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The ecology of female receptivity in the territorial butterfly *Hypolimnas bolina* (L.) (Nymphalidae): implications for mate location by males

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Abstract

Many male insects aggressively defend specific perching sites containing larval resources. There are three main explanations for how this behaviour could contribute to increased matings: perching males may aim to encounter (1) eclosing or freshly eclosed virgin females, (2) previously mated, ovipositing females, or (3) receptive females that visit these sites either specifically to mate or for other reasons. I evaluated these hypotheses by investigating the timing of post-eclosion female receptivity and the extent of polyandry within an Australian population of the butterfly *Hypolimnas bolina* (L.) (Lepidoptera: Nymphalidae). This species represents the group of butterflies in which males defend specific, geographically prominent, sites that overlap with the distribution of larval resources. Freshly emerged female *H. bolina* refrained from mating until their ovaries were close to maturation, resulting in a pre-mating period of 4–8 days. The presence of this substantial refractory period rules out the hypothesis that males defend pupation sites with the aim of mating with eclosing or freshly eclosed females. Secondly, almost 90% of females within the studied population carried only one spermatophore, a finding that mediates against the possibility that most perching males target (already mated) ovipositing females. The ‘rendezvous-site’ hypothesis is the most likely general explanation for territoriality in *H. bolina*; however, it remains unclear whether the distribution of larval hostplants per se has a primary influence on territory selection by males in this species.

Introduction

Many butterfly species exhibit conspicuous ‘sit-and-wait’ or ‘ambush’-style mate-searching tactics, whereby males perch at specific sites and fly out to investigate passing objects (reviewed by Rutowski 1991). This behaviour is often accompanied by aggression (Dennis and Shreeve 1988; Rutowski 1991), such that perching individuals do not tolerate conspecific males within their immediate vicinity (e.g. Wickman and Wiklund 1983; Rosenberg and Enquist 1991). Perching individuals may exhibit strong fidelity to specific locations, sometimes remaining in residence for several weeks (e.g. Rutowski 1992). Although the use of the term ‘territoriality’ as a descriptor of butterfly behaviour has been challenged (e.g. Scott 1974), it is now accepted that some males do attempt to expel conspecific rivals from specific areas, and this behaviour, coupled with site fidelity, fits the definition of this term (Baker 1972; Wickman 1985; Alcock 1988; Dennis and Shreeve 1988; Lederhouse et al. 1992).

There is a strong consensus among invertebrate ecologists that territorial perching behaviour of male insects is a mate-locating tactic (Thornhill and Alcock 1983). Several studies demonstrate the adaptive benefit of territoriality in butterflies (Shields 1967; Lederhouse 1982; Wickman and Wiklund 1983; Wickman 1985, 1988). However, although this behaviour is believed to lead to matings, the source of receptive females is almost always unknown (e.g. Rutowski 1992). This is due to the general dearth of quantitative information regarding aspects of female receptivity, including the timing of initial receptivity, lifetime remating frequencies and refractory periods (cf. Braby 1996), and the behaviour of receptive individuals (Rutowski 1991). This makes it difficult to fully understand an individual species’ mating system, and inhibits a complete appraisal of the ultimate influences upon mate-locating behaviour in insects (Rutowski et al. 1996).
The males of several territorial butterflies routinely defend places that either contain, or are immediately adjacent to, their larval hostplant (see Baker 1972; Rutowski and Gilchrist 1988; Rutowski et al. 1988; Rosenberg and Enquist 1991; Rutowski 1991; Lederhouse et al. 1992). There are three main explanations for how this behaviour could lead to increased encounters with females. First, individuals may defend sites where females are likely to be eclosing and taking their first flights (Rutowski and Gilchrist 1988; Rutowski et al. 1988). This ‘emerging female’ hypothesis predicts that females of these species will be receptive immediately upon eclosion, as with some species that practice pupal mating (e.g. Gilbert 1991). The second possibility is that males defend sites with the primary aim of encountering ovipositing females. This ‘ovipositing female’ hypothesis applies widely throughout insects (Thornhill and Alcock 1983), and obviously requires that females mate more than once throughout their lifetime. However, because the payoff of mating with non-virgin female butterflies is likely to be considerably less than mating with virgins (Wiklund and Forsberg 1985; Rutowski 1991), this strategy may be viable only for most males of those species in which females have high re-mating frequencies (such as Danaus plexippus [Nymphalidae]: Pliske 1973; Oberhauser 1989; Zalucki 1993). The remaining possibility is that males defend ‘rendezvous’ sites where adult females are likely to visit (either inadvertently or purposely) when receptive. This explanation has been proposed to explain the evolution of non-resource-based encounter sites in insects (e.g. Shields 1967), but it is not understood to what extent it might apply in the instances where defended sites also contain larval resources.

In this study, I use male territoriality in the nymphalid species Hypolimnas bolina (L.) as a model system for investigating the evolution of encounter-site selection in territorial butterflies. Males of this species perch at, and defend, geographically prominent areas of the environment, such as forest clearings, forest edges and along stream courses and paths (McCubbin 1971; Rutowski 1992; Kemp and Rutowski 2001). These sites generally contain larval foodplants (Rutowski 1992; Kemp and Rutowski 2001), although males do not obviously defend areas with the highest density of food plants (see below for a detailed description of reproductive ecology and site selection by males). Territorial behaviour is both seasonally and geographically consistent, and therefore appears to be the primary mating tactic of male H. bolina (Kemp 2001a). Perching individuals are highly aggressive (Kemp 2000a) and site tenacious (Rutowski 1992), and their behaviour is remarkably similar in most respects to that of other species in which males defend perching sites as a method of mate location (Rutowski 1991). This species therefore represents the group of butterflies in which males defend specific sites that overlap with the distribution of larval resources, but for which the source of receptive females – and the potential relevance of the distribution of larval hostplants – is largely unknown.

There is limited evidence that female H. bolina mate only once (Ehrlich and Ehrlich 1978; Rutowski 1992), which casts some doubt on the ‘ovipositing female’ hypothesis. However, the patterns and timing of female receptivity in this species have not been assessed in detail, and therefore a clear evaluation of the competing hypotheses is not presently possible. Here, I investigate the timing of ovarian maturation and mating, mating frequencies and the correlates of multiple mating in female H. bolina, to build a more complete picture of the mating ecology of this species. I aim to use these data to assess the relevance of each of the functional hypotheses (as outlined above) to territorial behaviour of male H. bolina (McCubbin 1971; Rutowski 1992; Kemp 2000a, 2001a; Kemp and Rutowski 2001). The hypothesis-testing aspect of this paper focuses on key predictions of the ‘emerging female’ and ‘ovipositing female’ hypotheses – that females should be
Female receptivity in *Hypolimnas bolina* 205

prepared to mate immediately upon eclosion, and that females should mate repeatedly, respectively. A failure to support either prediction will provide sufficient grounds for rejection of each respective candidate hypothesis.

Reproductive and behavioural ecology of *H. bolina*

*Hypolimnas bolina* is a truly polyphagous species, utilising at least 28 larval foodplant species world-wide (Vane-Wright *et al.* 1977). In North Queensland the most popular oviposition substrate is *Systrella nodiflora* (Asteraceae), a small (generally <0.50 m) annual shrub that grows abundantly along forest margins, banks, and disturbed areas (Kemp 1998). Ovipositing females seek out smaller plants (including fresh germinated seedlings and regenerative shoots from damaged [i.e. mown] plants), and generally lay eggs singly or in pairs on leaf undersides (Kemp 1998). Bouts of egg-laying are interspersed with flight, so eggs are potentially dispersed widely. Newly hatched larvae are relatively sedentary, but later-instar larvae wander from plant to plant and are capable of traversing tens of metres in a few hours (author’s unpublished data). At Cairns (16°53’S, 145°45’E), fifth (last) instar larvae have been found feeding upon *Ipomea triloba* (Convolvulaceae) and *Commelina cyanea* (Commelinaceae) growing amongst *S. nodiflora* (author’s unpublished data), which suggests that they may transfer between foodplant species. Coupled with the dispersive effects of female oviposition behaviour (Kemp 1998), this high degree of larval mobility suggests that pupae will probably be widely (and potentially unpredictably) dispersed.

Adult male *H. bolina* establish territories in disturbed areas such as forest edges and clearings, along paths and creek lines, and suburban yards (McCubbin 1971). In North Queensland, male’s territories generally contain, or are situated adjacent to, growth of *S. nodiflora*, *C. cyanea*, and occasionally *I. triloba* or *Sida rhombifolia* (Asteraceae; Rutowski 1992; Kemp and Rutowski 2001). However, these plants are widespread and extremely abundant (particularly throughout disturbed areas), and the most popular perch areas do not necessarily support the greatest growth of food plants, or the growth most suitable for oviposition (such as freshly germinated plants: Kemp 1998). Unlike some species (e.g. *Asterocampa leilia* [Nymphalidae]: Rutowski and Gilchrist 1988), *H. bolina* do not perch on the larval foodplant itself, but favour overhanging tree foliage or prominent vantage points such as tree stumps that overlook open areas (McCubbin 1971).

In a study at Cairns, Kemp and Rutowski (2001) found that male *H. bolina* favoured narrower, ‘bottleneck-like’ points along an otherwise homogeneous corridor through vegetation, regardless of spatial variation in the distribution of food plants. Thus, although territory distribution may broadly reflect, or overlap with, the distribution of larval hostplants, finer-scale site selection may be more closely determined by vegetative structure and its potential effects on vision in males and on patterns of travel by females (Kemp and Rutowski 2001). This aspect of male behaviour suggests that males aim to detect flying adult females, which is broadly consistent with all three hypotheses (as defined above) for territorial site selection.

Methods

Timing of initial receptivity

A total of 60 individual *H. bolina* (approximately 12 offspring of each of 5 females caught from Cairns in September 1998), were cultured on *Asystasia gangetica* (Acanthaceae) under constant conditions of 30°C and 13.5 : 10.5 L : D light regime (refer to Kemp 2000b for precise rearing protocols). *A. gangetica* yields close to optimum juvenile performance, and rearing conditions were selected to ensure that adults did not
enter reproductive dormancy (Kemp 2000b). Following pupation, individuals were transferred to a 0.7 × 0.7 × 0.7 m cage placed under dappled light conditions within a large outdoor flight enclosure (dimensions: 6 × 15 × 4 m). Pupae were then watched daily from approximately 06:00 to 14:00 hours to determine the time of pupal eclosion. No individual eclosed outside of these hours. Recently eclosed individuals were marked with an identifying number on both ventral hindwings (using a waterproof superfine Stabilo-OH Pen 841: Kemp 2000a) and placed on the undersides of palm leaves within the large enclosure. Individuals were then allowed to interact freely with minimal disturbance. Prior to liberating the first females, six field-caught male H. bolina were introduced into the enclosure to ensure that reproductively active males were available to females from Day 0. Further virgin (reared) males were subsequently liberated along with females so that the sex ratio was always highly male-biased, and virgin males were available at all times. Feeding stations were established within the cage, with both 20% honey solution and fermenting banana provided in separate paper-lined Petri dishes. These were changed daily.

To quantify the pre-reproductive period of newly eclosed females (generally) three individuals were randomly selected and dissected immediately upon eclosion (Day 0), at the end of the eclosion day (Day 1), the following day (Day 2), and every two days thereafter until Day 14. The length of the basal oocyte of each dissected female was measured (to the nearest 0.01 mm) using a calibrated eyepiece micrometer fitted to a dissecting microscope (40× magnification), and the bursa copulatrix was dissected to determine whether a spermatophore was present. Because the spermatophore structure in H. bolina is spherical, the height and length of dissected spermatophores were measured to the nearest 0.01 mm. Periodic searches of the cage were made (at roughly 15-min intervals) to locate mating pairs. The time of day that each female was first observed in copula was recorded, along with the time when each pair was last seen in copula, where possible.

Remating frequencies of females

Female H. bolina were captured opportunistically from sites in Cairns from September 1997 to May 1999. Individuals were immediately killed and dissected to determine ovarian status and to count the number of spermatophores within the bursa copulatrix. Because female butterflies receive a single, generally persistent spermatophore with each mating, a count of spermatophores should directly indicate mating frequencies of females (Pliske 1973; Ehrlich and Ehrlich 1978; Sims 1979; Lederhouse et al. 1989; Braby 1996). This technique is used here on the basis of results gained by Ehrlich and Ehrlich (1978), who found no evidence for degradation or absorption of spermatophores over time in seven Hypolimnas species, including H. bolina. Spermatophores were removed and measured (height and diameter; as above) to the nearest 0.01 mm. The number of chorionated eggs contained within the ovaries of each female was also counted, and wing wear assessed on a discrete five-point scale (Kemp 2000a) as an index of age.

Results

The timing of oogenesis and mating

Reared females eclosed with undeveloped ovaries (basal oocyte lengths of 0.06–0.16 mm), which matured gradually over a 10-day period (Fig. 1). The youngest gravid female (i.e. with chorionated oocytes) was dissected at the end of Day 4, while all females dissected after Day 10 were gravid. No female dissected before Day 4 carried a spermatophore, hence females appeared to delay mating until their ovaries had matured (Fig. 1). The pre-mating period of females in this study was 4–8 days (Fig. 1), a result that clearly contradicts the key prediction of the ‘emerging female’ hypothesis.

Females were first seen in copula as early as 08:00 hours and as late as 12:30 hours. The mean time at which mated pairs (n = 9) were first seen was 09:44 hours. The precise separation time was determined for only four of these mated pairs, and the observed period of copulation (a minimum estimate of total time in copula) in these last 60, 50, 60 and 6 min, respectively. Observation of two other pairs was suspended after 268 and 90 min respectively, and in one other pair after 24 h 30 min. A smaller-than-average spermatophore was transferred in the last-noted mating (cross-sectional area of transferred spermatophores = 4.20 mm2, 95% confidence interval of all other spermatophores = 4.45–5.89 mm2, n = 9). No caged female possessed more than one spermatophore, and all spermatophores were preceded in the bursa copulatrix by an amount of clear granular material.
Female receptivity in *Hypolimnas bolina*

Remating frequencies of females

Spermatophore counts of field-caught females failed to find support for the ‘ovipositing female’ hypothesis. Of 106 field-caught females, 94 (88.7%) contained one spermatophore, 10 (9.4%) contained two spermatophores, and two (1.8%) were virgin (overall mean = 1.10 ± 0.03 spermatophores). The distribution of spermatophores across females deviated significantly from random (Chi-square goodness of fit test to Poisson: $\chi^2 = 126.0$, $P < 0.0001$), tending to strongly uniform (variance : mean ratio = 0.10). The two virgin individuals were both in fresh condition, and one had fully regressed ovaries; the distribution of age classes for females carrying one and two spermatophores is given in Fig. 2.

As seen in caged females, varying amounts of clear, granular material were lodged anteriorly in the bursa copulatrix of 64 of the 104 field-caught non-virgins. The amount of this material was scored on a five-point discrete scale (absent, low, medium, high, extreme)
for 60 of these single-mated females (caught from August 1998 to May 1999). This parameter was negatively correlated with a score for wing wear (Spearman rank correlation: \( r_s = -0.35, n = 60, P < 0.01 \)), which suggests that granular secretion is depleted by the female over time. Dissected spermatophores ranged in width from 0.47 to 0.96 mm (mean = 0.71 ± 0.01 mm), and in height from 0.38 to 0.90 mm (mean = 0.67 ± 0.01 mm). Cross-sectional area of spermatophores was not related to wing wear (Spearman rank correlation: \( r_s = -0.09, n = 67, \text{n.s.} \)), which suggests that, unlike the granular material, the spermatophore structure is not significantly eroded over time.

Sampled females contained up to 270 chorionated oocytes (mean = 90.7 ± 7.4 oocytes). The number of eggs contained by females differed significantly between wing-wear classes (ANOVA: \( F_{4,60} = 2.97, P < 0.05 \)), with average egg number generally declining with age (Fig. 3).

**Discussion**

The data presented here clearly mediate against both the ‘emerging female’ and ‘ovipositing female’ hypotheses as large-scale explanations for perch-site defence in male *H. bolina*. First, because eclosing female *H. bolina* are not immediately receptive, males would not be rewarded by staking out emergence sites with the aim of encountering these individuals. Previous experiments (author’s unpublished data) have shown that adult *H. bolina* (both males and females) disperse at least 100–200 m from their immediate site of eclosion within several hours of emergence. The presence of a pre-reproductive period of 4–8 days (Fig. 1) suggests that the spatio-temporal distribution of initial receptivity by females (Wickman and Rutowski 1999) would be far removed from the time and place of eclosion. Second, because females remate only rarely, targeting ovipositing females should not prove a rewarding strategy for most males in the population. Mated females have reduced reproductive value (Wiklund and Forsberg 1985; Rutowski 1991), and insemination of such individuals may not guarantee paternity (Wedell and Cook 1998). The highest fitness returns in this species should therefore be realised by males that seek out and mate with receptive virgin females. These findings collectively suggest the ‘rendezvous site’ hypothesis as the most likely
Female receptivity in *Hypolimnas bolina* explains territoriality by males in this species. This does not exclude the possibility that males defend sites also used by females for oviposition or emergence; it simply suggests that territoriality by males is not primarily geared toward locating these individuals.

The ‘rendezvous site’ hypothesis is the most general of the three candidate hypotheses, and encompasses several more-explicit scenarios for the evolution of hostplant-related territoriality in insects. One such scenario is that receptive female *H. bolina* use larval foodplants as landmarks for the purposes of mate location, similar to the use of hilltops by many low-density or dispersed insect species (Lederhouse 1982; Wickman 1988). A simple decision rule, such as ‘fly to areas of larval hostplant’ when receptive, would allow females to simultaneously orient themselves toward both future oviposition sites and mating sites (Zalucki 1993). However, this strategy is expected to work best when larval foodplants grow in discrete patches (e.g. Zalucki 1993), which is clearly not true for *H. bolina* (Kemp and Rutowski 2001). Another, related, scenario is that females return to their place of emergence when receptive, and that males defend these sites, but do not aim to locate freshly emerging females *per se*.

At the other extreme, it remains possible that males defend geographic landmarks (such as clearings or sunspots), or sites where receptive females are ‘passively’ channelled due to other reasons, such as microclimatic preferences during dispersal (Rutowski 1991; Wickman and Rutowski 1999). In these cases, the territorial overlap with larval foodplants may be purely coincidental, and hostplant distribution may play no functional role in mate location itself. Most larval foodplants of *H. bolina* are classic ‘disturbance’ species that are widely and abundantly distributed throughout subtropical and tropical Australia. Decision rules of males that pertain to landmarks such as clearings, sunspots or vegetative corridors could easily result in the selection of territories that generally contain some growth of at least one larval foodplant species.

Clearly, further elucidation of the evolution of hostplant-related territoriality in *H. bolina* will require data on the behaviour and movements of virgin females during their first few days of adult life. Although these individuals are known to disperse at least from their immediate place of eclosion in their first few hours (author’s unpublished data), little more is known about their movements. This reflects a general lack of information on butterflies in general (Rutowski 1991), except for several species whose females mate immediately upon eclosion (e.g. Gilbert 1991). However, at least in North Queensland, it should be possible to gather data regarding larger-scale patterns of movement of female *H. bolina*, especially during periods of relatively high population density (see Kemp 2001b). These data would allow reconstruction of the likely spatial distribution of receptivity of females in this species (Wickman and Rutowski 1999), a process crucial for distinguishing between the remaining explanations for territorial site selection.

Compared with directly developing females of other butterfly species (e.g. *Coenonympha pamphilus* and *C. tullia* [Nymphalidae]; Wickman 1992), this study suggests that adult female *H. bolina* have a substantial pre-reproductive period (up to eight days). Long refractory periods are expected to cost females in terms of lost reproductive potential (Wickman 1992; Wickman and Jansson 1997), which implies that they should also serve a reproductive benefit (such that net reproductive success is maximised). This benefit may relate to the opportunity for mate choice by females (Wickman 1992; Wickman and Jansson 1997), or increased dispersal capability (Chew and Robbins 1984). In a study on two nymphalid species (*C. pamphilus* and *C. tullia*), Wickman (1992) demonstrated a relationship between adult pre-mating periods and longevity, with females of the shorter-lived *C. tullia* (average lifespan = 3.3 days) showing greater urgency to mate (i.e. a smaller pre-reproductive period).
Female *H. bolina* are relatively long-lived (non-diapausing individuals may live up to several months: author’s unpublished data), which suggests that the cost of delayed reproduction would be relatively lower (per unit time) than that experienced by shorter-lived species.

Finally, it is worth noting that this study addressed patterns of mating and oviposition only in directly developing female *H. bolina*. These individuals, like other widely dispersing butterfly species (e.g. *Agraulis vanillae* [Nymphalidae], *Anarita fatima* [Nymphalidae], *Colias eurytheme* [Pieridae] and *Pieris rapae* [Pieridae]: Chew and Robbins 1984, and references therein; *D. plexippus*: Oberhauser and Hampton 1995), eclose with immature ovaries that are matured over several days. The presence of a spermatophore is not required to stimulate this process of ovarian development (also see Ehrlich and Ehrlich 1978; Oberhauser and Hampton 1995). However, individual *H. bolina* eclosing from late March onwards in tropical Australia enter a state of reproductive diapause (which appears to be induced by changes in photoperiod: Kemp 2000b). These individuals retreat immediately to dispersed gullies and creek lines, and do not mate or develop their ovaries until early-mid spring (Kemp 2001b). Although it is not clear precisely when and where mating occurs after termination of diapause, overwintering individuals are highly dispersed (Kemp 2001b), and they have the potential to use the same hostplant-related encounter sites as their directly reproducing counterparts.

**Acknowledgments**

Many thanks to Professors Rhondda Jones, Ron Rutowski and Christer Wiklund for providing helpful comments on this manuscript. The research was supported financially by an Australian Postgraduate Research Award.

**References**


