H. erato* female (Photo by M. Cornish, 1998).
Figure 1.4 Scanning electron micrograph of a lateral view of the posterior abdominal segments, scent glands (SG) and stink clubs (SC) of a *H. charithonia* female (Photo by M. Cornish, 1998).
abdominal segment, below the scent gland, and at an apical angle to the ventral plate of the segment” (pg. 665). From this position, the stink clubs are directed backwards and outwards when the scent gland is exerted.

Müller (1878) further described the stink clubs as two laterally placed, club-like organs, resembling the shape of the haltere of dipters. Each stink club consists of a chitinous stalk about 1mm in length, with a terminal club covered in scales (Figure 1.5). These scales exhibit different forms in different species. Müller (1878) observed that the strengthened smell of the scent gland was readily detected in the excised tip of the stink clubs. He also noted that the nauseating smell of the female’s scent gland was very similar in scent to the carbylamine scent emitted from two scent glands within the male’s valvulae.

Eltringham (1925), a histologist by profession, related that rather than the stink club having its own gland, the lumen of its stalk opens into the scent gland itself. Furthermore, this female gland emitted a scent that was comparable to that emitted from the gland within the male’s valvulae. He also noted that the female everted her scent gland rapidly first handled but more slowly on subsequent interferences. On the second or later disturbances, the scent of the female regains its original strength only when the stink clubs are everted.

c) Function of the postmating odour of female Heliconius:

Until 1955, the witch hazel-like odour of certain female Heliconius species butterflies was primarily thought to be aposematic in function, with the odour affording the bearer protection from predation (Longstaff, 1912; Poulton, 1925; and Longfield, 1926). Crane (1955) was the first researcher who observed the use of the postmating odour in a sexual context. Eltringham (1925) had hypothesized a sexual function but had never witnessed its use. Crane stated that a protective function to the odour, if any, evolved directly out of a primarily sexual function. In her study of H. erato she reported that mated and virgin females have been observed to evert their scent glands during certain stages of courtship. However, the postmating odour was only detectable in mated females, and its presence deterred neither subsequent courtings nor at least one additional mating.

In 1976, another hypothesis regarding the specific function of the female postmating odour of certain Heliconius species appeared in the journal, Science. Gilbert (1976) performed experiments on female H. erato and H. charithonia. He described the function of the female postmating odour as acting as an ‘antiaphrodisiac’. This was based on behavioural observations not numerical data, and the author was clear to state that the evidence being presented was “anecdotal but highly suggestive”.

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Figure 1.5 Scanning electron micrograph of the anterior section of a stink club from a *H. charithonia* female (Photo by M. Cornish, 1998).
The observations which led him to his ‘antiaphrodisiac’ hypothesis, involved H. erato and H. charithonia males perched on conspecific pupae (Gilbert, 1976). Gilbert observed that pupal-perched males in captivity, were generally oblivious to any visual or tactile stimuli. However, when the abdomen of a mated female conspecific was brought to within a few centimetres of the perched males, the usually oblivious males were observed to leave the pupa. It was also noted that direct contact of the male antennae with the female abdominal glands was sometimes needed to dislodge the most persistent males. It was from these observations that he hypothesized the ‘antiaphrodisiac’ function of the female postmating odour.

d) Chemical analysis of the postmating odour:

It is generally accepted that most lepidopterous sex pheromones are chemical blends, and can often be described in terms of ‘major’ and ‘minor’ components (Steck et al., 1980).

Crane (1955) reported the results of chemical tests on the scent glands of mated H. erato females performed by Mr. E. C. Crocker of the Flavor Laboratory of Arthur D. Little, Inc. in Cambridge Massachusetts. The report issued, stated that the scent glands of a 16-hour-old female have a particularly sharp, strong phenylcarbamyamine (phenyl isocyanide, C₆H₅-N=C type) odour suggesting styrene (phenyl ethylene) and phenyl proprionaldehyde. The report further states that this odour must be due to a phenyl compound, with no more than 2 or 3 carbons on the side chain and possibly an oxygen or nitrogen.

Miyakado et al. (1989) performed another chemical analysis on isolates of the pheromone emitted from the female abdominal gland. They found the structure and absolute configuration of a macrocyclic lactone, which they identified as the major volatile component from the everted scent glands of Heliconius pachimus.

e) Structures similar to the scent glands of female heliconiines:

There are nymphalids that possess glands similar to those of the female heliconiines. Scott (1973) described similar glands in the argynnine genera Speyeria and Nymphalis. Parsons (1989) summarizes findings of studies that located similar abdominal scent glands in 5 European species of Vanessa. Similarly Parsons (1989) also reported that females of the European argynnine nymphalid genera Melitaea, Euphrydas, and some European satyrine nymphalid genera, such as Erebia, also bear dorsal glands. All of these glands are similarly positioned as in heliconiines, but there are no associated androconial clubs present.
Interestingly the males of the nymphaid subfamily Danainae possess scent producing structures known as hair pencils that bare striking resemblance in form and function to the stink clubs of the female heliconiines. The hairpencils appear in the same place on the male as the stink clubs on the female, can be forced out by carefully squeezing the abdominal tip of the male, serve a courtship function, and are hypothesized to be secretory (Brower et al., 1965). In the danaine genus *Parantica*, the hairpencils bear small apical leaf-like structures, which readily break off to produce small particles (Brower et al., 1965).

In regards to the behavioural use of female scent glands, it was reported by Boppré (1984) that female *Argynnis paphia* (L.), when receptive, would evert these glands by angling and bending her abdominal tip toward an approaching male. Conversely, Scott (1973) described the glands and female behaviour for *Gonepteryx rhamni* (L.) (Pieridae) and *Glutaphrissa saba* (Eucholidae). In both of these species, the glands are everted during courtship when the female is unreceptive and adopts a rejection posture.

**VIII. Why heliconiids are good study organisms**

There have been numerous and landmark studies conducted on the genus *Heliconius* over the years (see Brown, 1981 for review), and there are certain characteristics of the species that have made them a model organism for experimental research. First, their distribution throughout a wide variety of habitats in the tropics and subtropics make them amenable to a diversity of studies assessing habitat requirements and interactions. Second, due to their longevity, and the ease with which they are captured and marked, they are particularly suited to evolutionary, physiological, behavioural and community ecology studies (Benson et al., 1975; Boggs et al., 1981; Gilbert, 1991; Mallet, 1986). And finally, as some experimenters have explicitly stated (Crane, 1955; Beebe et al., 1960) most members of the genus have proven to be easily reared in captivity. Both the larvae and adults lend themselves to being examined in artificial environments and are relatively resistant to disease, parasitism and handling (Brown, 1972). Taken together these traits provide excellent opportunities for further captive studies on behaviour of all life stages.

Abstract:

Male courtship behaviours of two pupal mating *Heliconius* species were studied within a controlled environmental setting. Mated females of both species possess a postmating odour that is transferred to them, from the male, at the time of mating. The postmating odour has been hypothesised to function as an antiaphrodisiac pheromone. Previous behavioural observations collected from males perched on female pupae to the presence of the postmating odour led to the antiaphrodisiac hypothesis. It was observed that only the close proximity of a conspecific female in possession of this postmating odour was able to scatter pupal-perched males. Data collected from experiments conducted in the present study did not coincide with predictions made from the antiaphrodisiac hypothesis. Results from tests using three female types (virgins, and mated females with and without the postmating odour) indicated no significant difference in their ability to force males from 8-day-old female pupae. From the experimental results it was hypothesised that once a minimum threshold of disturbance was surpassed, pupal-perched males became more likely to leave female pupa.
Chapter 1 - Introduction

In insects pupal mating is an extremely rare mating strategy, and has only been reported in three insect orders (Thornhill and Alcock, 1983). Within the order Lepidoptera, the genus Heliconius provides an excellent opportunity to examine this mating system, as 42% of Heliconius species are pupal maters (Gilbert, 1991). Therefore, studying the Heliconius genus provides an opportunity for unique examinations of different pupal mating species, facilitating comparisons of the similarities and differences of the behaviours that surround this rare mating strategy.

Heliconius hewitsoni is a heliconiid species known to pupal mate. Extensive behavioural work has been done on H. hewitsoni in its natural habitat (Deinert, 1997). A generalized sequence of events illustrating the behaviours associated with pupal mating may be drawn from this study.

Males will patrol an area containing larval host plants upon which conspecific pupae may be located. Once these ‘patches’ are located, males will repeatedly visit throughout the pupation period. Gilbert (1976) reported that males of H. erato, another pupal mating heliconiid species, would inspect both young male and female pupae. However, once the pupae have aged (i.e., begin to discolour), males only inspected and alighted on female pupae. Gilbert hypothesized that there may be a male identification pheromone in operation which would protect older male pupae and newly emerged adults from harassment. A similar conclusion was reached by Bellinger (1954) for another heliconiid species, H. charithonia. In this species, males were able to distinguish male from female pupae, and only alighted on the females.

Once female pupae are located, patrolling males appeared to have the ability to detect how close these pupae are to eclosion. Deinert et al. (1994) reported that the nearer a pupa was to eclosion, the longer and more frequent the male visits became. Once a threshold age was reached, as early as 48 hours prior to eclosion, males began to land and perch on the female pupa. This perching was not a passive behaviour, as males fought for position and attempted to dislodge each other (Deinert et al., 1994). If not displaced, males would remain until the female eclosed.

When a female is ready to emerge, the pupal casing thins, and a male is able to pierce the pupal skin with his abdominal tip. Usually only one male punctures the pupal cuticle, although as many as three males puncturing the cuticle have been observed (Deinert, 1997). Irrespective of how many males pierce the cuticle, only one male will achieve copulation with the female. As she begins to eclose, the successful male engages the female’s genitalia, and subsequently she emerges in copula.

It has long been observed that adult females of most Heliconius species produced a distinct
odour (Müller, 1877). The odour had been observed in mated females, but not virgins, and was rarely, if ever, detectable in males. This mated female odour was described as resembling the scent of phenylcarbylamine or ‘witch hazel’ (Müller, 1877; Eltringham, 1925; Crane, 1955).

In his 1976 publication, Gilbert demonstrated that the female postmating odour, in two pupal mating races of H. erato, originated from the male. Mated females of H. e. chesteronii had a distinctively more fragrant odour than females of H. e. adanus. Seven interracial matings were obtained in an insectary and the odour of mated females was recorded. Gilbert found that it was the race of the male, regardless of the female’s race, that predicted the postmating odour of the female.

While no storage organ for the postmating odour is known, Gilbert hypothesized that the female scent gland was a likely candidate, as after mating the gland enlarges and intensifies in colour. He also hypothesized the mechanism by which the postmating odour is transferred. His hypothesis was that the female’s stink clubs would fit nicely into the gland-lined pouches inside of the male claspers outlined and illustrated by Eltringham (1925). From these pouches the pheromone or the necessary chemical precursors for pheromone synthesis, would be transferred to the female.

In the same publication, Gilbert hypothesized the function of the female postmating odour as an ‘antiaphrodisiac’. This conclusion resulted from experiments that were “anecdotal but highly suggestive”. He observed large clusters of male H. erato sitting on female pupae. It was also observed that these sitting males were oblivious to all visual and tactile stimuli, as even repeated hand battering failed to cause the males to leave the pupae. It was reported that males had only become “highly agitated, rapidly moving the antennae and palps, and generally dispersing in a matter of seconds” (pg. 420), when an abdominal tip of a mated H. erato female was brought to within centimetres of the cluster of males. Actual contact of the male antennae with the female abdomen was sometimes needed to dislodge the most persistent male, but all males left in the presence of a mated female abdominal tip. The same results were similarly obtained for H. charithonia, another pupal mating species. For both species, no males left female pupae in the presence of visual or tactile stimuli, and all males left in the presence of a conspecific female’s postmating odour. Therefore, based on Gilbert’s (1976) description of the phenomenon, I have designated this as the ‘all or nothing’ male response.

While Gilbert’s (1976) paper served to document the origin of the postmating odour, no subsequent attempts have been made to verify his findings, or to further elucidate the extent of any subtleties involved with the proposed antiaphrodisiac function for the female postmating odour. Although rare, antiaphrodisiacs have been described in other insect orders. From my review of the
In the literature, I found accounts of antiaphrodisiac pheromones in the following species: the mealworm beetle, *Tenebrio molitor* (Happ, 1969); the sweat bee, *Lassioglossum zephyrum* (Kukuk, 1985); the fruit fly, *Drosophila melanogaster* (Scott and Jackson, 1990); the tsetse fly, *Glossina morsitans* (Carlson and Schlein, 1991); and the pierid butterfly *Pieris napi* (Andersson, *et al*., 2001).

In order to better understand the existing hypotheses regarding the female postmating odour I decided to repeat Gilbert’s experiment (Gilbert, 1976) utilizing the same two pupal mating species, *H. erato* and *H. charithonia*. I hoped this would accomplish two goals. First, I could potentially replace anecdotal evidence with empirical evidence to either support or reject the antiaphrodisiac hypothesis. The second goal was to experimentally differentiate between two competing hypotheses concerning the role of the postmating odour. Contrary to Gilbert, Crane (1955) stated, after extensive field observations, that the postmating odour in *H. erato* females served a primarily sexual and secondarily defensive function. She based this conclusion on her observations that *H. erato* females evert their scent glands during courtship, and *H. erato* males will readily court and attempt to mate with mated female conspecifics. This conclusion is examined further in Chapter 2.

In order to accomplish these goals, experimental trials were conducted utilizing four stimulus types (3 female, and 1 tactile [hand battering]). Observations consisted of the male(s) behavioural reaction, recorded within two sequential time intervals of exposure, to each of the four stimulus types.
Chapter 1 - Materials and Methods

A. General Materials and Methods:

I. Environmental Parameters:

All butterflies used in experiments were reared at the Butterfly Conservatory in Niagara Falls, Canada, according to the Conservatory's preset environmental conditions. Environmental parameters were maintained by an Argus greenhouse computer monitoring system.

During the experimental period of 1997/98, temperature was maintained, from 07:00 to 19:00 hours at 25°C (± 2°C), with heating and cooling set points of 24°C and 26°C, respectively. From 19:00 to 07:00 hours, temperature was maintained at 22°C (± 2°C), with heating and cooling set points at 21°C and 23°C, respectively. Relative humidity was set at 75% (± 5%) and was maintained by an automatic mister system.

Circulating fresh-air streams were generated by a set of ridge vents and positive pressure fans. During the period of October to February, this system delivered approximately 150 ft³/min. of fresh air. For the period of March to October, the system delivered approximately one complete air change per minute, with the added assistance of an exhaust fan system.

II. Research Cages:

All research was performed in five isolation cages measuring 2.00m X 2.33m X 2.67m. All cages had concrete floors, 2 steel mesh-topped benches to support plant material, and were enclosed in fine gauge nylon mesh chosen to prevent the smallest first instar larvae from escaping. Access to the cage was gained through a steel framed nylon meshed door.

III. Larval hostplants:

Passiflora biflora (Passifloraceae) was propagated on-site and served the concomitant function of female oviposition plant and larval hostplant for both species. Cuttings from mature source plants were propagated and reared until the age of approximately 3 months. Maintenance of these plants included regular pruning, and automatic water and fertilization regimes designed by horticultural staff to induce maximum vigour and vegetative growth. Insect pests were controlled with species-specific biological control agents. After 3 months, healthy plants had reached the height of approximately 1m,
and contained numerous meristems and tendrils suitable for oviposition and larval feeding. I will subsequently refer to *P. biflora* plants of this quality as mature plants.

**IV. Breeding Stocks:**

Colonies of *H. erato* and *H. charithonia* were maintained by the entomological staff of the Conservatory using pupae received from butterfly breeders in Alajuela, Costa Rica and San Salvador, El Salvador. For each species, breeding stocks consisted of approximately 10 males and 20 females, replenished with fresh individuals from butterfly breeders every month to maintain vigour and genetic diversity of the progeny.

Each cage contained *Lantana camara* (Orange Lantana), *Pentas lanceolatea* (Egyptian Star Flower) and *Stachytarpheta franssii* (Porter weed) as a nectar source, while Yellow and Orange varieties of *Psiguria* spp. and a native North American *Heliotrope* sp. provided the source of pollen. All plants were maintained by the horticultural staff of the Conservatory. Maintenance included an automatic watering and fertilization system, pruning of dead plant material, monitoring biological control, as well as implementing a plant rotation schedule.

The plant rotation schedule was developed to provide butterflies maximum access to nectar and pollen resources before the plants became depleted of the necessary butterfly resources. As well as playing a key role in the normal development of adults, it has been demonstrated that the presence or absence of pollen plants, in some species may induce behavioural or physiological changes that affect mating (McNeil and Delisle, 1989). Additionally, at least one *P. biflora* was kept in all male and female cages. This provided the males with sensory information that is commonly associated with their natural mate location strategy. For mated females, this provided a potential oviposition site.

**V. Rearing of Larvae:**

Experimental individuals were collected as eggs from the Conservatory breeding stock. This was accomplished by introducing two mature *P. biflora* plants into the breeding stock cage. Females had access to these oviposition plants for approximately 48 hours. This provided sufficient time for oviposition, and additionally insured that larvae hatching from these egg batches had synchronized development.

After the 2-day oviposition period, these hostplants were removed to a larval development cage. Floors and benches of all breeding, research, and development cages were cleaned of frass daily,
power-washed, and disinfected once a month to prevent bacterial infection. Once the second instar was reached, larvae were transferred, 10 to 15 per plant, to another mature P. biflora to finish larval development in the same cage. These rearing hostplants were employed to provide the larvae with maximum access to fresh vegetative material thereby insuring minimum nutrient stress on larval development.

VI. Harvesting and sexing of pupae:

Larvae were allowed to complete development and pupate on the rearing hostplants in the larval development cage. Once the pupal skin had hardened (maximum 2 days), harvesting from the hostplant could be performed without the risk of damage. This was achieved by loosening the silken pad from which the pupa hung by scraping with a thumbnail along the point of attachment to the branch. This loosened the silk sufficiently to allow the pupa to be pulled free of the plant. This technique detached the pupa along with a portion of the silk pad, which remained attached to the cremaster.

Collections of pupae from the same egg batch were then sexed by examining the genital scar located on the terminal abdominal segments according to Beebe et al. (1960) and separated into same sex isolation cages to await eclosion. This was done to insure virginity and uniformity of exposure to the opposite sex for all experimental individuals.

Sexed pupae were pinned onto styrofoam emergence boards through the remaining silk attached to their cremaster. By piercing the remaining silk pad with a pin, the pupae could be attached to the boards. The boards were kept on an incline to allow all pupae to hang free from contact with each other and the styrofoam. Pupae were spaced 5 cm from their nearest neighbour to allow the emergent adult enough room to hang from the pupal case without risk of contact with any other butterflies.

VII. Treatment of adults:

After emergence, wing expansion and hardening, all individuals were marked on the hindwing using a fine tipped paintbrush and water based paint. By grasping the individual along the costal margin of the forewing, firm handling with minimum damage to wing scales was achieved. The marking code consisted of a letter denoting the egg batch from which the larvae were harvested, and a number denoting the individual within each batch. Furthermore, males were marked with white paint,
females with pink. This enabled the investigator to easily sex, identify and age each individual without the need for repeated handling.

Males and females were kept at approximately 15 individuals per cage. This density was chosen to avoid stress to both butterflies and plants. Males were kept in same sex cages, and not included in the experiment until they had reached sexual maturity (see section on age of experimental individuals).

B. Experimental Materials and Methods:

There are two separate parts within this chapter. The first consisted of a scanning electron microscope examination of the male and female genitalia of both *H. erato* and *H. charithonia*. The second consisted of behavioural experiments that examined the effect the female postmating odour had on pupal-perched males.

I. Scanning Electron Microscope Analysis of Genitalia:

All tissues collected were from newly emerged specimens approximately 2-6 hours old. To keep the usually inverted tissues of the females (scent glands and stink clubs) in view, specimen abdomens were ligated with string prior to drying. This provided enough hydrostatic pressure to evert the female scent glands and stink clubs. Similar techniques were used by Bronskill (1970) in examining female lepidopteran genitalia. For the males however, this procedure was found lacking. In order to view the internal structures of the male claspers, dried abdominal tips were first frozen in liquid nitrogen, and then cleaved with a razor blade.

Instead of critical point drying procedures, specimens were air dried in a glass dessicator for at least 72 hours prior to sputter coating. This was a trial procedure, which yielded excellent results, minimizing both time and money spent in tissue preparation.

Dried specimens were mounted on aluminum stubs with double-sided tape. Once mounted the tissue was sputter coated with gold-palladium. Coating was completed in three successive 20-minute periods, rotating the stubs between every period to insure complete coverage. Specimens were examined using a Hitachi S-2500 scanning electron microscope operated at 20 kV. Photographs were taken using Polaroid 55 Pos./Neg. film (See Figures 1.2 - 1.4).
II. Behavioural Analysis of the Female Postmating Odour’s Effect on Pupal-Perched Males.

A. Experimental Categories:

After emergence and marking, adult females were assigned at random to one of three treatment categories: (1) remain virgin, (2) be mated, or (3) have their stink clubs excised and then be mated. The females in these categories will subsequently be referred to as virgin, control and experimental females respectively.

Once categorized, each group of females was placed in a cage so that mated females were kept downwind of virgin females. Throughout the breeding area, a system of positive pressure fans, ridge vents, and exhaust fans, insured a constant supply and movement of fresh air. This single direction of circulated fresh air was utilized to reduce the risk of any unwanted pheromone transfer between females, thereby minimizing the chance of virgin females becoming exposed to the postmating odour of the mated females.

B. Experimental Manipulation:

The experimental manipulation consisted of excising the stink clubs of the experimental females prior to mating. From preliminary trials, it was concluded that 3-day-old females could undergo this manipulation with minimal effect on their behaviour and health. Complete recovery was defined as a return to normal observable behaviours such as flying, feeding on nectar and pollen, and social chasing.

The procedure for stink club excision entailed netting the desired female, and wrapping her wings with rice paper to insure minimal scale loss due to handling. A paper clip was then lightly applied across the costal margin of the wrapped wings to secure the individual and allow for easy handling. Once this was achieved, individuals were transported from the research cages to the lab.

Once in the lab, the secured individual was further restrained onto a styrofoam board. Restraint was achieved by taping the individual across the rice paper covered wings with masking tape. This held the individual firmly onto a piece of styrofoam. To keep the abdominal tip in working view, insect pins were used as braces. This was necessary as the natural reaction of the female in this situation was to retract her abdomen to within the folds of her wings. Pinning was facilitated by lightly blowing on the female; reflexively she extended her abdomen away from her wings. Once in this position a dissecting pin was placed into the styrofoam behind her exposed abdomen to prevent the
female from retracting her abdomen. Once secured in this manner, females were then placed under a dissecting microscope.

With watchmakers forceps, a stink club could be readily grasped by the base of the stalk at the point of insertion into the scent gland. Excision was accomplished by squeezing the forceps with enough pressure to sever the stalk of the stink club with minimal pulling. The same steps were repeated on the other side of the butterfly, thus removing both stink clubs. This procedure was adapted from similar techniques employed by Myers and Brower (1969) in the removal of male hair-pencils of the Queen Butterfly.

Following stink club excision, the female was monitored for any haemolymph leakage from the excision sites. If any was detected the female was deemed too physiologically compromised to continue in the experiment. When the procedure was completed, the female was returned to her cage to acclimate. Average time from netting to return to acclimate was approximately 5 minutes.

Based on personal observations following more than 100 dissections and subsequent observation periods, I am confident that there were no adverse behavioural or physiological effects noticed in a successfully dissected female.

C. How Matings were Obtained for Experimental and Control Females:

Having kept males and females isolated from each other, obtaining mating pairs was usually a very quick and easy procedure. A 6-day-old control (with stink clubs), or experimental (without stink clubs) female was placed into a cage containing only a nectar plant. Two randomly chosen, sexually mature males were then introduced. Once copulation had begun, the male that failed to achieve copulation was removed to prevent any harassment of the mating pair. Copulations were allowed to proceed until natural separation occurred. These methods, adopted from Boggs (1997b), proved to be a very successful means of attaining mating pairs.

These mating events were closely observed and the times until and the lengths of copulation were recorded. Once copulation was complete, the female was returned to her cage until she completed an entire day of ovipositing, generally the day following mating.

D. Age of Experimental Individuals:

The age of the males used in the behavioural section of the experiment varied; the only stipulations was that they were virgin and sexually mature. According to Crane (1957) *H. erato* males
become sexually mature no earlier than 48 hours post-eclosion. I observed that *H. charithonia* males were not able to successfully mate until a similar age was achieved. Thus, for both species this was the threshold I used for the minimum age of experimental males.

Ages for females of both species were kept uniform to aid in comparative studies. Virgin females were all 5 to 9-days-old. Mated females were 8 to 12-days-old. This provided the minimum time for all experimental manipulations, matings and subsequent recovery periods to take place.

E. *Experimental Pupae:*

The pupae that were involved in the experiment had been previously sexed (Beebe *et al.* 1960) and only female pupae were introduced into the male cage. Pupae were pinned through the remaining silk attached to their cremaster onto a *P. biflora* plant in the male cage. Beside each pupa was a numbered label, which identified the pupa and its age. Similar to previous studies (Edwards, 1881; Bellinger, 1954; Deinert *et al.*, 1994) males would begin to inspect the pupae immediately and begin to perch upon them in preparation for mating. Once the pupae had reached 8 days, one day prior to eclosion, the males were most interested in landing and remaining on the selected pupa. It was at this time that experimental trials commenced.

F. *Experimental Trials:*

Experimental trials began when either one or two males were perched upon an 8-day-old female pupa. Only males sitting on pupae that eclosed within 24 hours of the observations were included as valid trials. Trials recorded from pupae that did not successfully emerge the following day were omitted as male reaction to these pupae could confound the results. A male density of 15 butterflies per experimental cage was kept constant in all trials to control for the degree of intermale competition (Deinert, 1997).

The pupal-perched male(s) were exposed to different types of stimulus females and their subsequent behaviours recorded. There were three categories of stimulus females: virgin females, control females (mated with stink clubs), and experimental females (mated with stink clubs excised). I also included a hand battering treatment, which consisted of repeatedly striking the perched male(s) on the wings with the index finger of my left hand.

Females of each stimulus type were used twice in the experiment. In half of the trials, the stimulus females' abdomens were squeezed, in the other half they were not. To avoid repeated
measures, a single females abdomen was squeezed and non-squeezed in the presence of different males. This provided a means to examine the effect that the state of the females’ abdomen had on male behaviour, while minimizing the number of females needed. Squeezing was done to evert the scent organs of the females and concomitantly increase the intensity of the pheromone. Scent gland eversion was achieved by applying light pressure on the mid-sagittal section of the female abdomen with thumb and forefinger. This technique mimicked the hydrostatic pressure used by the female to evert her scent glands. Trials ended when all males had left, or the time limit (> 20 seconds) elapsed. All trials were videotaped with a tripod mounted Samsung video camera for subsequent analysis.

Experimental trials were grouped according to the type of stimulus, and whether or not a stimulus female was squeezed. The data tabulated from viewing the videotape were the duration of time until the male(s) reacted to the stimulus. Male reaction was recorded between the intervals of 0-10s or 11-20s of each trial, and scored as either dispersing from, or remaining on the pupa. These two time intervals were used across all trials to standardize the data. For all trials, duration until male reaction time was measured by the internal tape counter in the video camera.

Experimental predictions and expected results were determined from existing hypotheses (Gilbert, 1976). In the presence of the ‘antiaphrodisiac’ pheromone, i.e., the female postmating odour (mated females with stink clubs), all males should leave the pupa. In the absence of the ‘antiaphrodisiac’ pheromone (virgin and experimental females and hand battering), no males should leave the pupa. Therefore, as introduced earlier, I had predicted an ‘all or nothing’ response from the males in reaction to the pheromone.

G. Statistical Analysis

To test the effects that; (i) female abdominal state (squeezed versus non-squeezed); length of exposure to a stimulus (0-10s versus 11-20s); and (ii) female type (virgin versus control versus experimental) had on the rates of males leaving female pupae, sequential statistical tests were employed. Data were arranged in 2 X 2 or 2 X 3 contingency tables. Where there was contingency table cell counts of less than five, a Fisher’s Exact Test was employed (Zar, 1984). For contingency tables with all cell counts greater than five, Total $X^2$ Tests were employed.
Chapter 1 – Results

I. Percentage of males that left female pupae:

Raw data collected from this experiment are presented in Tables A.1-A.3. These data were used to calculate the percentage of males that left female pupae in the presence of trial stimuli, for both *H. erato* and *H. charithonia* (Figures 1.6 and 1.7). These graphs present the percentage of males that left pupae for the one and two male trials, and for both time intervals (0-10s and 11-20s) for all stimulus types. For the two male trials, percentages were calculated from replications in which one male left, as well as replications in which both males left. The percentage of males that left pupae was calculated using the total number of males present in all replications within the given interval. Therefore, if a male left in the 0-10s interval, it was excluded from the calculation in the 11-20s interval.

Figures 1.6 and 1.7 present experimental results that contradict the ‘all or nothing’ prediction from the antiaphrodisiac hypothesis. The trial categories without the postmating odour (hand, virgin and experimental females) all caused some percentage of males to leave (up to 54.5% for *H. erato* and 40% for *H. charithonia*). Conversely, the categories with the postmating odour (control females) failed to force all males to leave (in both species, there were 4 categories within the control female stimulus in which 0% of the males left female pupae).

II. Fisher’s Exact Test Analyses:

Further analysis was performed on the data in Tables A.1 and A.2 in order to detect any significant trends that would coincide with experimental predictions. The predictions were: (1) a squeezed female would force more males to leave than a non-squeezed female, and (2) more males would leave within the longer time interval compared to the shorter time interval.

A Fisher’s Exact Test (2-tailed) was performed on trial results for each female type of both species. Data used for statistical analysis were the number of trials in which any male left within the given time interval. Therefore, in the two male trials, whether or not one or two males left, both resulted in the same value being entered for statistical test purposes. This was done to eliminate the confounding factor of how one male dispersing affected the other’s decision to leave. The data from
Figure 1.6 Percent of *H. erato* males that dispersed over time in the presence of tactile and three categories of female stimulus. The number in () refers to the number of males that were present for each trial. The abbreviations 1-10s & 11-20s refer to the length of male exposure to the stimulus. Sample sizes are reported in Appendix A, Tables A.1 - A.3.
Stimulus Abbreviations

H - Hand Battering
Vr - Virgin Female
No Cl - Mated females without stink clubs
Cl - Mated females with stink clubs
Sq - trials in which the female’s abdomen was squeezed
No Sq - trials in which the female’s abdomens was not squeezed

Figure 1.7 Percent of H. charithonia males that dispersed over time in the presence of tactile and three categories of female stimulus. The number in () refers to the number of males that were present for each trial. The abbreviations 1-10s & 11-20s refer to the length of male exposure to the stimulus. Sample sizes are reported in Appendix A, Tables A.1 - A.3.
the trials were tabulated for each abdominal state category, time interval, and both one and two male trials.

To detect the effect of each experimental variable, further categorization was needed within each female type. Two separate sets of Fisher's Exact Tests were performed in order to control for experimental variables. The first set of tests (Table 1.1) examined the effect that the state of the female's abdomen (while controlling for time interval) had on males leaving female pupae. The second set of tests (Table 1.2) examined the effect that the time interval (while controlling for state of the abdomen) had on males leaving female pupae. This provided a controlled way to examine, within each female type, how the state of the female's abdomen and the length of male exposure to the stimulus affected male a males decision to leave a pupae. Two by two contingency tables were set up for both the one and two male trials. Within the forty-eight contingency tables constructed for both tests, five comparisons could not be calculated by the Fisher's Exact Test due to both compared values having zero sum column totals. These comparisons were arbitrarily given a designation of not significant.

No significant comparisons were found for either experimental variable by the forty-eight Fisher's Exact Tests (Table 1.1 and 1.2). Thus, the null hypotheses that the state of the female's abdomen, and length of exposure to the stimulus had no affect on males leaving female pupae, were not rejected. Therefore, within each female type, neither abdominal state of the female, nor length of male exposure to the stimulus, had a significant affect on males leaving female pupae.

III. Chi-Squared Analyses:

As neither state of the female's abdomen, nor time interval of stimulus exposure, had any significant effect upon males leaving female pupae within female types, the data within each female type were considered to have come from homogeneous sample populations and could therefore be pooled for each species (Zar, 1984).

To test the effect that female type had on males leaving female pupae, a set of $X^2$ Tests was performed on the pooled datasets for each female type. Two by three contingency tables were set up for both the one and two male trials from experimental data in tables A.1 and A.2. These tables and test results can be seen in Table 1.3 – 1.6. For both *H. erato* and *H. charithonia*, female type proved to be not significant in both one and two male trials (*H. erato* - 1 male: d.f. = 2, $X^2 = 0.75$, $p = 0.687$, $\alpha = 0.05$; 2 males: d.f. = 2, $X^2 = 1.81$, $p = 0.405$, $\alpha = 0.05$ and *H. charithonia* - 1 male: d.f. = 2, $X^2 = 0.48$, $p = 0.786$, $\alpha = 0.05$; 2 males: d.f. = 2, $X^2 = 0.13$, $p = 0.937$, $\alpha = 0.05$).

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Neither prediction drawn from the antiaphrodisiac hypothesis - the “all or nothing” response, or more fundamentally, that females with the postmating odour would left significantly more males than females without the odour - was supported by the results from this chapter.
Table 1.1 Results from the Fisher’s Exact Tests (2-tail) on *H. erato* and *H. charithonia* testing the effect that state of the female’s abdomen had on males dispersing from female pupae while controlling for time interval.

<table>
<thead>
<tr>
<th>Species (female type)</th>
<th>Time Interval</th>
<th>1 male (p-value)</th>
<th>2 males (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. erato</em> (virgin)</td>
<td>0-10s</td>
<td>No Stats.</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/2 vs. 0/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><em>H. erato</em> (virgin)</td>
<td>11-20s</td>
<td>0.429</td>
<td>0.138</td>
</tr>
<tr>
<td><em>H. charithonia</em> (virgin)</td>
<td>0-10s</td>
<td>0.467</td>
<td>1.000</td>
</tr>
<tr>
<td><em>H. charithonia</em> (virgin)</td>
<td>11-20s</td>
<td>0.523</td>
<td>0.491</td>
</tr>
<tr>
<td><em>H. erato</em> (control)</td>
<td>0-10s</td>
<td>1.000</td>
<td>0.077</td>
</tr>
<tr>
<td><em>H. erato</em> (control)</td>
<td>11-20s</td>
<td>1.000</td>
<td>0.200</td>
</tr>
<tr>
<td><em>H. charithonia</em> (control)</td>
<td>0-10s</td>
<td>0.205</td>
<td>0.467</td>
</tr>
<tr>
<td><em>H. charithonia</em> (control)</td>
<td>11-20s</td>
<td>0.429</td>
<td>0.444</td>
</tr>
<tr>
<td><em>H. erato</em> (experimental)</td>
<td>0-10s</td>
<td>No Stats.</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/3 vs. 0/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><em>H. erato</em> (experimental)</td>
<td>11-20s</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td><em>H. charithonia</em> (experimental)</td>
<td>0-10s</td>
<td>0.200</td>
<td>0.400</td>
</tr>
<tr>
<td><em>H. charithonia</em> (experimental)</td>
<td>11-20s</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

N.B. Numbers in brackets that appear below the No Stats. entries are the comparisons that could not be performed due to zero column sums.
Table 1.2 Results from the Fisher’s Exact Tests (2-tail) on *H. erato* and *H. charithonia* testing the effect that time interval had on males dispersing from female pupae while controlling for state of the female’s abdomen.

<table>
<thead>
<tr>
<th>Species (female type)</th>
<th>Female Abdominal State</th>
<th>1 male (p-value)</th>
<th>2 males (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. erato</em> (virgin)</td>
<td>NSq</td>
<td>No Stats. 0/2 vs. 0/2 NS</td>
<td>1.000</td>
</tr>
<tr>
<td><em>H. erato</em> (virgin)</td>
<td>Sq</td>
<td>0.061</td>
<td>0.080</td>
</tr>
<tr>
<td><em>H. charithonia</em> (virgin)</td>
<td>NSq</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td><em>H. charithonia</em> (virgin)</td>
<td>Sq</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td><em>H. erato</em> (control)</td>
<td>NSq</td>
<td>1.000</td>
<td>No Stats. 0/7 vs. 0/7</td>
</tr>
<tr>
<td><em>H. erato</em> (control)</td>
<td>Sq</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td><em>H. charithonia</em> (control)</td>
<td>NSq</td>
<td>1.000</td>
<td>No Stats. 0/5 vs. 0/4 NS</td>
</tr>
<tr>
<td><em>H. charithonia</em> (control)</td>
<td>Sq</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td><em>H. erato</em> (experimental)</td>
<td>NSq</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td><em>H. erato</em> (experimental)</td>
<td>Sq</td>
<td>1.000</td>
<td>0.559</td>
</tr>
<tr>
<td><em>H. charithonia</em> (experimental)</td>
<td>NSq</td>
<td>0.165</td>
<td>0.182</td>
</tr>
<tr>
<td><em>H. charithonia</em> (experimental)</td>
<td>Sq</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

N.B. Numbers in brackets that appear below the No Stats. entries are the comparisons that could not be performed due to zero column sums.
Table 1.3. A 2 X 3 contingency table presenting the number of *H. erato* trials, with one male present, in which a male left a female pupa (pooled within female type) in the presence of virgin, control and experimental females.

<table>
<thead>
<tr>
<th></th>
<th>Virgin</th>
<th>Control</th>
<th>Experimental</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leave</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Did Not Leave</td>
<td>12</td>
<td>5</td>
<td>12</td>
<td>29</td>
</tr>
<tr>
<td>Totals</td>
<td>16</td>
<td>7</td>
<td>14</td>
<td>37</td>
</tr>
</tbody>
</table>

Test result of the number of males that left female pupae in the presence of virgin vs. control vs. experimental females: $X^2 = 0.75$, d.f. = 2, $p = 0.687$.

Table 1.4. A 2 X 3 contingency table presenting the number of *H. erato* trials, with two males present, in which a male left female pupa (pooled within female type) in the presence of virgin, control and experimental females.

<table>
<thead>
<tr>
<th></th>
<th>Virgin</th>
<th>Control</th>
<th>Experimental</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leave</td>
<td>11</td>
<td>7</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>Did Not Leave</td>
<td>17</td>
<td>23</td>
<td>19</td>
<td>59</td>
</tr>
<tr>
<td>Totals</td>
<td>28</td>
<td>30</td>
<td>26</td>
<td>84</td>
</tr>
</tbody>
</table>

Test result of the number of males that left female pupae in the presence of virgin vs. control vs. experimental females: $X^2 = 1.91$, d.f. = 2, $p = 0.385$. 
Table 1.5. A 2 X 3 contingency table presenting the number of *H. charithonia* trials, with one male present, in which a male left female pupa (pooled within female type) in the presence of virgin, control and experimental females.

<table>
<thead>
<tr>
<th></th>
<th>Female Type</th>
<th></th>
<th></th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virgin</td>
<td>Control</td>
<td>Experimental</td>
<td></td>
</tr>
<tr>
<td>Leave</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Did Not Leave</td>
<td>22</td>
<td>15</td>
<td>20</td>
<td>57</td>
</tr>
<tr>
<td>Totals</td>
<td>27</td>
<td>20</td>
<td>27</td>
<td>74</td>
</tr>
</tbody>
</table>

Test result of the number of males that left female pupae in the presence of virgin vs. control vs. experimental females: $X^2 = 0.48$, d.f. = 2, $p = 0.786$.

Table 1.6. A 2 X 3 contingency table presenting the number of *H. charithonia* trials, with two males present, in which a male left a female pupa (pooled within female type) in the presence of virgin, control and experimental females.

<table>
<thead>
<tr>
<th></th>
<th>Female Type</th>
<th></th>
<th></th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virgin</td>
<td>Control</td>
<td>Experimental</td>
<td></td>
</tr>
<tr>
<td>Leave</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Did Not Leave</td>
<td>18</td>
<td>16</td>
<td>15</td>
<td>49</td>
</tr>
<tr>
<td>Totals</td>
<td>22</td>
<td>19</td>
<td>19</td>
<td>60</td>
</tr>
</tbody>
</table>

Test result of the number of males that left female pupae in the presence of virgin vs. control vs. experimental females: $X^2 = 0.18$, d.f. = 2, $p = 0.916$.  

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CHAPTER 1 – DISCUSSION

The purpose of this experiment was to examine the effect that the female postmating odour had on pupal-perched conspecific males. Predictions were that observed results would conform to those originally reported in the Gilbert (1976) study. In addition, by repeating Gilbert’s study, I hoped to achieve two goals. The first was to potentially replace anecdotal evidence with empirical evidence of male behaviour and reaction to female postmating odour. The second was to synthesize and examine these empirical data in order to test the first of two competing hypotheses regarding the function of the female postmating odour.

The data collected from this experiment satisfied the first goal of replacing the anecdotal evidence presented in the Gilbert (1976) paper with empirical data. Male behavioural reaction to female postmating odour was observed and recorded under controlled experimental settings.

As seen from the observed results presented in Figure 1.6 and 1.7, there was not an “all or nothing” male response to the female postmating odour. For both species, males were observed to disperse while in the presence of females with and without the postmatng odour. Therefore, it can be concluded from these data that there was not an obligate response from H. erato and H. charithonia males to the presence or absence of the female postmating odour of conspecifics.

Within female type tests:

The Fisher’s Exact Tests performed within each female type examined the effects that state of the abdomen and time interval had on males leaving female pupae. It had been predicted from the antiaphrodisiac hypothesis that abdominal squeezing (for the control females) would force significantly more males to leave a pupa. Abdominal squeezing everted the scent glands and concomitantly increased the concentration of the postmatng odour. Additionally, it was predicted that an increased exposure time to a stimulus would result in a higher number of males leaving female pupae. However, within each female type, neither state of the abdomen nor time interval, proved a significant factor affecting male leaving, for either species.

Although there were no statistically significant test results, there was an interesting result for the comparison between H. erato squeezed and non-squeezed, control females (when controlling for time). In the 0-10s time interval, with two males present, the p-value for this comparison was 0.077 (Table 1.1). This result, when viewed singularly, appeared to be consistent with the prediction of an
increased concentration of postmating odour dispersing more males. However, when viewed in the context of the other test results, the prediction lost its biological relevance.

According to predictions, both squeezed and non-squeezed control females should have forced the highest number of males to leave pupae compared to the other two female categories. In addition, the non-squeezed control females were predicted to force fewer males to leave pupa than the squeezed control females. Indeed the non-squeezed control females forced fewer males to leave than the squeezed control females. However, squeezed virgins actually forced a higher percentage of males to leave pupa than the squeezed control females (30.4% and 28.6% respectively) (Figure 1.6). Therefore, it was not the increased concentration of the postmating odour in the squeezed control females (or the postmating odour at all) that produced the nearly significant test result. Instead, it was that the non-squeezed control females were unable to left any males, compared to the non-squeezed virgin females (control - 0 out of 28, compared to virgin - 5 out of 30). This produced a near significant test result in the control female category and not in the virgin female category. Thus, the lone test result of near significance for H. erato control females, when viewed in the context of the rest of the test results, proved incongruous with predictions made from the antiaphrodisiac hypothesis.

Between female type tests:

The same dataset was further used to examine whether or not the different female types produced any significant differences in males leaving female pupae. Although there was not an obligate response of males to the presence or absence of the female postmating odour, there existed the possibility that females with the postmating odour forced significantly more males to leave female pupa than females without the postmating odour. However, for both species, in trials with one and two males present, no significant difference in male leaving was detected between female types (Table 1.3-1.6). Thus, the male decision to left was not significantly affected by female type. Therefore, the presence or absence of the postmating odour had no significant effect on male leaving in either one or two male trials for either species. This was a surprising and unexpected result. It neither coincided with the observations reported by Gilbert in the 1976 study, nor supported the predictions which came from Gilbert’s results.

Given the observed trends and the results of the statistical tests from this experiment, it must be concluded that the data collected do not support an antiaphrodisiac function for the female postmating odour at the pupal level, for either H. erato or H. charithonia.
Alternatives to the Antiaphrodisiac Hypothesis:
i) Pupal Sex Identification Pheromone Hypothesis:

If, as proposed, the female postmating odour was not acting as an antiaphrodisiac, then what could explain the observed trends in males that left female pupae for both species? As hypothesized by Gilbert (1976), the postmating odour may be a pupal sex identification pheromone that acquired the dual function of an antiaphrodisiac pheromone. The experimental data did not support the antiaphrodisiac hypothesis, but perhaps the postmating odour "convinced" the pupal-perched male(s) that they were on a male pupa instead of a female. In the papers that document pupal mating (Edwards, 1881; Bellinger, 1954; Deinert et al., 1994) it was always assumed that males were attracted by a scent given off by the female pupae. It seems equally likely that males were able to identify male pupae by scent, and subsequently avoided perching on these pupae. If the latter case were true, then if the female postmating odour was identified with maleness (the actual pheromone, or its chemical precursor, being donated from the male), its presence, in strong enough concentrations, could mimic the scent of a male pupa. In such a case, pupal-perched males would leave once they were convinced that they were on a male pupa.

Since the male has already invested considerable time and energy in locating, sitting on, and battling other males for pupae, he could lose a considerable energy investment if he were to prematurely leave female pupae. This risk, coupled with the fact that other males, who presumably retain some male odour, did not force other males to leave, indicated that there would have to have been a considerable amount of the pupal sex identification pheromone present to convince a male that he was now on a male pupa instead of a female. In support of this statement, I offer the personal observation of up to six H. erato and five H. charithonia males perched on a single female pupa for prolonged periods of time. Thus, any minimum critical threshold of the sex identification pheromone must be high.

There was a supportive trend for this hypothesis; more H. erato males tended to left in the presence of squeezed versus non-squeezed control females when there were two males present (Table A.1). The increased concentration of the postmating odour in a squeezed control female may have been enough to overwhelm the already present male odour from the other pupal-perched males, and to convince some or all the males that they were on a male pupa instead of a female pupa. This trend was not observed in the one male trials, but could have been a result of the smaller sample sizes. However,
for both species the pupal sex identification hypothesis does not explain the trends for males leaving pupae observed in all other categories of stimuli. The virgin and experimental female categories and the tactile category were all able to left some percentage of males despite lacking the postmating odour.

While no attempt was made in this thesis to ascertain how males identified conspecific female pupae, the observed trends of males leaving pupae in trials without the postmating odour did not coincide with predictions made from the pupal sex identification hypothesis. Although the observed trends appeared to indicate that males did not left due to pupal sex confusion, it was not the scope of this thesis to test all predictions made from this hypothesis. Therefore, it is recommended that further experiments be performed to more fully address the questions of how males identify the gender of conspecific pupa, and how the postmating odour effects this identification.

Experiments to test the pupal sex identification pheromone hypothesis could consist of measuring male inspections and landings on artificially scented and unscented female pupae. The frequency and duration of male interactions with scented and unscented female pupae could be compared to similar measurements gathered from males with male pupae. If the postmating odour had originally evolved as a male pupal identification pheromone, then it would be predicted that female pupae with the postmating odour should elicit responses from males that would be similar to those elicited from male pupae.

A source of the postmating odour would be essential to this experiment. I can suggest two ways to collect the female postmating odour. First, it could be collected from the excised stink clubs of mated females. Once a sufficient number of stink clubs had been acquired, these could be used to contact female pupae thereby applying the postmating odour. Alternatively, hexane washes and chemical isolation techniques could be used to isolated and perhaps characterize the postmating odour (Miyakado et al., 1989). This could yield a method for artificially synthesising the postmating odour.

ii) Minimum disturbance threshold hypothesis:

A different hypothesis that could explain the observed trends in male leaving is that pupal-perched males of both species were not oblivious to visual and tactile stimuli. Males do become increasingly reluctant to leave a female pupa as it nears eclosion, but contrary to the Gilbert (1976) observation, males could be forced to left by means other than the postmating odour. This hypothesis was termed the minimum disturbance threshold hypothesis. A prediction made from this hypothesis
null
was once a minimum threshold level of disturbance was surpassed, a pupal-perched male would be forced to leave.

During intermale competition on the pupa, males battle each other for primary position on the pupa. This position affords the holder the best chance of copulation once the female begins to eclose. Throughout this intermale battling, males risk physical damage to their wings and antennae, and some males can be forced to leave the pupa. When the probability of physical damage became high enough to compromise a male’s chances of achieving copulation, the male should left. Additionally, males might just get pushed off the pupa if they do not possess a position with a good grip. The presence of the experimental external stimuli (hand battering and the contact of female types) could have mimicked the pressures of intermale competition. It could have supplied the cues or stimuli associated with male jostling and its attendant risk of physical damage. Thus, a male might have been forced to left by an increasing threat of harm. Alternatively, the external stimuli might have forced males with loose grips to become dislodged. Either way, it was the disturbance caused by the external stimuli that was responsible for the observed trends of males leaving female pupae, not the presence or absence of the postmating female odour.

Although in contradiction to the generally accepted antiaphrodisiac hypothesis, the minimum disturbance threshold hypothesis provides a more compatible interpretation of statistical test results, and can explain the observed patterns in male leaving for all stimulus categories. Therefore, based on these data, I contend that the minimum disturbance threshold hypothesis is the most parsimonious and comprehensive hypothesis.

iii) Sources of Variability:

Controlling sources of variability in experiments is one of the advantages of captive over wild studies. However, absolute control of all sources of variability is seldom, if ever, achieved. In this study, there were two potential sources of variability: (1) pupal age, and (2) the age of experimental males.

Pupal age was controlled in this experimental to the extent that all experimental pupae were 8-days-old, with the added stipulation that each of these pupae produced live female butterflies the following day (Day 9). However, male leaving could have been affected by more subtle differences in age of these pupae (i.e. hours, or even parts of an hour prior to eclusion).
As pupae near eclosion, males become increasingly reluctant to leave. If male reluctance to leave increased as the pupae aged, there would be no reason to believe that this reluctance did not follow an increasing gradient right up to the time just prior to eclosion. Therefore, instead of pupal-perched males being oblivious to visual and tactile stimuli, as reported by Gilbert (1976), males become increasingly reluctant to leave a pupa due to visual and tactile stimuli as eclosion nears.

Because the exact time from the experimental trial until eclosion was not measured, this subtle difference in pupal age (further termed microage) could have contributed to the observed trends in males leaving female pupae. In other words, males on younger 8-day-old pupae may have been more easily forced to leave, compared to those on older 8-day-old pupae. However, the randomization of pupae in the experiment should have eliminated any directional bias in males leaving pupae created by the pupae's microage. Therefore, although I hypothesize that the microage of the pupae will affect a male's decision to leave a pupa, precautions were taken to prevent this from biasing the findings of this experiment.

The age of experimental males varied due to constraints associated with availability at critical experimental times. Thus, there was a larger age span than desired. This age span could have produced behavioural differences between younger and older males. All experimental males were kept virgin until being used in trials. An older male virgin could have exhibited a higher tenacity to cling to female pupae compared to a younger male virgin, as the older male has more to lose reproductively by missing a mating opportunity. This difference in male tenacity could have affected the patterns observed for males leaving female pupae. Therefore, even though all males had equal access to female pupae, and trials were randomly conducted on pupal-perched males, the variance in male age could have biased the results of this experiment.
Chapter 1 – Conclusions

Observations and predictions from Gilbert (1976) on the function of the female postmating odour were not supported by the data collected in this experiment. Pupal-perched males of *H. erato* and *H. charithonia* were forced from their pupae by means other than the hypothesized antiaphrodisiac pheromone. Statistical tests indicated that the presence of the female postmating odour had no significant effect on males leaving female pupae.

The interpretation of statistical test results and the observed trends from experimental data led to the development of a new hypothesis - the minimum disturbance threshold hypothesis. The stimuli used in the experiment mimicked the disturbance cues associated by males with those encountered during intermale competition on a pupa. Once a minimum disturbance threshold was surpassed, males became more likely to leave from a pupa. In this experiment, the disturbance took the form of tactile stimuli, or contact with female stimuli (mated females with and without stink clubs, and virgins).
Chapter 2: The effects of female postmating odour on male courtship behaviour, in two species of Heliconius butterflies.

Abstract:
Experiments were conducted to test the effects of the female postmating odour of H. erato and H. charithonia on male courtship behaviours. These experiments supplied the first empirical datasets that quantified the duration (measured as a courtship index) and frequency of male courtship behaviours in the presence of females with and without the postmating odour. The collected data were used to test predictions, drawn from two competing hypotheses, regarding the function of the postmating odour in terms of male courtship. The predictions drawn from the antiaphrodisiac hypothesis were not supported by the data. The mated females, both with and without the postmating odour, elicited nearly identical male measurements of duration and frequency of courtship behaviours. It was concluded that mated female rejection postures and behaviours were responsible for the significant difference detected in the comparison of courtship indices for H. erato virgins and mated females with the postmating odour.
Chapter II - Introduction

I. Courtship behaviour:

Most lepidopterans engage in precopulatory courtship behaviours. These behaviours serve a dual purpose. First, they are used to identify conspecifics and therefore prevent interbreeding. Second, since there are very few instances of forced copulation in lepidopterans (Wiklund, 1977), behavioural courtship displays provide the male a means of advertising his reproductive qualities to the female in an attempt to convince her to accept him as a potential mate. Male courtship displays also entail costs in terms of energy required for behavioural display, and time, as courting of one female reduces time available for locating and courting other mates. By measuring a male’s persistence in courtship displays and his willingness to initiate specific behaviours in the presence of different females, a quantitative index of male courtship behaviour can be generated. A quantitative index of male courtship behaviours would provide a comparable means of assessing a male’s desire to mate with a specific female.

II. Courtship Pheromones:

i) Courtship stimulating pheromones:

There are several common aspects of pheromones, which make them particularly useful in the study of courtship behaviours. First, in most lepidopteran species pheromones from both sexes are needed for successful copulation following courtship (Scott, 1973). Second, pheromones can be used over long distances to increase encounter rates between sexes (McNeil et al., 1999) or in close proximity to modulate mate choice (McNeil et al., 1999). Third, gender-specific behavioural responses are usually the same among species. Female pheromones generally elicit a male pursuit response, while male pheromones usually induce a female to land and accept the male (Scott, 1973).

The importance of male pheromones in achieving copulation was illustrated by Pliske and Eisner (1969) for the Queen Butterfly Danaus gilippus berenice (Danainae), Rutowski (1977) for the Small Sulphur Butterfly Eurema lisa (Pieridae), and Conner et al. (1981) for the moth Utetheisa ornatrix (Arctiidae). In all of these studies, males were experimentally deprived of scent producing structures and were subsequently unable to elicit receptive precopulatory postures in conspecific females.
ii) Courtship inhibiting pheromones:

As well as stimulating courtship, pheromones can also inhibit courtship. In the other known examples of courtship-reducing pheromones in insects, male courtship of females may be reduced (via chemical cues) in two ways. First, male-donated chemicals may function by acting as pheromone masks, i.e., by covering an attractive scent produced by the female. Second, a repellent pheromone (an antiaphrodisiac) may be transferred to the female, which inhibits male stimulation by female pheromones. The first method essentially hides the female by making her chemically invisible, the latter confers chemical visibility but renders the female unattractive to males.

Pheromone masks act by covering an attractive female pheromone and thereby preventing other males from responding to the female. In the mealworm beetle (Tenebrio molitor L.) males produce two pheromones. One acts as an excitant to attract females and the other acts as a mask (passed at the time of mating), which covers the female scent and thus inhibits the response of other males to this scent (Happ, 1969).

A similar pheromone is released by the beetle Trypodendron lineatum (Scolytidae). In this system, the male produces a pheromone, which masks a female aggregation pheromone, thereby inhibiting its attractive abilities (Borden, 1974).

Antiaphrodisiacs, which render the female repellent or unattractive to males, have been described for the Tsetse fly Glossina morsitans morsitans (Westwood), the fruit fly Drosophila melanogaster and the pierid butterfly Pieris napi (Andersson et al., 2000). In the Tsetse fly, a male-produced cuticular hydrocarbon is transferred to the female at the time of mating and inhibits male stimulation by female pheromones (Carlson and Schlein, 1991).

In Drosophila melanogaster, male-predominant hydrocarbons are transferred to the female at the time of mating (Scott, 1986) and reduce male courtship by mimicking male identification scents. Thus, these hydrocarbons act as antiaphrodisiacs by providing a sexually ambiguous signal to the male (Scott and Jackson, 1990).

In the pierid butterfly a male transferred an antiaphrodisiac compound (methyl-salicylae) to the female at the time of mating. This compound was emitted by mated females when courted and resulted in males quickly abandoning the females. It proved to be such a strong courtship deterrent that males refrained from courting virgin females who had been artificially covered with the antiaphrodisiac.
Gilbert (1976) proposed the antiaphrodisiac hypothesis as the mode of action for the postmating odour of female *H. erato* and *H. charithonia*. In that study, Gilbert extrapolated his results from the pupal mating stage to the adult courtship stage, suggesting that the pheromone functions to reduce male attraction to adult mated females. This contrasted with Crane’s (1955) hypothesis, that the postmating odour was primarily sexual and secondarily defensive in function, as it did not deter subsequent courtship or at least one additional mating. However, Crane’s study did not quantify the level of male courtship. Crane (1955) was able to observe males courting mated females, but made no attempt to detect or measure any reduction in courtship. Therefore, by collecting quantified male courtship measurements, I would be able to generate a novel dataset that would provide a means for supporting one of the two hypotheses regarding the function of the female postmating odour.

**III. Typical courtship behavioural sequence:**

In order to provide the context for the male behaviours that were collected and used to create a quantified measure of courtship, I have included a brief description of the typical courtship sequence of *H. erato* first published by Crane (1955).

A normal courtship sequence has two possible beginnings. First, a male that has visually located a female will begin chasing her closely. After a period of chasing, the female will usually settle on a leaf or other structure. If she does not, the male can urge the female to land by flying slightly above her and gently brushing her wings with his in an attempt to force the female downward.

Alternatively, a male approaches a female at rest. At a male’s approach, the female opens and closes her wings several times. If she is receptive, wing movement stops and both pairs are held closed over the back. The male then flutters (fans) in quick, repeated bursts close behind her, creating a gentle current of air against the female. During this stage, the male’s wings are never separated. This should be noted because the separation of the wings exposes the area of the male scent scales, and exposure is the means of disseminating these scales. The male claspers (harpes) at the tip of the abdomen remain tightly closed during this stage.

A receptive female responds to this fluttering in one of two ways. First, she responds by elevating the abdomen and evertting the scent glands. The stink clubs associated with these glands will only become exposed during the most excited female responses. Second, as the abdominal tip is elevated and the gland evertted, the female begins wing vibration. With the anterior margins of the forewings held closely together, the posterior portions are allowed to bell outward while the hindwings
are held slightly opened and begin to vibrate. These vibrations last for 1-2 seconds, with brief intervals between vibrations. Both male and female behaviours up to this point constitute Stage I of the courtship sequence.

Stage II may be omitted or strongly abridged by an excited male if the female is highly receptive. The male now shifts positions so that his fanning continues above and especially in front of the female. At this point, his fore- and hindwings become separated, exposing the scent scales, but the male’s claspers remain tightly closed. During Stage II the female continues to vibrate her hindwings as in Stage I.

The final sequence, Stage III, immediately precedes copulation. It begins with the male alighting and moving toward the female. He generally comes to rest with his eyes near the level of the female’s thorax. He continues fanning his wings with the friction surfaces exposed and she elevates her abdomen and extrudes the scent gland. The male now separates his claspers, and “J’s” his abdomen seeking to curve it between the posterior margins of the female’s hindwings in an attempt to engage her abdomen. If the female is receptive, her abdomen is lowered and the scent gland withdrawn. At this point, the male’s claspers grasp the lowered tip of the female’s abdomen, and engage her genitalia thereby initiating copulation. Once fully engaged, the male positions himself so he and the female are facing opposite directions.

Despite the lack of a published account for the exact behavioural sequence for *H. charithonia* courtship, Scott (1973) stated that closely related species usually perform similar courtship movements. In accordance with this statement, all the successful *H. charithonia* courtships observed in the course of this study followed the same behavioural sequence outlined for *H. erato*.

IV. Rationale for experiment:

In the following behavioural experiments, three sets of male measurements were used to answer the question of whether or not the male reduced his mating expenditure (courtship) in the presence of females with the postmating odour.

The first set of male measurements consisted of: (1) time until copulation, and (2) duration of copulation, in the presence of females with and without stink clubs. These were calculated to examine the effect on the male of the presence or absence of the female stink clubs. If the stink clubs are the organs responsible for transfer of the postmating odour, then the length of copulation might be affected by their absence. Additionally, if experimental females were adversely affected by the excision
procedure, it might be predicted that the time until copulation would be longer compared to control females.

The second set of male measurements was generated by calculating a male courtship index elicited by different females. A courtship index provided a quantitative measure of a male’s persistence in displaying specific courtship behaviours towards different females.

The third set of male measurements was collected by calculating the frequency of specific male courtship behaviours in the presence of different females. The courtship index was a good indicator of male persistence, but could not address a male’s willingness to initiate different behaviours. Therefore, the frequency of all male courtship behaviours was tabulated in the presence of females with and without the postmating odour.

According to the antiaphrodisiac hypothesis, several predictions were made regarding the persistence and frequency of male courtship behaviours elicited from different female types. First, virgins were predicted to produce the highest measure of male persistence and frequency of courtship behaviours. Virgins do not possess any postmating odour and should, therefore, be most attractive to males. Second, mated females with stink clubs were predicted to elicit the lowest courtship index and frequency of courtship behaviours. They possess the antiaphrodisiac pheromone and the organs associated with its dissemination. Third, mated females without stink clubs were predicted to elicit courtship indices and behavioural frequencies closer to that of virgins, as they neither had the postmating odour nor the structures associated with its dissemination. And fourth, if the postmating odour rendered the females male-like in odour, as in *Drosophila melanogaster* females (Scott and Jackson, 1990), it was predicted that the social index (persistence of male social behaviour elicited from other males) and courtship index of mated females with stink clubs would be similar.

Alternatively, the postmating odour may not act as an antiaphrodisiac pheromone. In this case it was predicted that male courtship indices and frequencies of courtship behaviours elicited from the 2 mated female types would be similar and those elicited from the virgin females would be significantly higher.
Chapter II - Materials and Methods

I. Environmental Conditions and Containment of Research Butterflies:

The care and maintenance of all individual stages of butterflies used in this experiment are outlined in the General Materials and Methods section of Chapter I. All plant requirements, research cages, and environmental parameters used in this experiment were identical to those previously outlined in Chapter I. Pupae were pinned according to sex and allowed to emerge in single sex holding cages. Once emerged, individuals were marked and moved to isolation cages according to research requirements.

For this experiment, four experimental groups were maintained: virgin males, virgin females, mated control females, and mated experimental females. A one-way flow of air through all cages was created by the forced-air fans and vents in the research area. Because of this one-way flow of air, mated females were kept downwind of males and virgins to prevent any unwanted pheromonal transfer between individuals.

II. Experimental Manipulation (Stink Club Excision):

The experimental manipulation for this study was the excision, prior to mating, of the stink clubs from the experimental female group (See Experimental Methods Section in Chapter I).

III. Sequence and Time Frame for Experimental Individuals:

All individuals used in this experiment were marked with water base paint as soon as their wings had hardened sufficiently to allow handling without damage (See General Methods Section in Chapter I).

Experimental females underwent stink club excision 3 days post-eclosion. This date was chosen as females manipulated earlier than this did not always fully recover. Control females were handled in the same manner but did not have their stink clubs removed. This was done to control for handling effects on both groups of females included in the experiment.

Two days were allowed for recovery from the experimental manipulation. If a female was observed to fly and feed (on both pollen and nectar) in a usual manner, she was deemed to have recovered and was able to continue in the experiment.
Matings of both experimental and control females were performed on day 5 (See how matings were obtained in General Methods Section of Chapter I). Data recorded from these matings included (1) time until copulation began, and (2) duration of copulation.

Mated females of each group were isolated and allowed to acclimate in cages with normal nectar, pollen and oviposition plants. Again two days were allotted for recovery, and females that were observed to be flying, feeding (on both nectar and pollen) and ovipositing at the end of the recovery period, were allowed to continue in the experiment.

IV. Age of Experimental Individuals:
The ages of all individuals are in days, and refer to the number of days since eclosion.

i) Control and Experimental Females

Control females were mated with their stink clubs intact. Experimental females were mated with their stink clubs excised. For each of the experimental stages (stink club excision and mating), there was a 1-day window designed to accommodate variations in weather and cooperation of males. Thus, all control and experimental females that were ready for trials were 7-8 days old.

ii) Virgin Females

The age of virgin females used in the trials ranged from 2 to 10 days. Females less than 2 days old were not expected to exhibit a full range of female courtship behaviours (Crane, 1957) and, therefore, were not used in any trials. Although a narrower age group would have been preferable, this wider range of virgin ages was the result of availability during the time the trials were conducted.

iii) Experimental Males

The age of experimental males ranged from 6 to 13 days, for both species. The breadth of this age category was required as males had to be sexually mature (3 days minimum), and coordinated with the age of the females that were used in the trials. It is generally accepted that sexually mature lepidopteran males do not exhibit any changes in courtship behaviour with age (Scott, 1973). However, older males (>13 days) were more likely to have suffered detrimental physical damage (e.g. wing wear), which could have confounded the results of the experiment by increasing intermale variability. Therefore, only males under 13-days-old were used in experimental trials.
V. Experimental Trials:

Three experimental trials were conducted for each male. Experimental trials were 30 minutes in length and consisted of observing and recording male behaviours toward an introduced female. A male was randomly chosen from the designated age batch and was released into the observation cage for a 15-minute acclimation period. This allowed any handling effects to subside before a female was introduced.

After the acclimation period, a randomly chosen experimental or control female was released into the observation cage with the male, and the first trial began. Once this trial was completed, the male was allowed another 15-minute acclimation period before the next female was introduced. The order of introduction of experimental and control females was reversed for every male to avoid any patterned responses due to order of introduction.

Another 15-minute acclimation period was allowed before a randomly chosen virgin female was introduced to the male. The introduction of the virgin always followed the two mated female introductions. This was necessary, as copulation would have prevented any further introduction of females to the male.

The reaction of the male towards each introduced female was the variable to be measured. Male behaviours were collected by the observer from within the experimental cage. Due to the rapidity of courtship behaviours, filming on videotape proved awkward and inaccurate. Therefore, it was decided to adopt the tape recording methods of Brower et al. (1965). Lengths of male courtship behaviours were timed on a V.W.R. stopwatch/timer. Duration and frequency of behaviours were then recorded on a hand-held General Electric cassette recorder. Behaviours were later transcribed and tabulated from these cassette recordings.

VI. Male Behaviours Recorded:

i) Male chases female (Stage I)

This is the first stage of the male aerial courtship sequence. The number of chases and the duration of each chase were recorded. Within each chase, the frequencies of male contacts and fannings of the female were also recorded. At this stage, both contacting and fanning of the female were the behavioural mechanisms by which the male urged the female to land in order for courtship to advance to the next stage. These behaviours may also serve to provide species-specific olfactory and visual information to the female (Scott, 1973).
ii) Male approaches female (Stage I & II)

An approach was recorded only if the female was at rest and the male was within a distance where he could make direct contact with her. Both the frequency and duration of each approach were recorded. An approach indicated a male’s willingness to continue the courtship sequence past the aerial stage.

Similar to chasing, the frequency of contacts and fannings were recorded within each approach. These two behaviours serve the same function as in the chasing stage of courtship, but indicate that the courtship has advanced to the ground stage (Stage II).

iii) Male lands beside female (Stage III)

The frequency and duration of each incident in which the male landed beside the female was recorded. Landing beside the female indicated a male’s willingness to continue with courtship leading to copulation. Within each landing, the frequency and duration of any copulation attempts were recorded. A copulation attempt was defined and scored if the experimenter observed the male bending his abdomen. This described the abdominal position the male adopted while trying to locate the female genitalia, and was the final male behaviour prior to copulation. If the female was willing to mate, copulation began. If the female was unwilling to mate, she spread her wings and kept her abdomen elevated. This was the typical rejection posture for both species.

iv) Male and female rests

Both frequency and duration of male and female rests were recorded. This allowed for the calculation of total flying and resting times. From these times, male behaviours could be standardized into number of occurrences per flying/resting minute.

VII. Data Collection and Tabulation:

i) Time until copulation began and copulation duration

It was important to test for any unwanted variations in male behaviour elicited by the control and experimental females. To this end, the time until copulation began and the duration of copulation were analyzed for control and experimental females. This provided an indicator as to whether there were significant differences between the two female types resulting from the excision of the stink clubs.
ii) Courtship Index

The courtship index (C.I.) was calculated according to that of Thompkins and Hall (1981) and Scott et al. (1988). This index was defined as the percent of a 30-minute trial that a male spent exhibiting courtship behaviour towards the female. All C.I. values are expressed as a percent. It has proven to be an accurate measure of male persistence once courtship has begun (Scott and Jackson, 1988).

For the virgin females, the C.I. was calculated as the percent of time the male exhibited courting behaviours during the trial. This meant until either the time of copulation, or the end of the 30-minute trial. For the control and experimental females the C.I. was calculated as the percentage of time the male spent exhibiting courting behaviours in the 30 minute trial.

Only male courting behaviours that were measurable by duration were suitable for this calculation. These behaviours were (1) male chasing female (2) male approaching female and (3) male landing beside the female for copulation attempts. The C.I. was a means to quantify the persistence of males in maintaining courtship behaviours toward different females. It also provided a means for statistically testing any differences in male courtship persistence between female types.

iii) Behavioural Frequency

Frequencies of male courting behaviours were calculated for each female type. This calculation reflected the number of behaviours per female flight minute (for aerial behaviours) or per female rest minute (for ground behaviours).

To test for differences in specific male courtship behaviours, the C.I. was insufficient. Tabulating and standardizing the frequency of male courtship behaviours allowed for the quantification of male effort involved in initiating each stage of courtship. It also provided a value to statistically test the null hypothesis of equal male courtship means among all female types.

iv) Social Index (S.I.)

As a general index of male social behaviour, a category of male behaviour towards another male was included in the experiment. The duration and frequency of male social behaviours towards other males were tabulated similarly to the methods used to calculate the C.I. and frequency of male courting behaviours. The only male courting behaviours that were not also social behaviours were chase and approach fannings, and copulation attempts.
VIII. Statistical Analyses:

All statistical tests were performed with values calculated from trials involving both species.

i) Copulation duration and time until copulation

A Mann-Whitney U test was used to test for any significant differences between the control and experimental females for: time until copulation occurred, and duration of copulation.

ii) Virgin female trials

In trials that involved virgin females, some resulted in matings while others did not. Therefore, in order to test if there was any significant difference in calculated male C. I. values between these two trial types, a two-tailed Student’s t-test was performed.

iii) Courtship index (C.I.) and behavioural frequency

A Kruskal-Wallis single factor analysis of variance by ranks was employed to test for homogeneity of male C. I. means among female types. If the null hypothesis was rejected by the Kruskal-Wallis test, it was followed by the Nemenyi test of multiple comparisons (Zar, 1984) to detect which means significantly differed. The identical test procedures were employed to test for significant differences in behavioural frequency means among female types.

iii) Social index (S.I.) and behavioural frequency

Male behavioural data collected from trials in which males were exposed to other males (S. I.) could not be compared statistically to male behavioural data collected from female trials (C. I.). However, non-statistical comparisons were made to highlight the differences in duration and frequency of social behaviours compared to courtship behaviours.
Chapter II – Results

I. Time Until Copulation and Copulation Duration:

Between March 1997 and January 1998, 30 trials that ended in copulation were observed for *H. charithonia*. The mean time until copulation (± S.E.) for control and experimental females was 5.66 (± 1.22) and 6.98 (± 1.11) minutes, respectively (Figure 2.1). The results from the Mann-Whitney U Test for equal means for the time until copulation began were not significant for *H. charithonia* (U = 346.5; U' = 493.5; N_{Control} = 28; N_{Expt} = 30; p = 0.2502; α = 0.05).

During the same period, the mean duration of each copulation was observed for *H. charithonia*. The mean duration of copulation (± S.E.) for the control and experimental females was 54.25 (± 4.26) and 46.97 (± 2.28) minutes respectively (Figure 2.1). Similar to the test for time until copulation, the results from the Mann-Whitney U Test of equal means for the duration of copulation were not significant for *H. charithonia* (U = 333; U' = 507; N_{Control} = 28; N_{Expt} = 30; p = 0.1753; α = 0.05).

Between October 1997 and January 1998, 13 trials that ended in copulation were observed for *H. erato*. Similar to *H. charithonia*, the mean time until copulation and mean copulation duration were calculated. For *H. erato*, the mean time until copulation (± S.E.) was longer than those recorded for *H. charithonia* (*H. erato*; control = 27.23 (± 7.17) and experimental = 18.08 (± 6.48) minutes) (Figure 2.2). However, the results from the Mann-Whitney U Test for equal means found no significant difference in the mean times for copulation to occur between the control and experimental females (U = 51.5; U' = 91.5; N_{Control} = 11; N_{Expt} = 13; p = 0.2459; α = 0.05).

The means times for the duration of copulation (± S.E.) for *H. erato* were also longer than those recorded for *H. charithonia* (*H. erato*; control = 69.73 (± 3.89) and experimental = 71.85 (± 14.04) minutes) (Figure 2.2). However, similar to the time until copulation, no significant difference was found by the Mann-Whitney U test for equal copulation duration means between the control females and experimental females (U = 60; U' = 83; N_{Control} = 11; N_{Expt} = 13; p = 0.5045; α = 0.05).

II. Virgin Female Trials:

The third and last female that all males in the experiment were exposed to was a virgin. This was necessary because males had to remain virgin themselves to participate in the experiment. If a
Figure 2.1 Graph of copulation duration and time until copulation for *H. charithonia* females with (control) and without (experimental) stink clubs. Standard error bars are included. Numbers that appear inside () are number of replicates (n). P values from Mann-Whitney U tests are presented. The capital letters denote the pairwise statistical results at the 95% significance level.
Figure 2.2 Graph of copulation duration and time until copulation for *H. erato* females with (control) and without (experimental) stink clubs. Standard error bars are included. Numbers that appear inside () are number of replicates (n). P values from Mann-Whitney U tests are presented. The capital letters denote the pairwise statistical results at the 95% significance level.
male mated before he was exposed to both the control and experimental females, his further use in trials would have produced confounding results.

For both species, males were exposed to virgins and permitted to mate in 10 trials. Not all of these trials ended in copulation (H. erato: no copulation, 60%; H. charithonia: no copulation, 30%).

Once copulation began, the collection of male behavioural data used in the calculation of courtship indices stopped. This created unequal trial lengths (due to copulation) that could potentially have biased the calculation of courtship indices. Therefore, a two-tailed Student’s t-test was performed to test for equal courtship index means between the mated and unmated virgin females. The comparison was not significant for both H. erato (unpaired t_{0.05, 2, 8} = 1.892; p = 0.0952) and H. charithonia (unpaired t_{0.05, 2, 8} = 0.741; p = 0.4790). These results indicated that trials that were shortened by mating, were not biased toward higher courtship indices (Scott and Jackson, 1990). Thus, male C. I. values for all virgin trials could be used in further analyses without the risk of biasing results.

III. Courtship Index:

As can be seen in Figures 2.3 and 2.4, there was a similar trend for both species. The virgin female stimulus produced the highest mean courtship index values, and the male stimulus produced the lowest (See table 2.1 for exact values). A non-parametric Kruskal-Wallis test was employed to test for any significant differences among the C. I. means of the female stimulus types. The null hypothesis of equal C. I. means was not rejected for H. charithonia (H = 1.435; d.f. = 2; p_{0.05, 10} = 0.4879). However, for H. erato the results of the test were significant and the null hypothesis of equal means was rejected (H = 10.851; d.f. = 2; p_{0.05, 10} = 0.0044).

The Kruskal-Wallis test was not able to detect which of the C. I. means significantly differed; therefore a Nemenyi test for multiple comparisons was performed. Results were significant for the virgin versus control female comparison (q = 4.598; q_{0.05, 8, 3} = 3.314) (See Table 2.1).

IV. Behavioural Frequency:

A Kruskal-Wallis test was employed to test the null hypothesis of equal means for male courtship behaviour among the female types. No significant differences were detected in mean number of male courting behaviours for H. charithonia (Table 2.2).
Figure 2.3 Graph of the mean courtship index values for *H. charithonia* males elicited from different stimuli. Sample sizes for all stimulus types were n = 10. P-values were from Kruskal-Wallis tests for homogeneity of means. Capital letters denote pairwise statistical comparisons at the 95% significance level. The male stimulus type was not included in any statistical tests.
Figure 2.4 Graph of the mean courtship index values for *H. erato* males elicited from different stimuli. Sample sizes for all stimulus types were $n = 10$. P-values were from Kruskal-Wallis tests for homogeneity of means. Capital letters denote pairwise statistical comparisons at the 95% significance level. The male stimulus type was not included in any statistical tests.
Table 2.1 Courtship index means (± S. E.) of *H. erato* and *H. charithonia* males elicited from control, experimental and virgin females, and results of Nemenyi tests for multiple comparisons.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stimulus Type</th>
<th>No. of Trials (n)</th>
<th>Mean Courtship Index (± S. E.)</th>
<th>Significance (0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. charithonia</em></td>
<td>Control</td>
<td>10</td>
<td>12.02 ± 4.07</td>
<td>a</td>
</tr>
<tr>
<td><em>H. charithonia</em></td>
<td>Experimental</td>
<td>10</td>
<td>12.42 ± 3.63</td>
<td>a</td>
</tr>
<tr>
<td><em>H. charithonia</em></td>
<td>Virgin</td>
<td>10</td>
<td>20.56 ± 9.04</td>
<td>a</td>
</tr>
<tr>
<td><em>H. charithonia</em></td>
<td>Male</td>
<td>10</td>
<td>2.07 ± 0.49</td>
<td>see discussion</td>
</tr>
<tr>
<td><em>H. erato</em></td>
<td>Control</td>
<td>10</td>
<td>6.14 ± 2.74</td>
<td>a</td>
</tr>
<tr>
<td><em>H. erato</em></td>
<td>Experimental</td>
<td>10</td>
<td>7.39 ± 2.07</td>
<td>ab</td>
</tr>
<tr>
<td><em>H. erato</em></td>
<td>Virgin</td>
<td>10</td>
<td>29.65 ± 9.95</td>
<td>b</td>
</tr>
<tr>
<td><em>H. erato</em></td>
<td>Male</td>
<td>10</td>
<td>1.28 ± 0.59</td>
<td>see discussion</td>
</tr>
</tbody>
</table>

The letter designations (a, ab, b) represent the results of Nemenyi test comparisons performed between female types. The cells that contain different single letters, represent statistically significant comparisons.
Table 2.2 Mean standardized behavioural frequency per stimulus type, results of Kruskal-Wallis test for unequal means and results from the Nemenyi tests for multiple comparisons for *H. charithonia*.

<table>
<thead>
<tr>
<th>Standardized Behaviour</th>
<th>Mean Frequency per Stimulus Type (± S. E.)</th>
<th>P-Value</th>
<th>Significant Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control [a]</td>
<td>Exper. [b]</td>
<td>Virgin [c]</td>
</tr>
<tr>
<td>Approaches / female rest minute</td>
<td>0.90 (0.26)</td>
<td>1.01 (0.44)</td>
<td>1.26 (0.45)</td>
</tr>
<tr>
<td>Approach fans / female rest minute</td>
<td>1.26 (0.43)</td>
<td>1.01 (0.47)</td>
<td>2.03 (0.94)</td>
</tr>
<tr>
<td>Approach contacts / female rest minute</td>
<td>1.11 (0.40)</td>
<td>0.77 (0.30)</td>
<td>1.99 (0.96)</td>
</tr>
<tr>
<td>Copulation attempts / female rest minute</td>
<td>0.15 (0.08)</td>
<td>0.02 (0.01)</td>
<td>0.62 (0.49)</td>
</tr>
<tr>
<td>Chases / female flight minute</td>
<td>1.98 (0.50)</td>
<td>2.04 (0.52)</td>
<td>2.27 (0.57)</td>
</tr>
<tr>
<td>Chase fans / female flight minute</td>
<td>3.59 (1.39)</td>
<td>2.57 (0.87)</td>
<td>3.02 (1.22)</td>
</tr>
<tr>
<td>Chase contacts / female flight minute</td>
<td>5.45 (2.12)</td>
<td>4.06 (1.31)</td>
<td>4.15 (1.32)</td>
</tr>
</tbody>
</table>

Abbreviations: NS refers to the results of the Kruskal-Wallis test at the 0.05 significance level being not significant; letters in [ ] below the female type denotes the pairwise comparison designation used in the Nemenyi tests; S. E. values are given in parentheses ( ) below the behavioural means. The number of trials (n) for all categories was 10.
However, for *H. erato* the null hypothesis of equal behavioural means was rejected for two male courting behaviours (Figure 2.5). These behaviours were: (1) male approaches per female rest minute (H = 7.416; d.f. = 2; p 0.05, 10 = 0.025) and (2) male approach fans per female rest minute (H = 7.440; d.f. = 2; p 0.05, 10 = 0.024) (Table 2.3). Both were stage II courtship behaviours.

The Kruskal-Wallis test indicated that for *H. erato*, mean male copulations attempts (p=0.1065) and male chase contacts (p = 0.0862) were nearly significant (Table 2.3). Although there were no significant differences detected for male behavioural means in *H. charithonia*, the mean number of copulation attempts (p=0.0513) proved very nearly significant (Table 2.2). The trend of near significance for male copulation attempts was particularly interesting as this behaviour is associated with the advanced stages of courtship (stage III). As such, copulation attempts prove to be an excellent indicator of male willingness to finish the courtship sequence and attempt to copulate with a specific female.

One of the key predictions from the antiaphrodisiac hypothesis was that the control females would elicit the lowest frequency of male courting behaviours, the experimental females a medium frequency, and the virgin the highest frequency. For *H. erato*, mean male copulation attempts (Figure 2.6) conformed to this prediction. Unexpectedly, *H. charithonia* control females elicited a higher frequency of copulation attempts than the experimental female (Figure 2.6). Although not statistically significant, this trend did not support the prediction that the control female would have elicited the lowest frequency of behaviours from males. Indeed, the fact that males of both species were observed attempting to copulate with control females indicated that the postmating odour did not prevent males from reaching the end of the courtship sequence. Moreover, for *H. charithonia*, the postmating odour appeared to be responsible for the increased number of copulation attempts elicited by the control female, compared to the experimental female.

Another trend emerged that did not follow predictions made from the antiaphrodisiac hypothesis. The observed trend involved the grouping of the male behavioural means elicited from the experimental females. It had been predicted that because these females did not have the postmating odour, they should elicit degrees of male courtship similar to that elicited from virgin females. It turned out that the experimental females elicited degrees of male courtship behaviour most similar to the control females. In *H. charithonia*, this pattern was repeated in the behavioural means of the following: male approaches, approach fans, approach contacts, and copulation attempts. In *H. erato*, it was repeated for all male behavioural means except approach contacts per female rest minute. Thus,
Figure 2.5 Graph of the mean number of male approaches and approach fans per female rest minute from *H. erato* males elicited from different stimuli. Standard error bars are included. Samples sizes for all stimulus types were n=10. P-values are from Kruskal-Wallis tests for homogeneity of means. Capital letters denote pairwise statistical comparisons at the 95% significance level. The male stimulus type was not included in any statistical test.
Figure 2.6 Graph of the mean number of male copulation attempts per female rest minute for *H. erato* and *H. charithonia* males elicited from different stimuli. Standard error bars are included. Samples sizes for all stimulus types were n = 10. P-values are from Kruskal-Wallis tests for homogeneity of means. Capital letters denote pairwise statistical comparisons at the 95% significance level. The male stimulus type was not included in any statistical tests.
Table 2.3 Mean standardized behavioural frequency per stimulus type, results of Kruskal-Wallis test for unequal means and results from the Nemenyi tests for multiple comparisons for *H. erato*.

<table>
<thead>
<tr>
<th>Standardized Behaviour</th>
<th>Mean Frequency per Stimulus Type (± S. E.)</th>
<th>P-Value</th>
<th>Significant Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (a)</td>
<td>Exper. (b)</td>
<td>Virgin (c)</td>
</tr>
<tr>
<td>Approaches / female rest minute</td>
<td>0.57 (0.19)</td>
<td>0.65 (0.13)</td>
<td>1.50 (0.38)</td>
</tr>
<tr>
<td>Approach fans / female rest minute</td>
<td>0.78 (0.29)</td>
<td>1.01 (0.48)</td>
<td>2.59 (1.17)</td>
</tr>
<tr>
<td>Approach contacts / female rest minute</td>
<td>0.64 (0.36)</td>
<td>1.86 (1.46)</td>
<td>1.28 (0.37)</td>
</tr>
<tr>
<td>Copulation attempts / female rest minute</td>
<td>0.03 (0.03)</td>
<td>0.09 (0.07)</td>
<td>0.85 (0.50)</td>
</tr>
<tr>
<td>Chases / female flight minute</td>
<td>2.64 (0.79)</td>
<td>1.61 (0.22)</td>
<td>3.34 (0.81)</td>
</tr>
<tr>
<td>Chase fans / female flight minute</td>
<td>1.27 (0.86)</td>
<td>0.94 (0.34)</td>
<td>1.67 (0.62)</td>
</tr>
<tr>
<td>Chase contacts / female flight minute</td>
<td>1.99 (0.96)</td>
<td>3.31 (1.41)</td>
<td>5.78 (1.75)</td>
</tr>
</tbody>
</table>

Abbreviations: NS refers to the results of the Kruskal-Wallis test at the 0.05 significance level being not significant; letters in [ ] below the female type denotes the pairwise comparison designation used in the Nemenyi tests; S. E. values are given in parentheses ( ) below the behavioural means. The number of trials (n) for all categories was 10.
although not statistically significant, there appeared a repeating trend of males behaving similarly towards control and experimental females, despite the presence/absence of the postmating odour.

V. Social Index (S. I.):

The mean S. I.’s calculated for *H. charithonia* and *H. erato* males (± S. E.) were 2.07 (± 0.49) and 1.28 (± 0.59) respectively. Statistical comparison of these values with C. I. values was not possible due to the different samples from which they were drawn. However, for both species, the mean S. I. value was lower than all mean C. I. values (Figure 2.3 and 2.4), indicating there was a trend for the duration of male behaviours elicited from females to be longer compared to those elicited from males. Even females with the postmating odour elicited a longer duration of male behaviour than other males.

In both species, the male behavioural means of all four social behaviours elicited from other males were lower than all the corresponding behavioural means elicited from females (Table 2.2 and Table 2.3). Thus, there appeared to be a trend for females to elicit a higher frequency of behaviours from males. The pattern of increasing duration and frequency of male behaviours elicited from females compared to other males, indicated that the increase was motivated by a sexual, rather than a social attraction.

An informal comparison between species indicated that *H. charithonia* males more frequently initiated approaches and approach contacts than *H. erato* (Table 2.2 and 2.3). However, for male social chases and chase contacts, both species were nearly equal in the mean number of behaviours elicited from other males.
Chapter II - Discussion

Lepidopteran mating behaviour exhibits qualities described by Trivers (1972) for species' with a disparity in parental investment. Males invest only a spermatophore and associated accessory fluids in future offspring and are the active sex in courtship initiation. In contrast, females must allocate energy to metabolic processes involved in egg maintenance and sperm storage, as well as energy costs associated with locating suitable oviposition sites (Rutowski, 1984).

For butterfly species in which the average lifetime female mating frequency is one, these behavioural differences have lead to gender-specific sexual selection pressures and create a conflict between the sexes (Blum and Blum, 1979). For males, selection acts to confer competitive advantage upon adaptations that would enhance a male's ability to locate and copulate with females. For females however, selection acts to evolve strategies or traits that communicate species identity, and any specific rejection strategies developed to repulse a persistent male (Scott, 1973), thereby allowing her the largest time period for oviposition and feeding (Wiklund, 1977).

Males are predominately the active participant in courtship, while females generally adopt an inactive role. A female has been observed to take an active role in courtship only when she remains unmated for many days (Crane, 1955; Wiklund, 1982) or when sperm from previous matings has been depleted (Rutowski, 1980; Rutowski et al., 1981). Therefore, the only types of interactions that result in copulation are between receptive males and receptive or slightly unreceptive females. This places selective pressure on the male to develop sexual characteristics to make the unreceptive female receptive.

In experiments investigating the effect of an antiaphrodisiac on Drosophila melanogaster males, Scott and Jackson (1990) state that in nature, antiaphrodisiacs and female movement may both decrease the probability of a mated female being courted. The normal response of Drosophila virgin females to unwanted courtship is to "flee or decamp" (Spieth, 1974; Ewing and Ewing, 1986). For mated females, however, this response might involve leaving a valuable feeding and oviposition site (Spieth, 1974). An alternative would be to decrease the chance of a male initiating courtship or to increase the chance of a male shifting courtship to another female (Scott and Jackson, 1990).

In butterflies, females have evolved behaviours to reduce and/or avoid unwanted male courtship. Evasive flight patterns or refusal flights have been described for Colias spp. (Pieridae), Pieris protodice (Pieridae), and for Papilio polyxenes asterius (Papilionidae), (Rutowski, 1978a; 1979;
and Lederhouse, 1981). Flutter responses (female rapidly flapping her wings) have been recorded for *Euphydryas editha* (Nymphalidae), and *Eurema lisa* (Pieridae) (Labine, 1966; Rutowski, 1978b). Female lepidopteran rejection postures have been documented to be induced by pheromonal (Lundgren and Bergström, 1975), physiological (Sugawara, 1979) and hormonal (Obara, 1982) cues.

*H. erato* females also possess behavioural mechanisms evolved to thwart unwanted male courtship. These behaviours may include wing flattening and/or abdomen raising, which denies the male access to the female’s genitalia, not remaining landed during Stage III of courtship, (Crane, 1957), and evasive flight patterns, evolved to inform the male of the female’s unreceptivity or to prevent the male from keeping her in sight (Scott, 1973). *H. charithonia* females displayed similar rejection behaviours as *H. erato* females (pers. observ.).

A male should persist in courtship until copulation, or until a critical decision threshold is reached. This threshold marks the point where energetic costs outweigh possible reproductive benefits. Once this threshold is surpassed, the male should discontinue courtship. From the results of this experiment, I suggest that the male’s decision to discontinue displaying courtship behaviours was minimally affected by the female postmating odour. Instead, males were affected by females adopting ground-based rejection postures.

Results of the courtship index (C.I.) experiments were different for the two species. However, in neither case did the results support an “all or nothing” antiaphrodisiac function for the postmating odour. Males of both species courted females with the postmating odour (control females). Thus, the prediction of an “all or nothing” function of the postmating odour was not supported.

For *H. charithonia*, the prediction of unequal courtship indices between females with and without the postmating odour was not supported by test results. There was no statistical difference detected between the mean C.I.’s of the three female types (Table 2.1). However, for *H. erato* there was a significant difference between the mean C.I.’s elicited by control females compared to virgins (Table 2.1). Thus, the mated females with stink clubs elicited significantly shorter bouts of courtship behaviours from males than did their virgin conspecifics.

One interpretation of this was that the postmating odour was functioning as an antiaphrodisiac, but there was not an absolute effect on male courtship. Experimental *H. erato* females (without stink clubs) did not significantly differ from virgins in the duration of courtship behaviours elicited from males, suggesting that if a postmating odour was transferred and disseminated by the stink clubs, removing them removed the antiaphrodisiac. Therefore, it was the presence of the stink clubs and the
concomitant presence of the postmating odour that was responsible for the reduction in the mean C.I. value that resulted in the significant difference between control and virgin *H. erato* females.

However, the mean C.I. value elicited by *H. erato* experimental females grouped more closely with the control females rather than virgins (Figure 2.4). Thus, there was a trend for both mated female types to elicit reduced C.I.’s compared to virgins. The presence or absence of the stink clubs and concomitant odour did not play a role in significantly reducing courtship duration, as the nearly equal mean C.I.’s of the *H. erato* control and experimental females indicated. Although no significant difference was detected in *H. charithonia*, the same trend of mated female mean C.I. values grouping together was observed (Figure 2.3).

Based on the these results, I suggest that rather than the postmating odour being responsible for the reduced C.I. of both mated female types, it was instead the mating history of the female and her behavioural reactions (adopting rejection postures) to the courting male that were responsible for the reduction. These rejection postures and behaviours produced the significant reduction in C.I.’s (control versus virgin, *H. erato*) and were responsible for the similar grouping of mated female mean C.I. values, for both species. Additionally I suggest that if there were a larger sample size, the standard error (± 9.04) of the virgin stimulus category for *H. charithonia*, would be reduced, and thus would have produced a significant difference between the mated females and the virgins.

Although not testable statistically, both species’ mean Social Index values (S.I.) were lower than all three conspecific mean C.I. values. This indicated that there was a trend for males to spend more time exhibiting courting behaviours towards females than they did exhibiting social behaviours towards males (Figures 2.3 and 2.4). If, as in *Drosophila melanogaster* (Scott and Jackson, 1990), the postmating odour was providing sexually ambiguous pheromonal cues to the male, we would have expected to see a pattern of only the control female’s mean C.I. value grouped with the male’s mean S.I. value. The control female was the only female type with the postmating odour capable of producing the ambiguous cues. However, for both species, this was not observed (Table 2.1).

The difference between the mean male S.I. value and both mated females’ mean C.I. values were very similar compared to the difference between the mean male S.I. value and the virgin C.I. value. For *H. erato*, the difference between the mean S.I. and the mean C.I. of the control, experimental and virgin females were: 4.86, 6.11 and 28.37 respectively. For *H. charithonia* the difference between the mean S.I. and the mean C.I. of the control, experimental and virgin females were: 9.94, 10.34 and 18.49 respectively. Additionally, no significant difference was detected between
the C.I.'s of control and experimental females in any test. This evidence does not support the hypothesis that the postmating odour was responsible for reducing the male’s courtship duration by rendering the female male-like in odour.

Similar to the results of the C.I. analyses, the results of the single behaviour analyses yielded different results for *H. erato* and *H. charithonia* (Tables 2.2 and 2.3). Contrary to predictions made from the antiaphrodisiac hypothesis, for *H. charithonia* the null hypothesis of equal means for courtship behaviours was not rejected. The three female types did not elicit any significantly different frequencies of male courtship behaviours.

For *H. erato* the null hypothesis of equal means for the frequency of each male courtship behaviour was rejected for two behaviours: (1) the mean frequency of male approaches and (2) the mean frequency of male approach fans. Both of these behaviours proved significantly different for the control versus virgin female comparison. Similar to the C.I. analyses, the control female significantly differed from the virgin, but the experimental female grouped more closely to the control rather than the virgin female (Figure 2.5). According to predictions made from the antiaphrodisiac theory, experimental and virgin females (neither having the postmating odour) were predicted to elicit similar frequencies for all male behaviours, and these frequencies should have been significantly higher than those elicited by the control female. This prediction was not supported by the results.

What caused the mean frequency of male courtship behaviours elicited by both mated female types to group together for the two significantly different behaviours? One explanation would be that the removal of the stink clubs prior to mating did not prevent the transfer or dissemination of the postmating odour. Thus, the observed grouping of control and experimental females was due to the presence of the postmating odour. This, however, does not coincide with the observation that the postmating odour was not detectable by the observer in *H. erato* experimental females (pers. observ). If the postmating odour was acting as an antiaphrodisiac, and was prevented from being transferred to the female by removing her stink clubs, then the experimental females should have grouped more closely with the virgins. However, they grouped more closely with the control females. Therefore, I suggest that the postmating odour had no effect on the frequency of male courtship behaviours.

Similar to the conclusions reached from the C.I. experiments, I conclude that it was the female behavioural reactions that produced the significant differences in control versus virgin comparisons, and the observed grouping of the mean behavioural frequencies elicited by the control and experimental females. Both of the significant behaviours were Stage II courtship behaviours (male
aerial, female on ground). At this stage the female can perform only the ground-based rejection postures of wing flattening and abdomen raising, whereas in Stage I of courtship, the performance of only aerial rejection behaviours are possible. Stage I behaviours (evasive flight patterns) are primarily attempts by the female to break from the male’s visual contact. In this experimental setting, these behaviours proved ineffective, as visual contact was easier for the male to maintain in the reduced flying space of the research cage.

In additional support of this trend was a behaviour that although not significantly different for either species, was very close to statistical significance. This nearly significant behaviour was the frequency of copulation attempts, a Stage III courtship behaviour (male and female on ground). Again, in both species, there was the general trend for the mated females to group together compared to the virgins. As a close range behaviour, and the final behaviour of the courtship sequence, copulation attempts are arguably the single behaviour most indicative of a male’s willingness to mate with the female. This was relevant for two reasons.

First, its near significance (H. erato, p = 0.1065 and H. charithonia, p = 0.0513) indicated that there seemed to be a trend for males to reduce the number of copulation attempts with both mated female types. This was evidenced by the control and experimental females grouping together for the mean number of copulation attempts compared to the virgin (Figure 2.6). Therefore, the presence of the postmating odour, in the control females, had negligible effect on the frequency of copulation attempts elicited from conspecific males.

Second, the very fact that the male is attempting to copulate with mated females reflects a high male persistence, and his unwillingness to respond to any signals given off by the unreceptive female up to this point. This is in concordance with the assertion that in this environmental setting, only the ground-based rejection postures were effective in communicating a female’s unreceptivity.

This observation also fits the described selection pressures acting upon male behaviour toward unreceptive females. It has been observed that mildly unreceptive lepidopteran females may be convinced to copulate after prolonged courtship by the male (Pieris protodice, Abbott, 1959 in Scott, 1973; Danaus gilippus, Brower et al., 1965; and Colias spp., Rutowski, 1978a). Therefore, to counterbalance the strong sexual selection acting upon males to be persistent in courtship, females must be equally persistent in exhibiting visual or behavioural cues to advertise their unreceptiveness.
Chapter II - Conclusions

From results of these experiments, I conclude that the postmating odour of *H. erato* and *H. charithonia* was neither acting to repel courting males from mated females, nor providing sexually ambiguous olfactory cues to the male. Instead, any observed reduction in courtship duration or frequency of courtship behaviours was due to the female’s adopting ground-based rejection postures.

Similar trends within and between species were seen for all tests. For *H. charithonia* there were no significant differences detected in either duration of courtship behaviours (C.I.) or in the mean frequency of male courtship behaviours between female types. The pattern seen in *H. erato* for mated females to group together compared to the virgin females was also seen in *H. charithonia*. Although I suggest a similar effect in both species, it appeared from the significance of the *H. erato* trends, that *H. erato* males were more influenced by a female’s adopting rejection postures than *H. charithonia* males.

These conclusions coincide with sexual selection theory. The opposing forces of sexual selection on males and females select for male behaviours that increase the male’s chances of copulating with females. Therefore, males persistent in courtship displays are afforded a selective advantage over non-persistent males. In opposition to the selective forces that act upon the male, the forces of selection favour female traits that advertise a females unreceptivity to males. Therefore, females that display rejection behaviours are afforded a selective advantage over females that do not display these behaviours.
Epilogue

I. A summary and future experiments from Chapter 1:

The results from both chapters yielded results that were not supportive of the antiaphrodisiac hypothesis as the function of the female postmating odour. In Chapter 1, results indicated that at the pupal mating level, female type had no significant effect on males leaving female pupae. Males were equally likely to leave female pupae when exposed to a disturbance from any female type or tactile stimuli. Conclusions from these experiments led to the generation of the minimum disturbance threshold hypothesis.

To compliment and verify the findings in Chapter 1, future experiments could be conducted to continue investigating the role of the postmating female odour and pupal mating. The experiments could be conducted identically to those outlined in Chapter 1, but would involve behavioural comparisons of the number of males that dispersed from female pupae in the presence of alternative stimuli. I suggest one such stimulus to be the use of conspecific males as a source of disturbance. This would be a way to further verify the minimum disturbance threshold hypothesis. The prediction that a male caused source of disturbance (rather than female) would result in patterns of pupal-perched male leaving, similar to those produced by all the female types in Chapter 1.

Given the unexpected outcome of the experiments in Chapter 1, the pupal gender identification hypothesis had to be addressed post hoc. The post hoc data analysis presented in Chapter 1 did not support the pupal gender identification hypothesis. However, for a strengthened argument I believe that future experiments specifically designed to test this hypothesis should be performed.

One means of identifying whether it is the male or female pupae that give off a chemical gender signal, would be to alter their scents. One such experiment could involve artificially covering female pupae with the scent from the male valvulae. It would be predicted that if male pupae are identified by scent (the chemical or precursor to the female postmating odour), then covering a female pupa (presumably odourless) with the scent from a male’s valvulae should confer ‘maleness’ to the female pupa and thus prevent males from landing.

II. A summary and future experiments from Chapter 2:

In Chapter 2, the effect of the postmating odour at the adult stage was examined. Similar to findings in Chapter 1, results of this Chapter did not support the antiaphrodisiac hypothesis as the
function of the female’s postmating odour. Results indicated that males would court virgins more than mated females. However, mated females with and without the postmating odour were courted equally. Thus, the postmating odour did not produce any significant difference in male courtship behaviour. It was concluded that the observed difference in frequency and duration of male courtship behaviour between the mated and virgin females was caused by the mated females adopting ground-based rejection postures and behaviours.

A test designed to further substantiate or refute these conclusions, an experiment quantifying male courtship behaviour elicited from a virgin female who had been artificially covered in postmating odour could be performed. The use of a virgin would eliminate the rejection postures and behaviours that are adopted by mated females, and thus more clearly show a male’s reaction to the postmating odour.

Although future research may provide support for the antiaphrodisiac hypothesis, based on the results from both sets of experiments, and my personal observations, I conclude that the female postmating odour of *H. erato* and *H. charithonia* was not functioning according to predictions made from the antiaphrodisiac hypothesis. Having said that begs a response to the question: what do I believe is the function of the female postmating odour?

In the literature I found a good number of references to the postmating odour (see literature review), but relatively few hypotheses predicting its function. The few hypotheses that did attempt to predict its function, dealt with how a predator or conspecific male would react to this pheromone, and none examined or hypothesized how another female would have reacted to the pheromone. Based on observations throughout these experiments, I would like to now present a novel hypothesis regarding the function of the female postmating odour.

I propose that the female postmating odour functions as an oviposition-deterrent pheromone. From this hypothesis, it would be predicted that during oviposition, along with an egg, a female would also deposit a small amount of postmating odour onto the leaves, via the stink clubs, of a suitable hostplant. This would provide her a means of quantitatively assessing the approximate number of eggs she has laid on the hostplant via pheromone concentration. Once the female detected a critical concentration of the pheromone, this would in turn signal that the optimum egg density had been reached. If a female were to surpass this optimum density, she would risk decreasing her fitness, as there would be insufficient (or suboptimal) plant material to feed all the emerging larvae. The larvae would face either starvation or malnutrition, or the risk of migration in an attempt to locate another
food source. Higher mortality would result from either situation, and therefore would not be selectively advantageous.

Other oviposition-deterring pheromones have been reported in: (1) *Pieris brassicae* (Lepidoptera: Pieridae) (Behan, *et al.*, 1978), (2) *Agromyza frontella* (Diptera: Agromyzidae) (McNeil and Quiring, 1983) and (3) *Ostrinia nubilalis* (Lepidoptera, Pyralidae) (Thiéry and Le Quéré., 1991). These pheromones have been shown to decrease the availability of hostplant material to conspecifics, thereby helping secure sufficient resources for resident larvae, by reducing intraspecific competition (Prokopy, 1981).

Individual *H. erato* females are well known to have a strong postmating odour compared to other heliconiine species. They are also known to have cannibalistic larvae. Thus, a stronger postmating odour would result in the critical egg laying threshold being reached sooner, and thus fewer eggs laid per plant. This would prove selectively advantageous, as it would reduce the risk of conspecific cannibalism thereby increasing the female’s fitness.

Detection of another female’s postmating odour on a potential hostplant would indicate that there are already older larvae on this plant. The presence of older larvae would constitute a twofold threat to the fitness of an ovipositing female. First, they could potentially consume the newly laid eggs or young larvae on the plant (for cannibalistic species). Second, their presence indicates a lower availability of food for the new larvae (both gregarious and cannibalistic species).

Gilbert (1976) stated that the evolutionary derivation of the antiaphrodisiac system could be understood if there was benefit to both sexes. There needed to be a benefit to both sexes in order for them to evolutionarily cooperate and evolve the structures associated with the production (male), transfer and dissemination of the pheromone (female). The benefits (predicted from the antiaphrodisiac hypothesis) proved not to be supported by the data in these experiments. However, the same evolutionary reasoning could be applied to predictions from the oviposition density hypothesis.

Both sexes stand to increase their fitness by evolutionarily cooperating with this scenario. The male, by expending his energy in producing and donating the pheromone, or its precursor, to the female, would be protecting his genetic investment by ensuring maximum larval survival. The female would cooperate evolutionarily by evolving structures associated with the transfer and dissemination of the postmating odour, as she too would be maximizing her reproductive effort via enhanced larval survivorship.
To test this hypothesis further, a simple experiment could be performed. The experiment would be designed to quantitatively assay the egg laying of females on hostplants with and without the postmating odour. Leaves of a hostplant, without eggs, could be dabbed with the abdomen of a mated female, and with the abdomen of a virgin. An assay that recorded the number of eggs laid per plant could be performed by releasing a mated female into the cage with both plants. Counting the eggs laid per plant would be a way of quantitatively testing that the prediction the postmating female odour functioned as an oviposition density control mechanism.
Bibliography


null


Brower, A. V. Z., 1997. The evolution of ecologically important characters in *Heliconius* butterflies


Press.


is brought into the males. Numbers that appear in () in the two male trials are total number of males present during trials.

Note: Abbreviations: S.C. refers to males without string clubs; NS refers to males with string clubs. No C. refers to males without string clubs. S refers to males without string clubs.

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<th>Abdominal Time</th>
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<th>Dispersal (2 males)</th>
<th>Expected (1 male)</th>
<th>Dispersal (1 male)</th>
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<th>Expected (n)</th>
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Table A.1. The number of H. erato males that dispersed from the female pupa in the presence of female stimuli. Table includes results from trials with one and two males present.
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Table 4.2. The number of *H. chamissonia* males that dispersed from the female pupa in the presence of female stimuli. Includes results from trials with one and two males present. Abbreviations are defined in Table A.1.
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From trials with one and two males present.

Table A.3. The number of males that dispersed from female pupae while in the presence of female stimul. Table includes results.