"Alternative mate location strategies in the speckled wood butterfly (Pararge aegeria) : an evolutionary ecological approach"

Vande Velde, Lesley

Abstract
The coexistence of alternative behavioural tactics represents a widespread biological phenomenon. Alternative reproductive tactics (ARTs) are a particular case that has interested evolutionary ecologists since long. The butterfly Pararge aegeria can serve as a model system in this context having two male mate-locating tactics occurring simultaneously within a population. Males adopting a “perching” tactic defend a sunlit patch on the forest floor and wait for receptive females. Males adopting the alternative “patrolling” tactic fly through the habitat actively searching for females. According to game theory, the coexistence of alternative tactics should occur when the costs and benefits of each tactic differ in function of intrinsic or environmental factors. The overall goal of this PhD-thesis is to better understand the costs and benefits of the alternative mate-locating behaviours in P. aegeria from a conditional strategy viewpoint. To do so, we used empirical data from the...

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Alternative mate location strategies in the Speckled wood butterfly (Pararge aegeria): an evolutionary ecological approach

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Summary

The coexistence of alternative behavioural tactics represents a widespread biological phenomenon. Alternative reproductive tactics (ARTs) are a particular case that has interested evolutionary ecologists since long. The butterfly *Pararge aegeria* can serve as a model system in this context having two male mate-locating tactics occurring simultaneously within a population. Males adopting a “perching” tactic defend a sunlit patch on the forest floor and wait for receptive females. Males adopting the alternative “patrolling” tactic fly through the habitat actively searching for females. According to game theory, the coexistence of numerous alternative tactics should occur when the costs and benefits of each tactic differ in function of intrinsic or environmental factors. The overall goal of this PhD-thesis is to better understand the costs and benefits of the alternative mate-locating behaviours in *P. aegeria* from a conditional strategy viewpoint. To do so, we used empirical data from the field, from observations under semi-natural conditions in outdoor flight cages and from experimental work in the laboratory.

The quality of the sunlit spots that are used as territories by perching males has been assumed to play an important role relative to male fitness. In a first step, we focused on territorial perching behaviour in order to better understand what makes a sunlit spot a territory for a *P. aegeria* male by understanding which characteristics of a sunlit patch are associated with male presence. We demonstrated that territory selection was principally based on characteristics of the
sunlit spot that facilitate both visual perception and thermoregulation. We also addressed to what extent abnormal weather conditions may disturb the well-documented seasonal variation in male morphology and sunlit spot selection by perching males in the field. Our results showed evidence of flexible behavioural responses to abnormal weather conditions, but also important inertia with respect to functional morphology.

In a second step, we did laboratory and semi-natural experiments to measure the relative physiological and metabolic costs of the behavioural tactics as they were expressed under controlled conditions. Flight is a highly energy demanding way of locomotion. In butterflies, different flight types may correspond to different energetic costs. In turn, eco-physiological costs and conditions of individuals are expected to play a significant role relative to different behavioural tactics that imply different flight types and performances. Mate locating tactics of males *P. aegeria* represent two very different types of flight performances (short flights with powerful acceleration vs. long flights at a lower speed). We found evidence in line with the idea of territorial perching being energetically costly. By varying the energy reserves of males, we demonstrated that the behavioural tactics may be constrained by the physiological status of the male and by the host plant quality during larval development.

Resources used for flight are no longer available for reproduction, as is often assumed in life history theory. Therefore, in a thirst step, we studied the impacts of the expression of the behavioural
tactics on male reproductive output. Despite a supported trade-off between resources allocated to spermatophore production and flight activity, there was no evidence of an effect of the behavioural tactics on male reproductive investment. Finally, we also investigated whether females were able to discriminate males based on male mating status, or whether they were able to compensate when they received a very small spermatophore. Our results showed that females did not avoid mating with recently mated males, but they could compensate from receiving a non-substantial spermatophore by remating.

Further research on the ratio of costs and benefits of ARTs will be required to fully understand the pay-offs of perching vs patrolling under different environmental and intrinsic conditions relative to ART expression. Moreover, the evolution of ARTs should also be considered in response to female choice and behaviour, which has been largely ignored in the field of male mate location in butterflies. A stronger focus on female behaviour is therefore warranted for future work on butterfly ARTs in the field and in the laboratory.
Résumé

La coexistence de tactiques comportementales alternatives représente un phénomène courant en biologie. Les tactiques reproductives alternatives sont un cas particulier qui intéresse les biologistes en écologie évolutive depuis longtemps. Le papillon Pararge aegeria peut servir de système modèle dans ce contexte étant donné qu’il présente deux tactiques alternatives de localisation des partenaires sexuels simultanément au sein d’une même population : les tactiques dites ‘perching’ et ‘patrolling’. Les mâles adoptant la tactique ‘perching’ défendent une tache de lumière formée sur le sol du sous-bois et y attendent les femelles sexuellement réceptives. Les mâles adoptant la tactique alternative ‘patrolling’ volent à travers l’habitat en recherchant activement les femelles. Selon la théorie des jeux, la coexistence de plusieurs tactiques alternatives devrait se produire lorsque les coûts et bénéfices associés à chaque tactique varient en fonction de facteurs intrinsèques ou environnementaux. Le but général de cette thèse consiste à mieux comprendre les coûts et bénéfices associés aux tactiques comportementales alternatives de localisation des femelles chez P. aegeria basé sur le concept théorique de la stratégie conditionnelle. Pour ce faire, nous avons utilisé des données empiriques issues du terrain, d’observations en conditions semi-naturelles dans des cages extérieures et d’expériences menées en laboratoire.

La qualité des taches de lumière utilisées comme territoires par les percheurs est supposée jouer un rôle relativement important pour la
fitness du mâle. Dans un premier temps, nous nous sommes concentré sur le comportement territorial du percheur afin de mieux comprendre quelles caractéristiques de la tache de lumière influencent la présence du mâle dans la tache et l’utilisation de la tache comme territoire. Nous avons démontré que la sélection du territoire est principalement basée sur des caractéristiques de la tache de lumière qui facilitent la perception visuelle ainsi que la thermorégulation. Nous avons également regardé dans quelle mesure des conditions climatiques exceptionnelles pouvaient perturber la variation morphologique saisonnière habituelle chez les mâles percheurs ainsi que la sélection des territoires à travers les saisons. Nos résultats ont mis en évidence une certaine flexibilité des réponses comportementales face aux conditions climatiques anormales, mais aussi une inertie importante concernant la morphologie fonctionnelle.

Dans un second temps, nous avons mené des expériences de laboratoire et en conditions semi-naturelles afin de mesurer les coûts physiologiques et métaboliques associés aux tactiques comportementales telles qu’exprimées en conditions contrôlées. Le vol est un mode de locomotion très coûteux en énergie. Chez les papillons, différents types de vols peuvent correspondre à des coûts énergétiques différents. Par conséquent, on s’attend à ce que les coûts éco-physiologiques et la condition des mâles jouent un rôle important au regard des différentes tactiques comportementales impliquant différents types et performances de vols. Les tactiques de localisation des femelles chez les mâles de *P. aegeria* sont associées à deux performances de vols très différentes (vols courts et puissantes
accélérations versus vols longs et constants). Nous avons mis en
evidence que l’expression de la tactique ‘perching’ est plus couteuse
en énergie. En variant les réserves énergétiques des mâles, nous avons
démontré que l’expression des tactiques comportementales peut être
contrainte par la condition physiologique du mâle et par la qualité des
plantes hôtes durant le développement larvaire.

Comme indiqué par la théorie d’histoire de vie, les ressources
utilisées pour le vol ne sont plus disponibles pour la reproduction. Par
conséquent, dans un troisième temps, nous avons étudié les impacts de
l’expression des tactiques comportementales des mâles sur leur
investissement reproducteur. Malgré un compromis observé entre les
ressources allouées à la production du spermatophage et à l’activité de
vol, aucun effet de l’expression de la tactique comportementale sur
l’investissement reproducteur du mâle n’a été mis en évidence.
Finalement, nous avons également regardé si les femelles étaient
capables de discriminer les mâles en fonction de leur passé
reproducteur, ou si elles étaient capables de compenser le fait de
recevoir un très petit spermatophage. Nos résultats montrent que les
femelles ne discriminent pas les mâles récemment accouplés, mais
qu’elles semblent être capables de compenser le fait d’avoir reçu un
très petit spermatophage en se réaccouplant.

Davantage de recherches sur le rapport coûts-bénéfices des
tactiques reproductives alternatives sont nécessaires afin de totalement
comprendre les profits d’être percheur ou patrouilleur sous diverses
conditions intrinsèques et environnementales. De plus, l’évolution des
tactiques reproductives alternatives devrait également être considérée en réponse au choix et au comportement des femelles qui ont été largement ignorés dans le contexte des tactiques de localisation des partenaires sexuels chez les papillons. Un intérêt plus important du comportement des femelles est donc justifié pour les travaux futurs sur les tactiques reproductives alternatives des papillons sur le terrain et en laboratoire.
I. General introduction

I.1 Alternative reproductive tactics (ARTs)

I.1.1 Theoretical concepts and origin of the ARTs

Darwin (1859) was fascinated by variation within and among all organisms. Since his careful observations, biologists have argued in line with the theory of evolution by natural selection that if variants show differential survival and if the characteristics are heritable, the phenotype of organisms may change. If one phenotype is on average less successful than the other phenotype(s), then it will affect the frequency of that phenotype, and ultimately the further existence of it. Over the course of time, natural selection may modify phenotypes of species in ways that permit them to succeed in their environment (i.e. survival selection). Evolutionary ecology takes natural selection to explain the adaptation of organisms to their environment as its field of study. Its aim is to understand the ecology of organisms from an evolutionary perspective, so evolutionary ecologists ask questions about how organisms adapt to their environment, and the consequences of adaptive change for interactions among and within species. It examines the selective pressures imposed by the environment and the evolutionary response to these pressures.
However, many animals develop features that do not help them to survive, but they may help to maximize reproductive success (i.e. sexual selection) (Darwin 1871). Sexual selection is natural selection operating on factors that contribute to an organism's mating success. Sexual selection may operate at the intersexual level through one sex (usually the female) choosing among the opposite sex which has developed some attributes that make them more attractive and at the intrasexual level by one sex (typically males) competing (aggressively) among themselves for access to the limiting sex.

A major objective in evolutionary ecology is to understand the processes by which alternative phenotypes are created and maintained within and among populations. Consistent variation in the reproductive behaviour of males and females (i.e. alternative reproductive tactics, ARTs) and its maintenance at a stable frequency in a population has attracted much interest in this field. ARTs are likely to evolve under scenarios of disruptive selection. This can be the case when there are different reproductive niches that favour divergent tactics and specialisation for exploiting each niche (Taborsky et al. 2008; Taborsky & Brockmann 2010). For instance, males may wait for females at specific places that they will defend, or they may search for females in wider areas. Differences in reproductive tactics may then imply differences in flight performances and/or morphological adaptations (Brockmann 2008). The evolution of ARTs is also influenced by the interactions between the sexes, as well as by competitive interactions within a sex (Alonzo 2008). When acquiring mates is costly, selection may favour males to avoid costs of
competition by adopting an alternative way to maximize their success. ARTs for mate searching often involve a division between high-investment or high-risk but sedentary, non-dispersing tactics and low-investment or low-risk but active, dispersing, searching tactics with longer-range movements (Brockmann 2008). Changes in female behaviour and in micro-distribution may alter the benefits and costs of male mate locating behaviour and in this way shapes the evolution of male ARTs (Alonzo 2008). For instance, when receptive females represent a rare resource and their distribution is unpredictable, males may benefit to wait females within specific sites rather that actively search for them in wide areas (e.g. Dennis & Shreeve 1988). Male density will also affect searching costs and also influence their choosiness (e.g. Gotthard et al. 1999). Typically, when the costs of searching increase, choosiness should decrease (Real 1990; Reynolds and Gross 1990).

Many populations contain more than one phenotype within a sex in animal mating systems (Gross 1996). ARTs are described for several taxonomic groups, from insects to mammals (for taxonomic review of ARTs see Oliveira et al. 2008). Phenotypic differences may involve diverse attributes, including behavioural, physiological, and morphological ones (Moran 1992). Maynard Smith (1982) introduced game theory and the concept of the evolutionarily stable strategy (ESS) in part to help explain the evolution of such variation in phenotypes. An ESS is a strategy which, when a certain frequency of the population adopts it, is unbeatable reproductively compared to a given set of alternatives. An equilibrium is also possible when two or
more strategies are evolutionarily stable against each other. Commonly, game theory offers three means by which phenotypic diversity within a sex may arise: if individuals adopt pure strategies with a genetic polymorphism; if individuals adopt a single mixed strategy and spend a certain portion of time in one behaviour, and then switch to another; and if individuals adopt a single conditional strategy and change their behaviour through specific environmental or status conditions (Cade 1980; Gross 1996). Each of these strategies has a clear set of conditions that are necessary if they are to be maintained in the population.

In pure strategies (also named alternative strategies) the average reproductive success derived from both phenotypes is equal providing by frequency-dependent selection. It implies that the mixture of alternative tactics is evolutionarily stable, and any departure from an equilibrium will be countered by selection re-establishing the equilibrium frequency (Cade 1980; Gross 1996; Taborsky & Brockmann 2010). Pure strategies are rare but were suggested by research on several fish (Gross 1991a,b; Ryan et al. 1992), a bird (Lank et al. 1995), a marine isopod (Schuster & Wade 1991), a lizard (Sinervo & Lively 1996) and insects (Tsubaki et al. 1997; Tsubaki 2003). In these cases, the various phenotypes are the result of polymorphic genotypes (i.e. fixed genetically). For example, in the small marine isopod Paracerceis sculpta, there are three alleles at a single autosomal locus that are thought to result in larger fighter males, intermediate-sized males that mimic females, or small sneaker males (Schuster & Wade 1991). Males of the damselfly Mnais costalis
differ in wing colouration and adopt either a bourgeois tactic by defending potential oviposition sites, or a satellite tactic where females are opportunistically pursued (Tsubaki et al. 1997). A captive rearing experiment suggested that this male polymorphism is genetically controlled by a single-locus, two-allele autosomal polymorphism (Tsubaki 2003). Fixed tactics are expected if conditions change either rarely during a lifetime, or if the change occurs unpredictably (Shuster & Wade 2003).

In a mixed strategy males spend a certain portion of their active time adopting one behavioural tactic but there is a probabilistic switch to display an alternative tactic. Again, alternative tactics should yield on average equal fitness by frequency-dependent selection (Maynard Smith 1982; Gross 1996; Taborsky & Brockmann 2010). In contrast with the conditional strategy (see below), the shift in behaviour does not correspond to any environmental or individual factor. Consequently, this strategy is expected under unpredictable environmental conditions (Cade 1980). But, as detecting environmental events inducing the shift may be difficult, it increases the difficulty distinguishing between a mixed and conditional strategy (Plaistow et al. 2004). While a mixed reproductive strategy is theoretically conceivable, there is little empirical support for such a strategy operating at the within-sexual level (e.g. Brockmann et al. 1979; Alcock et al. 1977; but see Plaistow et al. 2004).

Under a conditional strategy, individuals make a ‘decision’ relative to environmental conditions or competitive abilities (Cade 1980; Gross
The average fitness of the tactics should be unequal, but the chosen tactic should result in higher fitness for the individual relative to the alternative (Hazel et al. 1990; Gross 1996). The status-dependent selection model (Figure 1) predicts one (or multiple, see Lee 2005) switch-point in status where the average payoffs of individuals adopting both tactics is equal. For the conditional strategy to be maintained there must be a status-dependent fitness trade-off between the tactics. It means that above the switch-point, individual with high status will benefit from adopting one tactic, while those below that point, with low status, will benefit more from adopting the alternative tactic (Fig.1; Hazel et al. 1990; Gross 1996; Taborsky & Brockmann 2010). Under these conditions, we cannot say that individuals below the switch-point make the “best of a bad job” by practicing the alternative tactic with lower payoff as all individuals do the best tactic according to their status (Hazel et al. 1990). The ‘decision’ concerning the type of tactic performed may be reversible with males shifting their behaviour depending on the conditions (e.g., Davies 1978; Alcock & Houston 1987; Alcock 1997; but see Brockmann 2001; Taborsky & Brockmann, 2010) or individuals males may be morphologically “locked in” to specific tactics at certain life stages (e.g., Sinervo & Lively 1996; Cook et al. 1997; Emlen 1997; but see Brockmann 2001; Taborsky & Brockmann 2010). Males may perform one tactic all their adult life, but this might still be a conditional ‘decision’ based on social or environmental events when the individual was immature (Cade 1980; Taborsky & Brockmann 2010). It is widely believed that conditional strategies are the most common form of intrasexual phenotypic diversity (Gross 1996;
Brockmann 2008; Taborsky & Brockmann 2010). Nevertheless, a conditional strategy will not be favoured when non-territorial males obtain almost as many matings as successful territory holders, because in this case there is little benefit to being conditional (Plaistow et al. 2004).

**Figure 1.** The alternative reproductive tactics X and Y have status-dependent fitness functions, where the fitness of a given tactic depends on the status of the individual adopting the tactic. The fitness functions are different between the alternative tactics, but at the point of intersection both tactics have equal fitness ($s^*$). Individuals of high status obtain greater fitness through phenotype X than through phenotype Y while individuals of low status obtain greater fitness through phenotype Y than through phenotype X. (Based on Gross 1996)

There is a growing number of papers demonstrating the heritability of such a switch point and the ability to respond quickly to selection (e.g. Hazel et al. 1990; Gross & Repka 1998; Shuster & Wade 2003; Emlen 2008). For the conditional strategy to evolve, it is important that there is heritable genetic variation for the tactic switch point (i.e., the response to the environmental cue) to be subject to natural selection.
(Hazel et al. 1990; Shuster & Wade 2003; Emlen 2008). For example, in the scarab dung beetles of the genus *Onthophagus*, male fighting ability increases with both adult body size and with horn size. Males that exceed a critical threshold of body size develop a pair of long, curved horns on their heads, while smaller males remain hornless. Horned males aggressively defended tunnel entrances containing breeding females while hornless males employed nonaggressive sneaking behaviours when faced with competitively superior males. Sneaking behaviours adopted by small, hornless males require high degrees of manoeuvrability that allow these males to access females inside tunnels and copulate with the female before they have reached the surface (Eberhard 1982). In the beetle *Onthophagus acuminatus*, the switch point of male dimorphism was found to be heritable (Emlen 1996).

I.1.2. Empirical examples of conditional ARTs in insects

Alternative reproductive tactics (ARTs) occur in most orders of insects, across a wide range of mating systems, and during all steps in the reproductive process (locating a mate, gaining access to the mate, copulating, and post-copulatory behaviour) (Brockmann 2008). Nevertheless, empirical studies showed that most ARTs occurred for mate searching (see examples in Brockmann 2008) with males adopting a territorial tactic to monopolize access to females and other males using a “sneaky” tactic in which they try to mate with females without defending a resource.
Conditional male mating strategies have been studied extensively in relation to male traits, such as body size and resource-holding potential (Andersson 1994; Marden 2000). In mate competition a small male may employ a behavioural tactic that involves sneaking, while a larger male employs a behavioural tactic that involves fighting (Gross 1991a). One commonly cited example is that of the bee *Centris pallida* (Alcock 1979, Alcock et al. 1977). Large males 'patrol' specific areas close to the ground apparently using olfaction to locate newly molted females and defend the area against other males, while smaller males 'hover' well above the ground and chase after females missed by the larger conspecifics.

There is typically a large-male mating advantage associated with the ability to defend a territory (Andersson 1994; Andersson & Iwasa 1996). This pattern is, however, not universal. Several studies reported no effect of size (e.g. Marden 1989; Strohm & Lechner 2000) and other studies have even reported an apparently paradoxical small-male mating advantage in territorial species (Convey 1989; Hernandez & Benson 1998; Voigt et al. 2005; De Block & Stoks 2007).

Age difference is also an important condition that may affect male reproductive behaviour (Howard 1978; Andersson 1994). Due to their relatively reduced opportunity for future survival and reproduction, older males are generally selected to increase their expenditure in sexual competition (Forsythe & Montgomerie 1987; Kemp 2002a,b, 2003). Moreover, females might also prefer older males since an older phenotype may indicate the presence of genes that contribute to
survival for extended periods (Cade 1980). Howard (1978) pointed out that older males are more experienced in competition and will, therefore, present an advantage relative to younger males, all other factors being equal. On the contrary, Forsyth & Montgomerie (1987) have shown that old males of the damselfly *Calopteryx maculata* are displaced from their territories by younger males. As the energy reserves of male declined with age, Marden & Waage (1990) and Plaistow & Siva-Jothy (1996) suggested that any correlation between age and the likelihood of winning likely reflects a decrease in lipid reserves with age; therefore lipid reserves were a better predictor of the outcome of winning fights than age in this species. This example illustrates that reproductive tactics are often complex and may involve the interaction of several organismal variables (Cade 1980).

Conditional strategies may also be the result of environmental heterogeneity, including ambient temperature (Heinrich 1986; Wickman 1985a, 1988; Alcock 1994), rapid decline of female receptivity (Kon et al. 1986; Dennis & Williams 1987), population density (Cade 1980) or localisation and quality of territories. For instance, males of the odonate species *Leucorrhinia intacta* shift their behavioural tactic (territorial versus transient) based on the availability of perches relative to oviposition substrates and tend to adapt their choice relative to the frequency of the tactic adopted by other males (Waltz & Wolf 1988).

Previous theoretical studies, and some empirical studies, suggested that the factors affecting female availability should influence male
reproductive behaviour (e.g. Hirota et al. 2001; Rutowski et al. 1996). Larison (2007) showed that males of the damselfly *Protonoeura amatorial* change their tactic use in response to light conditions. Under strong light conditions, males preferentially hover over the water and attempt to grab females that are ovipositing in floating debris while males use the alternative sit-and-wait tactic under low-light conditions. However, there were confounding factors in this study as he also observed that the density of ovipositing females was higher under high-light conditions. This indicates that physical factors may exert indirect as well as direct effects on the behavioural tactic used for locating females.

I.1.3 The case of butterflies

In the process of obtaining mates, male butterflies are described by Scott (1974) as adopting two main behavioural tactics: perching or patrolling. Perching is a ‘sit-and-wait’ tactic in which males sit at characteristic sites and investigate passing objects while waiting for females, whereas patrolling is an active search behaviour for receptive females over wider areas. According to Dennis & Shreeve (1988) the pattern of mate-locating behaviour in butterflies reflects ancestral links with the structure and dynamics of resource distribution. Patrolling is assumed to be the ancestral behaviour as it can be performed under widespread and unpredictable resource distributions. By contrast, perching males will only achieve matings if receptive females are able to locate males at specific places (Dennis & Shreeve 1988) or if detection and interception of the females is easier in these
places (e.g. Bergman et al. 2007). Therefore, perching is an adequate tactic if mating occurs only in particular sites of the habitat (Dennis & Shreeve 1988). Therefore, they suggested that perching behaviour may have evolved only in habitats in which topographic features provided predictable vantage points for acquiring mates. Males may encounter females directly on or near emergence sites when it exists dense larval food plants spots and when the density of larvae per plant is high (e.g. *Euphydryas chalcedone*; *Asterocompa leila*). Many species locate females in specific landmarks such as patch on the bare ground (e.g. *Vanessa atalanta*), sunlit spots (e.g. *Pararge aegeria*) or trees (e.g. *Incisalia iroides*; but see Rutowski 1991 for more examples).

Perching behaviour has often been confused with territoriality, the two terms occasionally being used as synonyms (cf. Scott 1974; Dennis & Shreeve 1988). Territorial species engage in aerial displays or contests which can result in physical damage to the contesting individuals (Baker 1983; Wickman & Wiklund 1983; Alcock 1983; Dennis & Williams 1987). However, in several perching species, males may never physically defend the areas in which they perch (e.g. *Cupido minimus* Morton 1985; *Maniola jurtina* Dennis personal observation). Males also vary in their fidelity to particular areas (Dennis & Shreeve 1988). Males of the mobile *Aglais urticae* and *Inachis io* move between perching sites throughout their adult lives (Baker 1972), while certain males of *P. aegeria* and *C. minimus* may use a set of perching sites within a small area for a period of days, or
General introduction

Although the mode of male mate location behaviour can be species-specific, there are several species in which both behavioural types can co-occur within the same population (e.g. *P. aegeria* Wickman & Wiklund, 1983, *C. pamphilus* Wickman 1985a,b, *Lasiommata megera* Dennis 1982; Wickman 1988, *Strymon melinus* Alcock & O’Neill 1986, and *Chlosyne californica* Alcock 1994). Males may sometimes shift between perching and patrolling tactics at different times of the day (cf Scott 1974; Shreeve 1990, 1992). This observation raises the question about the factors that favour the adoption of perching and patrolling behaviours. Ide & Kondoh (2000) used a game model to investigate which conditions favoured perching behaviour if both sexes had two alternative mate-locating tactics. Their model predicted that perching behaviour is favoured when: (i) the cost to perch is
sufficiently lower than that of patrolling, (ii) the cost to females of visiting a perch site is sufficiently lower than staying at an emergence site, or (iii) searching efficiency is higher in a perch site than at an emergence site.

Conditional changes in behavioural tactics are widespread in butterflies (cf. Davies 1978; Dennis 1982; Wickman & Wiklund 1983; Shreeve 1984; Wickman 1985a, b, 1988; Alcock & O’Neill 1986; Dennis & Williams 1987; Alcock 1994; Van Dyck et al. 1997a, b; Van Dyck & Matthysen 1998). Butterflies are winged heliotherms, which means that their body temperature is greatly affected by solar radiation incidence on their body and convective heat gain from the environment (Clench 1966) and their flight performance directly depends on the temperature of the thorax, which contains the flight muscles (Heinrich 1993; Merckx et al. 2006). Consequently, their mating behaviour is strongly affected by their thermal physiology (Willmer 1991; Ravenscroft 1994; Stutt & Willmer 1998). In a number of butterfly species the tactic used depends on ambient temperature (e.g. Wickman & Wiklund 1983; Shreeve 1984; Wickman 1985a, 1988; Alcock 1994). Many studies have shown that butterflies engage in territorial behaviour when circumstances are spatially or temporally suitable to regulate body temperature (Ravenscroft 1994; Rutowski et al. 1994, 1996; Bitzer & Shaw 1995; Ide 2002b). When thermal resources are not homogenously distributed – as for instance, in forests – basking sites such as sunspots may provide rendezvous sites where territoriality and perching may be favoured (Davies 1978; Kemp & Wiklund 2001). Nevertheless, the
importance of owning a territory may vary with the weather and between seasons (Ide 2002a,b). Because flight requires high body temperatures (Heinrich 1986), flight ability rises through the day in parallel with air temperature so that territory ownership is devalued under warm weather conditions (Wickman & Wiklund 1983; Wickman 1985a; Stutt & Willmer 1998). Consequently, Wickman (1985a) observed that male individuals of Coenonympha pampilus switch from territorial perching in the morning to patrolling in the afternoon. Wickman & Wiklund (1983) found that the duration of contests between males P. aegeria declined with season. As it was previously shown by Shreeve (1984), this correlated with the rise in air temperature, but also with a decrease in the number of receptive females.

Adoption of sit-and-wait tactic is more advantageous than searching tactic when receptive females represent a scarce resource in the habitat (Dennis & Williams 1987). Consequently, population density (Dennis 1982; Alcock & O’Neill 1986) and receptive female distribution (Dennis & Williams 1987; Rutowski et al. 1996; Hirota et al. 2001) are decisive conditional factors that may influence the mate location behaviour of butterfly males. Low population density should favour perching and high density should favour patrolling (Scott 1974; Willmer 1991). In territorial species, this switch in behavioural tactics should be interpreted in terms of the economics of territory defence, with rising cost of defence under high density (Wickman & Wiklund 1983; Shreeve 1987; Rutowski 1991). Wickman & Wiklund (1983) observed an increase of the frequency of patrolling with the number of
active males, and therefore, with the intensity of male-male interactions in the population. On the other hand, Scott (1974) and Ehrlich (1984) predicted that, when females are scarce, perching at topographic localities would save energy and time for both sexes, and consequently, represent the best tactic to ensure early and successful mating. By contrast, when populations are dense, search times are short and patrolling males will locate females more rapidly than perchers at a particular locality. Contrary to the predictions based on thermal constraints, some males shift from morning patrolling to afternoon perching (e.g. *Ochlodes venata* Dennis & Williams 1987; *Lethe diana* Ide 2004). This is assumed to be an adaptive response to the rapid decline in the reproductive value of females over the course of a day (Dennis & Williams 1987).

Merckx & Van Dyck (2005) showed that habitat fragmentation may alter cost-benefit ratios of alternative mate-location tactics. Mean ambient temperature as well as densities per unit of habitat were higher in agricultural fragmented landscapes compared to continuous woodland. Consequently, they observed a higher frequency of patrolling and also of intermediate behaviour between perching and patrolling in the agricultural fragmented landscape than in the woodland. Convective cooling may also affect body temperature and thus the duration of flight bouts. In highly fragmented, agricultural landscapes, convective cooling by the wind was showed to be stronger than in continuous woodland (Merckx et al. 2008).
While some species switch between behavioural tactics under biotic or abiotic conditions, individuals of other species may show a high persistency in the behavioural tactics they adopt (e.g. Wickman & Wiklund 1983; Van Dyck et al. 1997b). Tactics that are fixed for the adult life are often generated by condition-dependent developmental mechanisms where the decision on the life-history traits trajectory depends on environmental or social conditions during the immature stage (Tsubaki & Brockmann 2010). The condition-dependent switch may also be influenced by a size threshold during the larval stage (Emlen 2008) or in seasonal environments, by the time of birth of an individual in relation to environmental oscillations (the ‘birthdate effect’, Taborsky 1998). Van Dyck & Wiklund (2002) have showed that males *P. aegeria* that emerged in spring had a more pronounced perching design than males emerged in summer. Because the perching flight performance demands greater aerial control and agility, perching males should exhibit traits associated with high acceleration ability and speed, whereas patrolling males should exhibit traits associated with flight endurance (Wickman 1992; Van Dyck 2003; Wiklund 2003). Consequently, not all individuals are able to obtain or hold a territory and the choice of adopting a particular behavioural tactic is dependent on intrinsic conditions (i.e. morphological or physiological traits). Several studies have shown that perching species had larger thorax/body mass ratios, higher wing loadings, and higher aspect ratios (wing span squared divided by wing area) than patrolling species (Chai & Srygley 1990; Wickman 1992; Van Dyck & Matthysen 1998). All these morphological characteristics allow
perching males to exhibit a high flight capacity and manoeuvrability (Berwaerts et al. 2002).

Studies on Odonata have demonstrated that physiological traits are likely to play a strong role determining a male’s ability to become territorial (Marden & Rollins 1994; Marden & Waage 1990; Plaistow & Siva-Jothy 1996; Plaistow & Tsubaki 2000). Depleted energy reserves constrain males to adopt non territorial behaviour as they do not have enough fat to fight (Marden & Waage 1990; Plaistow & Siva-Jothy 1996). As short powerful take-off flights characterising perching behaviour are the most energy demanding (Dudley 2000), we could expect perching males to be more dependent on their lipid reserves (i.e. capital and income breeding). Nevertheless, few studies on butterflies have investigated the importance of energy sources (i.e., lipid resources) relative to the probability to adopt a behavioural tactic (Kemp 2002a; Takeutchi 2006; Martínez-Lendech et al. 2007) and at this stage, do not show such a relation between lipids and territorial ability in butterflies.
I.2. Costs relative to the expression of ARTs in flying insects

I.2.1 Physiological costs

Active insect flight muscles have the highest metabolic rates of any known animal tissue (Downer & Matthews 1976), flapping flight being the most energetically demanding mode of animal locomotion (Bartholomew & Casey 1978; Dudley 2000). In most species of Diptera and Hymenoptera carbohydrates constitute the predominant substrate for flight, whereas in many species of Lepidoptera and Orthoptera this substrate is used in combination with lipids; carbohydrates are used at the initiation of flight, whereas lipids are the main fuel during sustained flight (Beenakkers et al. 1984; Candy et al. 1997). In the majority of insects, lipids are usually found in the fat body and are principally presented as triacylglycerols (Beenakkers et al. 1985; Canavoso et al. 2001). Lipid storage is mainly the result of transfer of dietary fat from the midgut to the fat body during the feeding period. In addition, lipid storage can result from de novo lipid synthesis in the fat body from carbohydrates (Beenakkers et al. 1984, 1985; Canavoso et al. 2001).

Energetic costs may influence the way animals defend territories and compete for mates (Davies & Houston 1984). For instance, lipid reserves represent a limiting resource in some territorial species of Odonata and Diptera because it determines whether, and for how long,
a male is able to defend a territory (e.g. Plaistow & Siva-Jothy 1996; Plaistow & Tsubaki 2000; Kemp & Alcock 2003). Entering into an escalated fight and chasing a male from a territory corresponds to considerable use of the energy reserves of a territorial male (Plaistow & Siva-Jothy 1996). Larger fuel reserves would enable increased endurance in aerial interactions (Takeuchi 2006) and therefore enable efficient perching tactic. Powerful take-offs of perching males are known to be the most energy demanding (Dudley 2000). Moreover, males that defend non resource-based territories have relatively reduced foraging opportunities (e.g. Kemp & Wiklund 2001; Kemp & Alcock 2003) compared to patrolling males. Consequently, perching tactic is assumed to be the most nutritional dependent, as well as the most costly behavioural tactic to adopt. Therefore, we could expect different mating tactics to be condition determined with only males with large initial lipid reserves to be able to defend a territory and males with limited lipid reserves to be constrained to adopt the less costly, patrolling tactic.

Short, powerful take-offs and high acceleration ability versus flight endurance characterise perching and patrolling flight, respectively. Resting and active metabolic rate associate to these flight performances can be used to investigate the energetic costs of alternative tactics (Hack 1997a, 1998). Individuals spending high energy levels for activity have higher resting and flying metabolic rates than less active one. These active individuals often present several adaptations that increase the efficiency of metabolism during activity (e.g. increase density or surface of mitochondria or increase
size of energetically active organs) or that increase the maximum speed at which the metabolic rate can be increased or decreased (Reinhold 1999). High metabolic costs of subsistence in flying males should affect reproductive effort, either by decreasing the energy available for reproductive activity (e.g. mate location, spermatophore production) or by increasing foraging time (Hack 1997a,b). Consequently, variability in metabolic costs between individuals may represent an important factor shaping alternative mating tactics.

I.2.2 Reproductive costs

Life-history theory predicts trade-offs between resources invested in reproduction, in flight activity and in somatic maintenance. A useful system in which these particular trade-offs can be studied in insects is wing polymorphism. Numerous studies of wing-polymorphic species have shown that long-winged females typically have a lower fecundity than short-winged, flightless females (reviewed by Roff & Fairbairn 1991). Such trade-offs have been particularly studied in females (oogenesis-flight syndrome Johnson 1963 but see Hughes et al. 2003; Jervis et al. 2005; Gibbs et al. 2010 for examples in butterflies). It is probably because it is generally assumed that males have a reduced gametic investment relative to females. However, in several male insects and contrary to vertebrates, sperm production signifies a considerable cost (Dewsbury 1982; Svärd & Wiklund 1989). It is particularly the case for males that transfer during the copulation a spermatophore to the female that contains sperm and accessory gland products (i.e. Lepidoptera, Orthoptera). In these species, male
ejaculates are costly to produce as shown by the fact that copulation durations were longer and ejaculates smaller in matings involving recently mated males (Svärd 1985; Bissoondath & Wiklund 1996; Lauwers & Van Dyck 2006).

Flight performances are assumed to vary considerably between alternative mate-locating tactics. Aerial contests of territorial individuals are known to be particularly energy costly (Marden & Waage 1990; Marden & Rollins 1994; Plaistow & Siva-Jothy 1996; Takeutchi 2006). Consequently, we may expect energy invested in intensive flight activity for mate location no longer being available for other aspects of reproduction (i.e. spermatophore production, courtship, guarding) and therefore results in lower fitness.

Ejaculates transferred by lepidopteran males contain a significant amount of protein, which is likely to be important to females and can therefore be considered as parental investment (e.g. Wiklund et al. 1993; Boggs 1981b, 2003). Males that transfer large ejaculates appear to be rewarded with increased paternity both in polyandrous (e.g. Bissoondath & Wiklund 1996) and monandrous species (e.g. Lauwers & Van Dyck 2006). Indeed, despite the fact that polyandrous butterfly species transfer larger spermatophores that contain more sperm compared to monandrous species (Svärd & Wiklund 1989; Gage 1994), Lauwers & Van Dyck (2006) demonstrated that female of the monandrous species *P. aegeria* that have received a small spermatophore from a recently mated male produced less offspring than females that received a larger spermatophore from a virgin male.
This highlighted that receiving a small spermatophore may result in a fitness cost for both sexes, especially in monandrous species. However, little is known about flight-reproduction trade-offs among male insects and its consequences for male and female fitness.

1.3 Aims & outline

The coexistence of alternative reproductive tactics is common in animals and often involves a distinction between territorial, sedentary tactics and non-territorial, searching tactics. The butterfly *Pararge aegeria* represents such a case. Males adopt two alternative mate-locating tactics that co-occur simultaneously in a population: the territorial perching tactic and the patrolling tactic (Scott 1974). Perching males sit within sunlit spots on the forest floor where they adopt a sit-and-wait tactic with short inspection flights and several aggressive interactions with potential intruders, while patrolling males fly through the habitat searching for females (Wickman & Wiklund 1983; Shreeve 1987; Van Dyck et al. 1997a). From a conditional strategy viewpoint, alternative reproductive tactics with unequal mean fitness may evolve when there is a fitness trade-off. Then, some males will benefit from adopting the perching tactic, whereas expressing the patrolling tactic would be more advantageous for other males relative to environmental or intrinsic conditions. In order to better understand the co-occurrence of ARTs within populations, we need to focus on the costs-benefits ratio of each behavioural tactic under various conditions.
The overall goal of this PhD-thesis is to better understand the costs and benefits of the alternative mate-locating behaviours in *P. aegeria*. The work consisted of three main steps (Figure 2).

| Part I: Benefits | Chapter III | Hypotheses 1: Holding a territory facilitates the detection of females. Which characteristics of a sunlit spot are significant to a perching male in order to serve as a territory? |
| Part II: Physiological costs | Chapter IV | Hypotheses 2: Sunlit spot characteristics vary among seasons. How do male design and behaviour change with seasonal environmental changes? What happens under extreme seasonal weather conditions? |
| Part III: Reproductive costs | Chapter V | Hypotheses 3: Fast take-offs are highly energy demanding. Is territory holding more energy demanding than non-territorial behaviour? |
|                      | Chapter VI | Hypotheses 4: High costs of territory holding. Is behavioural tactic expression constrained by lipid reserves accumulated during the larval stage? |
|                      | Chapter VII | Hypotheses 5: Trade-off between flight and reproduction. Is male reproductive investment of perching males reduced compared to non-territorial males? |
|                      | Chapter VIII | Hypotheses 6: Receiving a small spermatophore affects female fitness. Are females able to avoid receiving a small spermatophore or can they compensate in some way? |

Figure 2. Structure of the PhD dissertation resuming the main hypotheses and successives questions approached in each chapter (see details in the text).
In a first step, we focussed on the potential benefits to defend a territory by perching males. For this part, we studied territory holders in more detail, particularly under field conditions (Chapter III, IV). Several studies show or assume that territorial males have on average a higher mean reproductive success than non-territorial males (Davies 1978; Wickman & Wiklund 1983; Bergman et al. 2007; Fisher & Fiedler 2001). By releasing and following receptive virgin females, Bergman et al. (2007) showed that perching males of P. aegeria achieved on average twice as many matings than patrolling males under the experimental conditions in outdoor cages. They suggested that the higher reproductive success of territorial P. aegeria males resulted from better female detection within sunlit territories. Therefore, we may expect an effect of the availability and the quality of sunspots on the success and the choice of adopting one of the two behavioural tactics. In a mixed woodland, we observed that some sunlit spots are invariably defended while others were never occupied. Bergman & Wiklund (2009) showed that males of P. aegeria defended preferentially larger sunlit spots on the forest floor for visual detection reasons. Nevertheless, as adult butterflies are flying heliotherms, in Chapter III, we assessed whether territory site selection was also influenced by the thermal conditions relative to the characteristics of the sunlit spots. Environmental conditions have a strong impact on all behaviours of flying heliotherms, including mate-locating behaviour of males. Weather conditions, as well as the characteristics of the sunlit spots vary between the seasonal cohorts of the long flight period of P. aegeria (i.e., from March to October). Consequently, in Chapter IV, we also focused on temporal variation
in territory selection through the season, and particularly, on consequences of extreme seasonal weather conditions on functional flight morphology and behavioural responses of perching males.

In a second step, we evaluated the physiological costs of holding a territory versus a non-territorial behaviour under controlled environmental conditions (Chapter V, VI). There is evidence of a certain consistency in mate locating behaviour in individual *P. aegeria* males (Van Dyck et al. 1997b). Previous studies have demonstrated morphological differences between perching and patrolling males (see Van Dyck 2003 for a review). These differences can be seen as intrinsic traits that condition the mate-locating behaviour. The type of flight performance strongly differs between perching and patrolling tactics; perching corresponds to high frequency of take-off flights and aerial combats with high levels of manoeuvrability while patrolling corresponds to longer, enduring flights through larger area at a lower speed (Wickman 1992; Van Dyck 2003 and references therein). Fast take-off flights are known to be amongst the most energy-demanding types of aerial locomotion (Dudley 2000). Hence, the physiological status of the individual male is also expected to affect the mate-locating behaviour. In this part, we tested the energetic costs of territory holding in *P. aegeria*. In Chapter V, lipid use and metabolic rate of males adopting the different mate-locating tactics were assessed to better understand the physiological costs underlying these alternative tactics. In this chapter, the impacts of some other male life-history traits (i.e., male body size and age) on the physiological status of males were also evaluated. In Chapter VI, we investigated whether
differences in initial lipid reserves (i.e., the capital breeding budget) is related to the behavioural tactic. Considering the high energetic cost of territorial defence (e.g., Plaistow & Siva-Jothy 1996; Plaistow & Tsubaki 2000; Kemp & Alcock 2003), we predict a relationship between lipid mass and mate-locating tactic in the sense that males having higher lipid mass are more likely to adopt the territorial tactic.

In a last step, in accordance with life history theory predicting a trade-off between resources invested in flight and reproduction (Stearns 1989; Roff 1992), we focussed on the reproductive costs induced by the different behavioural tactics and its consequences on male and female fitnesses (Chapter VII, VIII). The reproductive investment of male butterflies represents a non-negligible cost (e.g., Svärd 1985; Bissoondath & Wiklund 1996; Lauwers & Van Dyck 2006). In Chapter VII, we tested the effect of different flight activity levels and behavioural tactics as well as of male and female mate traits (i.e., body size and age) on male reproductive investment. In this chapter, we explicitly tested whether brothers tended to adopt a similar behavioural tactic suggesting a heritable component of mate-locating behaviour. In Chapter VIII, we focused on the influence of male mating history (i.e. virgin or mated) on females behaviour to minimise fitness costs. In monandrous species like *P. aegeria* (Svärd 1985), receiving a small spermatophore from a recently mated male represents a cost for the female (e.g., Lauwers & Van Dyck 2006). We tested whether females are able to discriminate between males that differ in their mating history, and whether there is some compensation in case a female received a small spermatophore.
In the general discussion (Chapter IX), we discuss the wider implications and interpretations of our results, in combination with the literature, to update our understanding of the coexistence of alternative mate locating behavioural tactics within *P.aegeria* populations based on a conditional strategy viewpoint. We discuss the genetic versus environmental influences on behavioural tactics expression and the benefits and costs of each behavioural tactic and the trade-off between flight and reproduction.
II. Methods and material: summary and illustrations

II.1. The study species: Pararge aegeria tircis

*P. aegeria* is a temperate-zone satyrine butterfly (Lepidoptera: Nymphalidae) with a distribution range covering Europe and North Africa (Figure 2d. Hill et al. 1999). Two subspecies are recognized: *P. a. tircis*, which is distributed across northern and central Europe and characterised by yellow wing spots, and *P. a. aegeria*, distributed in southern Europe and characterised by orange wing spots (Weingartner et al. 2006). Throughout Europe it is a non-threatened, widespread species (Maes & Van Dyck 1999, Asher et al. 2001). Recently, its distribution expanded northwards probably in response to global warming (Hill et al. 2001). In Belgium and The Netherlands, *P. aegeria* has also increased both in distribution and abundance over the few recent decades (Van Dyck et al. 2009).

The species is multivoltine and has a complex life cycle with different developmental pathways that correspond to different seasonal cohorts (Figure 2e.; Wiklund et al. 1983; Nylin et al. 1989, 1993, 1995; Van Dyck & Wiklund 2002). A first generation results from individuals that hibernated at the pupal stage (resulting in adults from March to May) or the larval stage (adults from May to June). The second generation results from directly developing individuals (adults fly from July to October). Overwintering pupae are described as the
progeny of the first emergence of the second generation and overwintering larvae as the progeny of the last emergence. Both temperature and daylength are important in determining whether individuals enter diapause or develop directly (Shreeve 1986; Nylin et al. 1989). The advantage of pupal diapause relates to an early start the next year with the possibility of producing two generations of offspring and a lower mortality risk from freezing (Wiklund & Friberg 2011). However, the pupal stage seems to be more vulnerable to parasitism than the larval stage (Shreeve 1986). On the other hand, at this stage, no information is available, to the best of our knowledge, about the direct costs induced by hibernating versus direct development, but, Wiklund & Friberg (2011) showed lower mortality (i.e. a higher peak of population) and bigger individuals in the summer (i.e. for individuals of the direct development) suggesting lower costs for the direct developmental pathway.

*P. aegeria* is a medium-sized butterfly (male wing length: c. 22 mm; fresh male body weight: c. 50 mg; Van Dyck & Matthyssen 1998). Females are on average larger than males. The dorsal wing surface is light to dark brown with small yellow spots (Figure 2a. Van Dyck 2003). Seasonal (Van Dyck & Wiklund 2002) and geographic (Merckx & Van Dyck 2006) variations in these features have been observed. Black eyespots are present in the submarginal zone of the dorsal hindwings (Shreeve 1987). Several morphological traits of males and females have a genetic basis (Van Dyck et al. 1998; Berwaerts 2004). Berwaerts et al. (2008) also showed a significant
heritable basis for take-off flight performance (i.e. fast acceleration) in males.

*P. aegeria* is a butterfly that principally occurs in different types of woodland where sunlight can penetrate to the forest floor and along woodland edges (Tolman & Lewington 1997). Recently, it also occurs in more fragmented agricultural landscape with hedgerows and other habitat types with some kind of woodland aspect (Merckx et al. 2006). Habitat fragmentation may alter microclimates (Merckx et al. 2008), and morphological variation (Berwaerts et al. 1998; Merckx & Van Dyck 2006) as well as variation in mate-locating behaviour in this species (Merckx & Van Dyck 2005).

Females usually mate only once, although a small proportion may re-mate (Wickman & Wiklund 1983). Eggs are laid on different grass species under rather humid and shady conditions (Shreeve 1986). The cryptic larvae rest under the grass blades, and feed on the edges of the leaves (Figure 2b). The green or brown pupae are formed on the host plant or on other vegetation (Figure 2c). The green pupae of *P. aegeria* are formed in green surrounding and the brown pupae in brown-grey surrounding (Van Dyck et al. 1998; Vande Velde L. personal obs.). Van Dyck et al. (1998) have observed larger adults resulting from green pupae suggesting a cost of pupal pigmentation. Adults feed mainly on honeydew and to a lesser extent on fluids of trees, rotten berries, and nectar. Both males and females frequently interrupt their flights for dorsal solar basking to regulate body temperature within a relatively high range (32-34°C) allowing full
activity (Shreeve 1984; Van Dyck & Matthysen 1998). Males locate females by perching or by patrolling behaviour, which represent different flight types. Perching requires multiple short, explosive flights with high levels of acceleration and manoeuvrability in order to approach a passer-by in a fast way, whereas patrolling is done by long-lasting flights at constant and lower speed (Van Dyck et al. 1997b; Berwaerts et al. 2006). In woodland, there is a clear dichotomy between perching and patrolling. In fragmented agricultural landscapes, males have been shown to make more and wider local movements, associated with patrolling and with an intermediate mate-locating behaviour combining the traits of both perching and patrolling tactics (Merckx & Van Dyck 2005).

*P. aegeria tircis* is an interesting model to study ARTs in butterflies as both behavioural tactics co-occur in one population. Moreover, the ecology and life history of the species are well known (Watt & Boggs 2003). *P. aegeria* has in particular become a popular model organism for studies of insect thermal and behavioural ecology, especially in the framework of male-male competition (e.g., Davies 1978, Kemp et al. 2006a,b), alternative mate locating tactics (e.g., Wickman & Wiklund 1983; Shreeve 1987; Van Dyck et al. 1997a,b), and flight ecophysiology (e.g., Berwaerts et al. 2002, 2006; Merckx et al. 2006; Shreeve 1984; Stutt & Willmer 1998; Kemp et al. 2006).
Figure 2. *Pararge aegeria tircis*: a. Morphology of adult males and females (Tolman & Lewington 1999); b. caterpillar; c. pupa (green colour form); d. actual geographical distribution of *P. aegeria* in Europe (Hill et al. 1999); e. phenology of adults (first generation from April to June with individuals resulting from a winter diapause in the pupal (first emergence) and caterpillar stage (last emergence); second generation from July to September with individuals from direct development).
II.2. From field to lab experiments

During this study, we have recorded empirical data from the field, from observations under semi-natural conditions in outdoor flight cages and from experimental work in the laboratory.

The data of the chapters III and IV were collected on the field. Behavioural observations and measurements were done in sunlit spots in the woodland ‘Bois de Lauzelle’ in central Belgium (Ottignies/Louvain-la-Neuve, 50°67’N, 4°59’E). This mixed woodland (220 ha) is principally composed of three habitat types: (i) mature, deciduous stand dominated by beech *Fagus sylvatica* and oak *Quercus robur*; (ii) coniferous stand dominated by spruce *Picea abies* and larch *Larix decidua*; and (iii) woodland tracks.

For all the other chapters, a breeding stock was established with founding females from the Lauzelle woodland population. The breeding was performed under standard conditions in a climate room (Light-Dark: 16h-8h; 24°C during the day and 16 °C at night). Females were allowed to oviposit on potted tufts of the host grass *Poa trivialis* in small cages (0.30 m$^3$) in the laboratory. *P. trivialis* was grown on a standard soil mixture in a greenhouse. Eggs were collected and transferred to Petri dishes. Hatched larvae were placed on the potted host grass (5-6 larvae per potted plant) covered with a fine-meshed netting. They were allowed to develop directly without any diapause. Pupae were individually placed in small transparent jars (125 ml) until adult eclosion (Figure 3).
In order to assess the behavioural tactics of the males (see Chapter V, VI and VII), they were placed into two identical semi-cylindrical outdoor cages (9.0 x 3.7 x 1.8 m). Camouflage netting with some holes on the cages created dappled light conditions like in a forest with sunlit patches. The floors of the cages were covered by wood chips and there were small, green artificial trees that were used as perch by the butterflies (Figure 4). There were 6 males per outdoor cage per session. We did multiple observation sessions per group of males to score the behaviour of each male. This allowed assigning each male to one of two types: 1) a permanent percher (i.e., high fidelity to a sunlit patch, high level of aggression, high frequency of short inspection flights), and 2) a non-percher (i.e., males that were
rarely or never observed to defend a sunlit patch and that regularly flew through the cage, eventually alternated by resting on the netting). Some males also showed a mixed behaviour (i.e., males that defended a sunlit patch during one period in their stay in the cage, but behaved as a non-percher for most of the time in the cage). These males were used in Chapters V and VII as lipids or spermatophore traits were compared in function of the intensity of territorial defence, but were not included in the chapter VI as we observed the impact of male status on the probability to adopt the extreme territorial versus non-territorial tactics. From the human’s perspective, the forest conditions created in the outdoor cages seem far from natural conditions. However, from the butterfly’s perspective, the contrast of light resulting from the holes in the camouflage netting appears sufficient to induce natural perching behaviour as demonstrated in previous studies on *P. aegeria* behaviour (Leimar et al. 2003; Merckx et al. 2003; Kemp et al. 2006a). Nevertheless, it is less clear for the patrolling behaviour, limits of the cages preventing long, continuous flights. Biais induced by these semi-natural conditions were taking into account for results interpretations and discussed in concerned chapters.
Physiological and metabolic costs relative to the behavioural tactics and other individual traits were estimated in the laboratory (see Chapter V, VI). Lipid extractions were performed by placing individuals in refluxing diethyl ether in a Soxhlet apparatus (Figure 5). The method simply consists of comparing male dry body mass (without wings) before and after lipid extraction to obtain the total lipid mass remaining in the individual (Marden 1989).
Figure 5. Lipid extraction procedure: schema of the Soxhlet apparatus. Butterfly bodies were placed individually in paper filter bags. The bags were placed in refluxing diethyl ether of a Soxhlet apparatus for 8 h to extract all lipids. The figure shows the details of the flux of the diethyl ether through the Soxhlet apparatus placed on a warm source; the vapour of the solvent rises and cools down in the condenser. Lipids were extracted from the individuals placed in the paper thimble and fall down on the bottom of the balloon.

The resting metabolic rate of males was obtained by measuring the CO₂ emission of butterflies within a flow-through system using dual-sensor oxygen analyser (Sable Systems International Oxzilla II) connected to a CO₂ analyser (Sable Systems CA-10a) (Figure 6). After having placed the individual in the metabolic chamber (volume:
25 cl), we flushed it with CO$_2$-free and H$_2$O-free air. Water and CO$_2$ were removed from the chamber by air going through three successive columns containing silicagel, drierite and ascarite, respectively. When all CO$_2$ was removed, we shifted the air flow on a shortcut circuit during 10 min (‘baseline’; period between M1 and M2 on Figure 6c). During this period, the individual was maintained at rest (without active flight or walking) in the closed chamber. After 10 min, the chamber was flushed again at a flow rate of 630 ml/min (Sable Systems International subsample TR-SS3 pump) and the CO$_2$ gas accumulated during this period in the chamber was measured (i.e. CO$_2$ gas corresponding to air consumption by the butterfly during 10 min; period between M2 and M3 on Figure 6c). Ambient temperature was kept stable at 30 ± 0.1°C (Physitemp Model BAT-12 digital thermometer).
Figure 6. Respirometry procedure; a. & b. picture and scheme of the air flow-through respirometer (Sable Systems International). Water and CO$_2$ were removed from the recipient by air going through three successive columns containing silicagel (1), drierite (1) and ascarite (2), respectively. After, air was flushed by the TR-SS3 pump (3) to the metabolic chamber (4) and the CO$_2$ analyser (5); c. The CO$_2$ graph obtained after 10 min of baseline (i.e. air flush through shortcut circuit; between M1 and M2) and next the air flush through the chamber (i.e. measure of CO$_2$ consumption of the butterfly at rest during 10 min; between M2 and M3).
Reproductive costs related to the behavioural tactics were estimated by measuring the spermatophore mass, the number of eupyrene sperm (i.e., fertile sperm) and the copulation duration of males in the laboratory (see Chapter VI, VII, VIII). Mating sessions were performed in small cages (0.3 m³) under standardized climate room conditions (see above) (Figure 7a,b). Spermatophore traits were obtained by dissection of the female abdomen frozen directly after copulation. Spermatophores were removed from the bursa copulatrix of females and weighed (Figure 7c,d). Next, the spermatophore wall was ruptured by forceps to release the sperm that was gently stirred with a fine needle. Eupyrene sperm bundles were coloured with 4′,6′-diamidino-2-phenylindole and counted using a Epifluorescence Polyvar microscope (Figure 7e). For practical reason, we have chosen to use this method to observe eupyrene sperm in order to increase counting precision. Counting eupyrene sperm without fixation and coloration was possible, but resulted in low measurement accuracy. Consequently, apyrene sperm was not considered as it necessitates further dilutions impossible after fixation of eupyrene sperm.
Figure 7. Reproductive experiments: a. pairs were placed in small cages (0.3 m$^3$); b. Mating; c. Corpus bursae (x 12; Binocular); d. Spermatophore after dissection from the bursa (x 25; Binocular); e. Eupyrene sperm bundles coloured with 4',6'-diamidino-2-phenylindole (x 100; Epifluorescence Polyvar).

II.3. Statistical approach

In chapter III to VII, we mainly used AICc model selection to determine the influence of various factors on the response of interest. This approach has many advantages over more classical stepwise approaches that end up with a unique "best model" with a sometimes unrealistic dichotomy between important and non important explanatory variables (while several other models could be comparable) and in which the standard errors and model coefficients are biased. Indeed, repeated null hypothesis testing gives misleading and no longer valid p values. The alpha level must be adjusted with
any method but it becomes very rapidly extremely low and hardly usable as the number of tests increases.

In all cases, we fitted the global model and chosen submodels (consisting of selected possible combinations of the explanatory variables) and computed for each model the AICc value and the AICc weight (see details below). Except in chapter III, no single model was clearly more supported than the others (i.e. a large number of models had similar AICc support from the data), then, we used the model averaging approach to quantify the effect of each explanatory variable by its AICc weight.

We discuss here the philosophy of the AICc model selection and model averaging based on Anderson (2008) and Burnham & Anderson (2002) and the way we interpreted the results.

II.3.1. AICc model selection

The AIC (Akaike information criterion) is an estimator of the information lost when you represent the full reality (“the truth”) by a model. The AIC is based on the log likelihood of the model penalized for the number of parameters included in this model:

$$AIC = -2 \log(L(\hat{\theta} | x)) + 2K$$

where K is the number of parameters in the statistical model, and L(\theta) is the maximum value of the likelihood function for the estimated model.
The ‘best model’ is closest to full reality and, therefore, the goal is to find the model where AIC is smallest.
Nevertheless, AIC may perform poorly if there are too many estimated parameters in relation to the size of the sample. AICc values correspond to AIC values corrected for small samples. Consequently, it is recommended to always use this criterion in practice:

$$AICc = AIC + \frac{2K(K + 1)}{n - K - 1}$$

where \(n\) is the sample size.

It is not the absolute size of the AICc value but the relative values (i.e. delta AICc) that are important for the interpretation. AICc value is the distance from each of the models to full reality, whereas the \(\Delta AICc\) values relate to the distance between each of the models to the best one (i.e. this value corresponds to the difference of the model AICc and the minimum AICc in the set of models considered):

$$\Delta_i = AICc_i - AICc_{\text{min}}$$

This transformation forces the best model to have \(\Delta AICc = 0\), while the rest of the models have positive values. The larger the \(\Delta AICc\), the less plausible is fitted model \(i\) as being the best approximating model in the candidate set. A widely used empirical rule to determine the models to take into account is to consider the models with \(\Delta AICc < 2\). Even if this rule might be useful, the AICc weight of each model (\(W\)) gives a way to calibrate the \(\Delta AICc\) values. The weight of a model is the probability for a model to be selected as the best model in this set of models and conditional to the dataset used, based on the AICc criterion (hence the sum of AICc weight = 1):
The model weights allow to precisely quantify the respective probability of the different hypotheses (models) via evidence ratios \( (E) \). An evidence ratio of special interest is between the estimated best model (min) and some other model \( i \):

\[
E_{\min i} = \frac{w_{\min}}{w_i} = e^{(-\frac{1}{2} \Delta_i)}
\]

For example, assume the evidence ratio = AICc weight min / AICc weight \( i = 4 \). This measure indicates that support for the best model is 4 times that of the model \( i \). Evidence ratios allow to quantify precisely the model selection uncertainty, i.e. the probability for each model to be selected as best model, but we should not use these as threshold values.

### II.3.2. Model averaging

We might ask why we want to base the entire inference on the (estimated) best model when there is usually uncertainty in the selection as to be the “best” model. The model averaging approach permits to consider a set of “probable models” instead of the unique “best model”. This allows to use the entire information of the whole set of models without any need of a threshold. The method consists of calculating the AICc weight of each variable, which is computed as the sum of the AICc weight of all the models where this variable appears. This variable weight is an estimate of the relative importance of each explanatory variable in terms of predictive power. Model
averaged coefficients are estimated as the mean of each parameter from each model, weighted by the model weight. A similar approach is used to estimate the standard errors of the model coefficients that takes into account model selection uncertainty and was not conditional to one model.

Nevertheless, interpretation problems of variables weights arise when each variable is not present in the same number of models; a specific case is the variables (e.g. interaction terms) that are present in fewer models, because such variables are a priori penalized, their AICc weight being obtained as a sum of fewer terms; this is particularly true when model selection uncertainty is high. We therefore used a permutation test (N. Schtickzelle and G. San Martín y Gomez, unpublished) to help the interpretation of the variables weights by assessing whether the observed weight of each explanatory variable is significantly higher than expected under the null hypothesis (no relation between the response and the explanatory variables). This test comprises randomly shuffling the response variable column (500 random permutations for analyses in Chapter IV, 1000 random permutations for analyses in Chapter V to VII), disrupting any relationship that could exist with explanatory variable, and fitting the full set of models to compute the weight of all explanatory variables. From this simulated distribution of the AICc weight of each variable under the null hypothesis, a p-value was computed for each explanatory variable, representing the probability to obtain a value equivalent or higher than the observed AICc weight of the variable if
it has no predictive value on the response. In our study, variables with a p-value < 0.05 were considered as significant.

For some explanatory variables (mainly interaction terms with p-value between 0.04 and 0.10) we sometimes observed a high incertitude of the parameter estimate (i.e. standard error ≥ parameter estimate) while they were classified as (marginally) significant by the model averaging. This apparently paradoxical observation could be the result of a parameter estimate being quite different in the different models where it is included; this can happen when two explanatory variables are not independent from each other, leading for example to a positive estimate for the variable A when variable B is included, and a negative estimate for A when B is absent. Because the standard error of the multimodel averaged parameter estimate includes the model selection uncertainty, i.e. the variation of the parameter estimate between different models, it will be estimated at a relatively large value. But at the same time, the AICc weight of the variable can be high if the variable has a “significant” effect in highly supported models, independently of the direction of its effect (positive or negative). In practice, as the support of these variables was generally marginal, we did not focus on these factors in the discussion.
Chapter III

III. Body temperature and territory selection by males of the speckled wood butterfly (*Pararge aegeria*): what makes a forest sunlit patch a rendezvous site?

Lesley Vande Velde, Camille Turlure and Hans Van Dyck

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In this chapter, we studied territory selection in *Pararge aegeria* in order to better understand which sunlit spot-related traits perching males select in this context. Holding a territory may represent a benefit as female detection and flight activity within temperate, structurally heterogeneous forests are expected to be facilitated on such warm and well irradiated spots. Although this chapter does not focus on a comparison between the behavioural tactics, it provides a more detailed insight into the conditional factors that are of significance for the perching tactic. This insight will be important for later chapters.

**Author contribution:** Study design: L.V.V. and H.V.D.; Field/lab work: L.V.V.; Data analysis: L.V.V. and C.T.; Interpretation and writing: L.V.V., C.T. and H.V.D.

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Abstract

Insects locate mobile resources like prey items or mates using either sit-and-wait (‘perching’) or active (‘patrolling’) searching tactics. The sit-and-wait tactic can be accompanied by defending and monopolizing a site through territorial behaviour. The present study focuses on the territorial perching behaviour in males of the speckled wood butterfly (*Pararge aegeria* L.). Recent studies suggested that the selection of territories (i.e. sunlit patches on the forest floor) is driven by structural characteristics of the site that affect male visual detection. However, given that adult butterflies are heliothermic organisms and that forests provide a diverse array of light environments, it seems likely that thermal aspects may also be used for territory selection. We tested whether used and unused sunlit patches differed in thermal profile under field conditions in a Belgian woodland. We also used dummy butterflies to quantify variation in operative thoracic temperature and to calculate heating rates within (i.e. different vegetation structures) and between patches. Sunlit patches occupied by a territorial male were larger, and were more frequently characterized by low vegetation structures compared with empty sunlit patches. It took longer to reach optimal thorax temperature (starting from a fixed suboptimal body temperature) in small patches compared to large patches. We suggest that aspects of visual detection need to be combined with thermal aspects to fully understand territory selection in the speckled wood butterfly, as synergetic and/or trade-off effects of ambient temperature, solar radiation, and canopy/vegetation structure may be involved.
III.1. Introduction

Animals, including insects, usually adopt a variant of two basic tactics for locating mobile resources like prey or mates. They either sit-and-wait or they search for resources (Huey & Pianka 1981). The spatial and temporal distribution of the resources may favour one of the two behavioural tactics to maximize foraging or mate-locating efficiency and, ultimately, fitness. Environmental conditions other than the resources per se (e.g. ambient temperature) may further affect, and even constrain, the use and success yielded by waiting or searching tactics. Waiting can be accompanied by defending and monopolising a site through territorial behaviour (Baker 1983; Maher & Lott 1995).

Males of several insect species occupy and defend encounter sites as part of their mate-locating behaviour (Thornhill & Alcock 1983). Butterflies are no exception; males locate mates by perching on a particular site or by patrolling behaviour, referring to the sit-and-wait vs. search dichotomy, respectively (Scott 1974). In some butterflies, males are either perchers or patrollers, whereas in others both tactics coexist within a population but frequencies may vary with environmental conditions (Shreeve 1992; Wiklund 2003). However, the evolution of perching, and more particular territorial perching, would require a higher predictability of receptive females at particular encounter sites in the environment. Under certain circumstances, males may defend resource-based territories. Such sites can be associated with larval or adult food resources, with thermal requirements or with female emergence sites (Dennis & Shreeve...
When there are no obvious resources that spatially or temporally concentrate receptive females at certain identifiable encounter sites, males may alternatively settle at non-resource based encounter sites (Parker 1978; Thornhill & Alcock 1983). Such sites typically include well-defined topographical structures (e.g. hilltops – Lederhouse 1982) or vegetation structures (e.g. tree tops – Alcock & O’Neil 1987) as easily identifiable landmarks. Although rendezvous sites can be relatively easy to identify at the species level, the selection criteria of individual males for choosing a particular spot have received less attention. It has, however, been noticed that the very same sites can be used repeatedly across the season and even across successive generations suggesting consistent territorial preferences (e.g. Alcock 1983). Perch availability, proximity of competing males, female behaviour, as well as environmental conditions such as temperature, sunlight conditions, and wind speed may affect territory selection by males (e.g. Switzer & Walters 1999). Although it is a significant component of habitat use in males and females, few studies on insects have quantified the environmental factors affecting territory selection (Dennis et al. 2003; Turlure & Van Dyck 2009; but see, for example, Ravenscroft 1994; Rutowski et al. 2001).

Here, we address the issue of territory selection in the speckled wood butterfly (Pararge aegeria L.). In woodland populations, perching males occupy sunlit patches on the forest floor and often fight over residency (e.g. Wickman & Wiklund 1983; Kemp & Wiklund 2004). As in many other species, receptive females are not expected to have a random distribution in space and time (Parker 1988; Rutowski 1991; Wickman 2009).
Hence, male mating success of territory holders will be influenced by territory location relative to female movement patterns (Rutowski 1984; Wickman & Rutowski 1999; Wiklund 2003). Moreover, butterflies are winged heliotherms and their flight performance directly depends on the temperature of the thorax, which contains the flight muscles (Heinrich 1993; Merckx et al. 2006). Take-off flight performance from a sitting position on a perch is energetically highly demanding (Dudley 2000) and thorax temperature has been shown to have a significant impact on take-off flight performance in *P. aegeria* (Berwaerts & Van Dyck 2004). Butterflies can, however, alter their behaviour including substrate choice, position, and activities, in order to maintain their thoracic temperature inside a relatively narrow range over a wide range of air and substrate temperatures. This is highly relevant for mate location behaviour (e.g. Rutowski et al. 1994), and males may hence select sunlit patches based on characteristics that favour behavioural thermoregulation in order to respond quickly to passing females or intruding males.

Woodlands typically represent considerable microclimatic variance within and between structural patches, including clearing and edge effects (Chen et al. 1999). Particularly, the diverse arrays of sunlit environments greatly vary in light intensity and spectral composition (Endler 1993). The structural complexity of forests (e.g. clearings, dense stands, stands with varying canopy cover, tracks) may force a butterfly to move through many different ambient light and temperature conditions in a short period of time (Douglas et al. 2007) and may offer different functional environments for territory holding males among different sunlit patches within the same forest. Since
vision plays a significant role in butterfly mate location (Rutowski 1991, 2000; Rutowski et al. 2001), sunlit patch characteristics may also affect visual performance of territory holders and ultimately their mating success. Several studies suggest higher mating rates in territorial males compared to non-territorial patrolling males (e.g. Davies 1978; Fischer & Fiedler 2001; Bergman et al. 2007). However, Shreeve (1984) found that patrolling *P. aegeria* males located females at a faster rate than perched males, but flights were restricted by the necessity to maintain high body temperatures. Recent experiments with *P. aegeria* show evidence for the hypothesis of more efficient visual detection in large sunlit patches (Bergman & Wiklund 2009a). They conclude that light conditions improve the ability of males to detect and pursue conspecifics, which explains why *P. aegeria* males defend territories in large rather than small sunlit spots in forest habitats.

We agree on the significance of environmental characteristics of sunlit patches for visual detection, but its role may not exclude complementary functional aspects that relate to thermoregulation. Therefore, we test whether used and unused sunlit patches differed in thermal profile in a woodland population of *P. aegeria*. We test the hypothesis that larger sunlit patches are preferred by males for thermal reasons. The study by Bergman and Wiklund (2009a) was carried out in northern Europe (Sweden), whereas we worked at lower latitude (Belgium) representing different climatic conditions. Moreover, they did not study thermal properties of sunlit patches. We used dummy butterflies to quantify variation in operative thoracic temperature within patches (i.e. different substrates) and between patches in a
standardised way (Rutowski et al. 1994). We discuss the results on thermal aspects in relation to vision and other functional aspects in order to develop a better understanding of sunlit patch selection in this territorial woodland butterfly. Finally, we discuss perspectives for further behavioural work in this context.

**III.2. Material and methods**

*Study species*

The speckled wood (*Pararge aegeria* L.) is a satyrine butterfly that primarily occurs in woodlands. The species is multivoltine and has a complex life cycle with different developmental pathways that correspond to different seasonal cohorts (Wiklund et al. 1983; Van Dyck & Wiklund 2002). Adults feed mainly on honeydew and to a lesser extent on fluids of trees, rotten berries, and nectar. Females usually mate only once (Wickman & Wiklund 1983). Eggs are laid on different grass species under rather humid and shady conditions (Shreeve 1986). Both males and females frequently interrupt their flights for solar basking to keep body temperature high for full activity (Shreeve 1984; Van Dyck & Matthysen 1998). For a detailed discussion of the mate-location behaviour in males we refer to Van Dyck (2003).

*Study area*

This study was conducted in the summer of 2008 in a mixed woodland (220 ha) in central Belgium (Bois de Lauzelle, Ottignies/Louvain-la-Neuve – 50°67’74”N, 4°59’78”E). Hence, this
summer cohort of adults was individuals that developed directly without a larval or pupal diapause. Field data were collected in three different vegetation types that have sunlit patches available to *P. aegeria*: (i) mature, deciduous stand dominated by beech *Fagus sylvatica* and oak *Quercus robur*; (ii) coniferous stand dominated by spruce *Picea abies* and larch *Larix decidua*; and (iii) woodland tracks.

*Characteristics of sunlit patches*

Between 6 August and 18 September, we characterised several environmental characteristics of a total of 86 sunlit patches (36 in deciduous forest, 20 in coniferous forest and 30 on woodland tracks). We only considered sunlit patches that existed for at least 2 hours and that were > 0.5 m². During days with fine weather conditions that would allow butterfly activity (> 16°C, low degree of cloudiness), we recorded the following characteristics of a sunlit patch: (i) the length *L* and the width *W* of each sunspot were measured using a yardstick (precision: ± 0.01 m) and the area of the sunlit patch was calculated assuming an ellipsoid area (i.e. Area = *L*·*W*·π/4); (ii) the vegetation within the sunlit patch was characterized using three categories that reflect the successional stage 1) litter, herbs and low shrubs (< 0.5 m; short herb layer SHL), 2) tall herbs and shrubs (0.5-1.0 m; tall herb layer THL), and 3) shrubs and small trees (>1.0 m; shrub layer SL); (iii) the substrate temperature of the main substrates within the sunlit patch and the air temperature, at 1 m height within the sunlit patch and outside the sunlit patch under completely shaded conditions, were measured (Physitemp thermometer with a fine probe, type BT-1, accuracy: ± 0.1 °C); (iv) the canopy cover was measured with a
convex spherical densitometer (Model A) at three randomly chosen points in the sunlit patch, and the average value was calculated, for several sunlit spots of each forest habitat type.

We recorded whether a sunlit patch was occupied or not by a territorial *P. aegeria* male. In case there was a male sitting on the patch, we tested his willingness to return to the patch after some disturbance by throwing a small stick in the patch (see Van Dyck et al. 1997b). This allows distinguishing between patrolling males that briefly visit a patch and territorial perching males. Additionally, a subset of the 18 occupied sunlit patches was visited repeatedly over several days. The presence of a territory holder in each of the sunlit patches was recorded. This allowed us to calculate a measure of the persistence of territory occupancy (i.e. the ratio of the number of presences on the number of visits).

*Thermal properties of sunlit patch*

Optimal thoracic temperature for active flight in *P. aegeria* males ranges between 32 and 34.5°C (Shreeve 1984; Van Dyck & Matthysen 1998). To estimate the thermal significance of each sunlit patch (n = 86, see above), we recorded the time needed to raise thoracic temperature from suboptimal (i.e. 25°C) to optimal (i.e. 33°C) using dummy *P. aegeria* butterflies that were placed at a relevant substrate in the sunlit patch. A dummy is a recently killed *P. aegeria* male specimen with the wings dorsally spread to mimic dorsal baskiing, with the thorax connected to a thermometer to monitor body temperature via a hypodermic needle containing a thermocouple (Physitemp, type MT-29/1B, accuracy: ± 0.1 °C). Although such
dummies do not exactly reflect body temperatures as in living butterflies, they are highly useful models to quantify relevant variation in thermal conditions under field conditions in a standardised way (Heinrich 1986). For details and further justification of this method we refer to Rutowski et al. (1994) and Merckx et al. (2008).

**Statistical analyses**

As the three ambient temperature measures for a sunlit patch (see above) were highly correlated (Pearson correlation tests: all $P < 0.0001$), we used only air temperature inside the sunlit patch for the analyses to avoid problems of multicollinearity. Logistic regression models were used to analyze the presence of *P. aegeria* territory holders relative to the characteristics of the sunlit patch (i.e. area, substrate structure, air temperature, and interactions between forest habitat type and these factors). Linear regression models (with Poisson distribution, log link function) were used to relate the persistence of territory occupancy of a sunlit patch to its characteristics (i.e. area, substrate structure and interaction between forest habitat type and these factors). Finally, linear regression models (with normal distribution) were used to analyze dummy butterfly heating rate relative to sunlit patch area, substrate structure, air temperature and interactions between forest habitat type and these factors). For the three analyses, we computed each time all possible models, and the best one was selected using the AIC criterion (models with $\Delta$AIC $< 2$) and the parsimonious principle (Burnham & Anderson 2002). The factor ‘forest habitat type’ was not included as a main factor as the number of occupied versus unoccupied sunlit spots was
experimentally determined in each habitat type. Means are given ± S.E.

III.3. Results

Sunlit patches in deciduous and coniferous parts of the forest had similar degrees of canopy cover, whereas it was significantly lower for sunlit patches on woodland tracks ($F_{2,20} = 28.05$, $P < 0.0001$; Fig. 1).

![Figure 1. Mean canopy cover (measured with a convex spherical densitometer) in sunlit spots according to forest habitat type. Means and 95% confidence intervals are given.](image)

The logistic regression models for sunlit patch occupancy by a *P. aegeria* territory holder (Tables 1 and 2) showed: (i) a higher incidence for larger sunlit patches (the area of the sunlit patches
ranged from 0.5 to 314.2 m$^2$ and was of 24.98 $\pm$ 7.33 m$^2$ and 12.98 $\pm$ 2.62 m$^2$ for occupied and unoccupied patches, respectively, despite a relative incertitude on the parameter estimate; (ii) a higher incidence under warmer conditions; and (iii) a lower incidence for a vegetation with more abundant shrubs and small trees compared with an earlier successional vegetation structure. The best model also showed an interaction effect between forest habitat type and the substrate structure composition. However, the interpretation of this interaction effect is somewhat complicated by unavoidable bias in vegetation structure among the forest types (i.e. most of the substrate structure of the coniferous forest was tall herb layer), as the distribution of the vegetation or substrate structure among the three forest habitat type was not homogeneous ($\chi^2 = 21.24, P < 0.0001$; Fig. 2).
### Figure 2

Substrate structure in the sunlit spot selected according to forest habitat types. THL, tall herb layer; SHL, ground/short herb layer; SL, shrub/tree layer.

<table>
<thead>
<tr>
<th>Habitat Type</th>
<th>SL</th>
<th>SHL</th>
<th>THL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Woodland pathway</td>
<td>30.0%</td>
<td>43.3%</td>
<td>26.7%</td>
</tr>
<tr>
<td>Deciduous forest</td>
<td>11.1%</td>
<td>72.2%</td>
<td>16.7%</td>
</tr>
<tr>
<td>Coniferous forest</td>
<td>9.5%</td>
<td>85.7%</td>
<td>4.8%</td>
</tr>
<tr>
<td>Variables in the model</td>
<td>k</td>
<td>AIC</td>
<td>ΔAIC</td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>----</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Area + Air temperature + Substrate structure + Substrate structure x Habitat type ***</td>
<td>9</td>
<td>117.98</td>
<td>0.00</td>
</tr>
<tr>
<td>Area + Air temperature + Substrate structure + Area x Habitat type + Substrate structure x Habitat type ***</td>
<td>11</td>
<td>115.98</td>
<td>1.00</td>
</tr>
<tr>
<td>Air temperature + Substrate structure + Substrate structure x Habitat type</td>
<td>8</td>
<td>120.66</td>
<td>2.68</td>
</tr>
<tr>
<td>Area + Air temperature</td>
<td>3</td>
<td>120.98</td>
<td>3.00</td>
</tr>
<tr>
<td>Area + Air temperature + Substrate structure + Air temperature x Habitat type + Substrate structure x Habitat type</td>
<td>11</td>
<td>121.16</td>
<td>3.18</td>
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<td>121.24</td>
<td>4.26</td>
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<td>121.46</td>
<td>4.48</td>
</tr>
<tr>
<td>Air temperature</td>
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<td>121.00</td>
<td>5.02</td>
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<td>Intercept only</td>
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<td>121.22</td>
<td>5.24</td>
</tr>
<tr>
<td>Substrate structure + Substrate structure x Habitat type</td>
<td>7</td>
<td>121.49</td>
<td>5.51</td>
</tr>
<tr>
<td>Area + Air temperature + Area x Habitat type</td>
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<td>123.95</td>
<td>5.97</td>
</tr>
<tr>
<td>Area + Air temperature + Substrate structure</td>
<td>5</td>
<td>124.11</td>
<td>6.13</td>
</tr>
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Table 1. Logistic regression models of sunlit spot occupancy according to sunlit spot characteristics (i.e. area, substrate structure, air temperature and interaction between forest habitat type and these factors). The table presented for each model: the list of variables used, the number of parameters (k), the AIC value of the model and the difference (Δ) of AIC with the lowest-AIC model. Supported models are indicated by *** and selected best model is in bold.

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Parameter estimate</th>
<th>S.E.</th>
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Table 2. Factors affecting probability of sunlit spot occupancy (estimated using best model from Table 1). For categorical variables, the estimate expresses the difference from the reference level (fixed to zero). THL, tall herb layer; SHL, ground/short herb layer; SL, shrub/tree layer.

Territory occupancy persistence based on the repeated visits of a selection to sunlit patches could, however, not well be explained by the parameters tested in our study since the best model was the one with only the intercept (Table 3).
Table 3. Logistic regression models of sunlit spot occupancy persistence according to sunlit spot characteristics (i.e. area, substrate structure and interaction between forest habitat type and these factors). The table presented for each model: the list of variables used, the number of parameters (k), the AIC value of the model and the difference (Δ) of AIC with the lowest-AIC model. No model was supported since the model with intercept only had the lowest AIC value.

<table>
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</table>

The time to reach an optimal operative thorax temperature starting from a fixed suboptimal temperature was on average 98.52 ± 14.08 s and 80.44 ± 13.23 s in an unoccupied versus occupied patch, respectively. Linear regression models (Tables 4 and 5) showed: (i) faster heating in dummy butterflies with higher air temperature; (ii) higher heating rate on ground/short herb layer structure compared to tall herb layer and shrub/tree layer; and (iii) faster heating in dummy butterflies placed in large sunlit patches, despite a relative incertitude on the parameter estimate.
<table>
<thead>
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Table 4. Logistic regression models of the heating time of dummy butterflies in sunlit spots according to sunlit spot characteristics (i.e. area, substrate structure, air temperature and interaction between forest habitat type and these factors). The table presented for each model: the list of variables used, the number of parameters (k), the AIC value of the model and the difference (Δ) of AIC with the lowest-AIC model. Supported models are indicated by *** and selected best model is in bold.

Table 5. Factors affecting the heating time of dummy butterflies in sunlit spots (estimated using the best model from Table 4). For categorical variables, the estimate expresses the difference from the reference level (fixed to zero). THL: tall herb layer. SHL: ground/short herb layer. SL: shrub/tree layer

III.4. Discussion

Butterflies are winged heliotherms, which renders environmental conditions highly significant for their (flight) behaviour (Kingsolver & Watt 1983; Dennis 1993). This is particularly true in temperate-zone regions where ambient temperature only rarely approaches optimal operative thorax temperatures for butterflies (Shreeve 1984). Moreover, *P. aegeria* are active under the typical dappled light environments of forests offering strong thermal heterogeneity between sunlit and shaded forest parts. Not all sunlit
patches are as likely to be occupied by a perching male. Here, we show evidence of thermal differences between sunlit patches that were used by perching males and those that were not.

During short inspection flights through the sunlit patch and during fights and pursuit flights, thoracic temperature of males will decrease due to convective cooling (Van Dyck & Matthysen 1998). Dorsal basking is used to heat up again (Clench 1966). Reducing the time spent basking is advantageous for a perching male since a fast take-off flight towards passing females and intruders requires a starting position with the wings closed; butterflies close their wings when they have reached their optimal body temperature. Optimal body temperature will improve flight performance (Berwaerts & Van Dyck 2004). The use of sunlit patches increased with ambient temperature within the observed range (i.e. 16-30 °C) suggesting that the ability to intercept mates and intruders would increase with ambient temperature. We did not study the morphology here, but males that already settle on sunlit patches under relatively cool conditions may represent a non-random subsample. Adult morphology in *P. aegeria* differs between seasonal cohorts, and populations, that have to deal with different thermal conditions (Van Dyck & Wiklund 2002; Merckx et al. 2006).

Our study also showed an effect of vegetation structure within a sunlit patch on male body temperature and heating rate. Heating up was faster in the lower open parts of the vegetation. This vegetation was mainly composed of the low growth forms of brambles (52% of the short herb category). Whether bramble leaves differ in their reflectance spectrum relative to other plants and, hence, in local
microclimate, requires further testing. Few males were observed basking on the ground, although this represented 19% of the first vegetation structure type. However, such a substrate may be more often used under cooler conditions like in spring time (Van Dyck et al. 1997b); here we only collected data under late summer conditions. In summer, a wider range of surfaces provides suitable substrates for thermoregulation (Hardy & Dennis 2007). Alternatively, different substrates may also signify differential predation risks, but little is know on this aspect.

Similar to the study by Bergman and Wiklund (2009a) focusing on visual performance in Swedish *P. aegeria*, we found larger sunlit patches tending to be more frequently used by perching males in our Belgian population. They argued that a minimal area of illumination is required for a male to accurately and efficiently perceive and intercept females and intruders against a complex and dappled background (Rutowski 2000; Bergman et al. 2007). This suggests that sunlit patch size mainly has an effect on mate and intruder detectability since Bergman et al. (2007) found no support of female preference for landing in large sunlit patches. As our results tend to show a faster heating in larger sunlit spots, we argue that the novel insight on visual perception in differently sized sunlit patches does not exclude a complementary role of thermal properties for territory selection in *P. aegeria*. Males may even have to deal with trade-offs between characteristics of sunlit patches that facilitate thermoregulation and visual perception. If females mostly fly at > 1 m height, males may benefit from avoiding low perches to improve their visual detection probabilities (Rutowski et al. 1991; Rutowski 2000).
But higher perches may be less suitable for heating up rapidly. Such a trade-off will be more severe on days with higher wind speed, or in zones closer to the edge of the forest that have stronger wind impacts than more central zones (e.g. Wright et al. 2010). Cooler, higher substrates may be mainly used at the middle part of the day and less so during the morning or later in the day (Hardy & Dennis 2007). It illustrates the condition-dependent nature of substrate use within patches and the use of sunlit patches that vary in vegetation structure in a thermally complex environment.

The presence of trees in the sunlit patch, and hence a reduced visual overview of the territory, may also represent a cost or risk of dealing with a second resident on the patch. This cost will vary among sunlit patches as a function of the vegetation structure. We, for example, observed two males occupying the same sunlit patch where some kind of visual barrier (high shrubs and trees) occurred in the middle of the patch (L. Vande Velde, pers. obs.). When a short flight was induced, males noticed each other and engaged in long fight corresponding to escalated fights between males that both have the motivation of a resident male (Davies 1978; Kemp & Wiklund 2004). Although most studies on mate location in *P. aegeria* have focused on what is happening at the understorey level, at least part of the story is taking place at the canopy level (Dennis et al. 2009; L. Vande Velde, pers. obs.). Whether there is also perching and patrolling going on at the canopy level is, however, poorly understood.

Since ambient temperature, canopy cover, levels of solar radiation, and vegetation structure vary throughout the spring and summer season of this multivoltine butterfly, there is clearly scope for
further work on territory use in *P. aegeria*. Here, we only focused on (late) summer conditions. The relative importance of heating and cooling will vary with seasonal conditions (e.g. Dennis & Sparks 2005; Hardy & Dennis 2007), but not necessarily in a simple linear way, especially as operative temperature is influenced by interaction effects of ambient temperature, solar radiation, canopy and understorey vegetation structure, and the morphology of the butterflies (Dennis 1993; Van Dyck & Matthysen 1998). Future work should incorporate this seasonal aspect on the relative roles of thermoregulation and visual detection for territory selection in *P. aegeria*. 
IV. Flight morphology and territory selection
under abnormal seasonal weather conditions in the butterfly *Pararge aegeria*

Lesley Vande Velde, Gaëlle Rigaux, Gilles San Martin y Gomez and Van Dyck Hans

[Chapter IV is an unpublished manuscript]

In Chapter III, we have demonstrated that perching males selected sunlit spots based on characteristics that facilitate both visual perception and thermoregulation. Since perch site characteristics may vary with environmental conditions, we analysed perch selection through different seasons. More specifically, we studied how abnormal weather conditions may impact male flight morphology and perch selection behaviour relative to predictions from the literature under normal seasonal conditions.

**Author contribution:** Study design: L.V.V. and H.V.D; Field/Lab work: G.R. and L.V.V.; Data analysis: L.V.V., G.S.M. and G.R.; Interpretation and writing: L.V.V. and H.V.D.

Thanks are due to Nicolas Schtickzelle for statistical advice.
Abstract

Seasonal plasticity in morphology and life history traits is commonly observed among multivoltine insects. Seasonal environmental patterns are in principle predictable, so that insects may anticipate during development on their future adult environment in an adaptive way. In the Speckled wood butterfly (*Pararge aegeria*), a previous study has shown that males of the spring cohort have a more pronounced morphological design that relates to territorial perching behaviour than summer males. This matches with the seasonal decline of territoriality in line with increasing ambient temperature from the spring to the summer (signifying a gradual decrease of the value of a sunlit spot on the forest floor as a warm site to meet females). Here, we explored variation in flight morphology and in territory selection in the spring and the summer cohort of *P. aegeria* in a year with abnormal weather conditions (i.e. exceptionally dry and sunny spring followed by an exceptionally wet and cloudy summer). This allowed testing to what extent these abnormal climatic conditions would alter morphology and territorial perching behaviour (sunlit spot selection) of males compared to normal seasonal variation. Our results showed evidence of flexible behavioural responses in males to abnormal weather conditions, but also important inertia with respect to functional morphology. Males generally showed similar morphological traits than observed under normal seasonal conditions. By contrast, territory selection behaviour deviated from what was expected under normal seasonal conditions; sunlit spots were used as hotspots in summer while they seem to be selected to facilitate visual perception in spring.
We discuss the observations relative to male morphological and behavioural plasticity in the context of more frequent extreme weather conditions with the global warming.
IV.1. Introduction

In the temperate-zone region, climatic conditions are of particular significance to small ectotherms organisms like many insects (Heinrich 1986). Multivoltine insects (i.e. with more than one generation a year) have the opportunity to respond differently to the predictable seasonal pattern of environmental conditions. Seasonal adjustments may include life-cycle regulation, larval and adult morphology and behaviour, and they are typically the result of phenotypic plasticity (Nylin et al. 1989; Wiklund et al. 1991). Environmental cues that are used to develop the appropriate seasonal phenotype include photoperiod and temperature (Danilevski 1965; Beck 1990). In insects, there are several cases of seasonal diphenism (e.g. Shapiro 1976; Nylin et al. 1989; Nylin 1992; Wiklund et al. 1991; Nylin & Gotthard 1998 for a review), and seasonal variation is often assumed to reflect adaptive variation (Nylin et al. 1995).

Butterflies are popular study systems to address seasonal plasticity in morphology and life history (including development and life cycle regulation) (e.g. Nylin 1994; Gotthard & Nylin 1995; Nylin & Gotthard 1998). In all life stages, butterflies are mainly ectotherms. The temperature experienced during larval development is known to play a significant role for growth rate and hence for the final size of the butterfly. Higher temperatures generally produce faster growth and smaller individuals at maturity (e.g. Atkinson 1994; Sibly et al. 1997). Adult butterflies are more particularly heliotherms, so their activities can be hampered under cool and cloudy weather as their body temperature will influence the performance of the flight muscles, and
body temperature is strongly influenced by solar radiation on the exposed body parts (Clench 1966; Dennis 1993). Consequently, morphological traits related to thermoregulation may vary between spring and summer cohorts with seasonal plasticity in basal wing colour (e.g., higher degree of melanisation in cold spring compared with summer; Kingsolver & Wiernasz 1991) as a well-studied example. But seasonal variation also exists in morphological traits that relate to flight performance including body size, wing loading and wing shape (e.g. Van Dyck & Wiklund 2002). For instance, in the butterfly *P. aegeria*, both males and females have larger relative thorax mass, higher wing loading, and longer wings in the spring cohort compared to the summer cohort (Van Dyck & Wiklund 2002). While males and females showed the same pattern, the functional explanation of this seasonal effect may differ between the sexes. Under low temperature conditions, females need to invest relatively more in morphological traits to achieve a similar flight performance (e.g. dispersal and egg spreading) in spring than in summer (Azevedo et al. 1998), while spring males showed a more pronounced ‘territorial perching’ design than summer males in the context of mate-locating behaviour (Van Dyck & Wiklund 2002).

Several behavioural aspects of an adult butterfly’s life differ with season, but here we focus on territorial perching behaviour and the associated territory selection in males of the butterfly *Pararge aegeria* (L.). Both morphological design (e.g. Wickman 1992) and thermal conditions (e.g. Wickman 1988; Rutowski et al. 1994; Fischer & Fiedler 2001) have been shown to be of significance to mate-locating behaviour and to territorial perching in particular. In several
butterflies, there is evidence of weather-related changes through thermal constraints in the occurrence of territorial perching behaviour both at the diurnal level as well as the seasonal level (e.g. Wickman & Wiklund 1983; Rutowski et al. 1996; Ide 2002a,b). Individual *Coenonympha pamphilus* males compete, for example, for territories under low temperature conditions and are more likely to leave their territories to patrol over a wider area when temperatures are higher (Wickman 1985a). Similarly, in the multivoltine butterfly *Lethe diana* males show territorial behaviour mainly in spring (Ide 2002a,b). When territorial perching areas are of significance as sites to heat up (such as sunlit spots in a forest) for maintaining body temperatures well above air temperature to permit full flight activity (Shreeve 1987; Rutowski et al. 1991, 1994), such sites are most valuable at low air temperature. Under such conditions males are more likely to try monopolizing such a “hot spot” by adopting a sit-and-wait tactic (i.e., territorial perching tactic). Because flight requires high body temperatures (Heinrich 1986), flight ability is facilitated with the advancement of the season as air temperature increases. Accordingly, under higher ambient temperature, males should benefit from leaving territories to adopt a non-territorial searching tactic (patrolling tactic) in order to intercept females in flight. Then, the importance of sunspots as mating rendezvous sites and territory would decrease as ambient temperatures increases (Wickman & Wiklund 1983; Dennis 2004).

Temperature does not only affect the probability of perching and patrolling, it may also affect the way butterflies perch or patrol. Rutowski (2000) showed, for example, changes in the type of perching sites with temperature. Early in the morning and in cool
spring weather, warm sites facilitate flight activity and they can be essential for territorial defence, and ultimately, for the acquisition of receptive females (Dennis 2004). As bare ground substrates are warmer than vegetation surfaces, males prefer perching on the ground under low ambient temperature in the early spring (Dennis & Sparks 2005; Hardy & Dennis 2007). Cooler, higher substrates are likely to be used as the season warms up (Hardy & Dennis 2007). In addition to thermal properties, several studies of territorial insects also addressed the issue of visibility relative to territory site choice (Rutowski et al. 1991; Rutowski et al. 2001; Bergman et al. 2007; Bergman & Wiklund 2009a). For instance, males of *P. aegeria* selected preferentially larger sunlit spots that were shown to facilitate female detection (Bergman & Wiklund 2009a). Nevertheless, thermoregulation and visibility are not mutually exclusive factors. Vande Velde et al. (2011a) (Chapter III) demonstrated that thoracic heating time of males was faster in large sunlit spot that would also facilitate visual perception.

Seashore seasonal environmental patterns are in principle predictable, so that insects may anticipate during development on their future adult environment by using environmental cues and phenotypic plasticity to track seasonal variation in an adaptive way. Solar radiation and ambient temperature increase through the season reaching highest values in late-spring and summer, respectively (Anderson 1964). However, for forest insects these seasonal changes will interfere with bud burst and the closing of the canopy. *Pararge aegeria* males of the first generation have been shown to develop a more ‘territorial perching’- design (i.e. larger relative thorax mass, wingloading and
aspect ratio) than do summer males (Van Dyck & Wiklund 2002); this matches with a seasonal decline of territorial defence from late spring to summer in this species (Wickman & Wiklund 1983). In spring, sunlit spots at the forest floor or vegetation may be viewed as hotspots in which a male’s flight ability for take-off and aerial fighting is maximized and were females come to warm up (Wickman & Wiklund 1983; Dennis & Sparks 2005). During this cold period, sunlit spots should be selected in order to minimise the time required for reaching an optimal body temperature, and hence, optimal activity. Thoracic temperature of an active P. aegeria is within the range of 32-34.5° (Shreeve 1984; Van Dyck & Matthysen 1998). We may therefore expect a positive correlation between the use of sunlit spots and ambient temperature within it. In the summer, the difference between air and optimal body temperature decreases which, in turn, facilitates movement into the woodland (Shreeve 1984). In this period, sunlit spots are expected to be principally selected based on characteristics that increase visual perception and only secondarily on aspects that may help thermoregulation (Dennis & Sparks 2005).

Thermal biology is getting more and more attention within this era of climate change. Gradual increase in temperature has attracted much attention, but climate change also includes the increase of the frequency in extreme or ‘abnormal’ weather conditions to meteorological standards (Easterling et al. 2000; Bale et al. 2002; Menzel et al. 2006). In some parts of Europe drought events are suggested to become increasingly frequent (Morecroft et al. 2002). Infrequent and severe climatic events may have a strong biological impact including sudden distributional or phenological shifts and local
extinctions (Easterling et al. 2000; Parmesan et al. 2000; McLaughlin et al. 2002). Extreme weather events may also alter host plant quantity and quality which in turn may affect foraging success, competition, morphology and reproductive behaviour (Parmesan et al. 2000; Talloen et al. 2004). For instance, Gibbs et al. (2011) showed that drought-stressed hostplants affect survival rate, development time, morphology and reproductive output by affecting host plant quality in males and females of *P. aegeria*.

In this study, we used field data on flight-related morphology and sunlit spot territory selection in the Speckled wood butterfly (*Pararge aegeria*) to explore the impact of abnormal seasonal weather conditions in a behavioural, mate-locating context. Our measurements and field observations were obtained in central Belgium in 2010. This year was characterized by an exceptionally dry and sunny spring followed by an exceptionally wet and cloudy summer (Table 1). These conditions allowed testing whether these abnormal climatic conditions may differently impact morphology and behaviour of males compared to expectations under normal seasonal variation that predict males to have a more pronounced ‘perching design’ in spring (Van Dyck & Wiklund 2002) and defend sunlit spots that facilitate heating up in spring and that would principally facilitate detection of females and intruders in summer (e.g. Dennis & Sparks 2005). To do so, we compared morphological traits known to be important for thermoregulation (degree of basal wing melanisation; Van Dyck & Matthysen 1998) and for flight performance associated with acceleration and manoeuvrability, which has been shown to be functionally significant to territorial perchers (wing loading, body
mass, aspect ratio). As seasonal phenotypic variation in *P. aegeria* was shown to respond principally to photoperiod (Van Dyck & Wiklund 2002), we do not expect strong differences in seasonal morphology under abnormal climatic conditions compared to normal seasonal variation. However, we cannot exclude the role of other factors than photoperiod for phenotype production and plasticity on the field (e.g. host-plant quality – Kooi et al. 1996; Pellegroms et al. 2009; Gibbs et al. 2011). We also studied sunlit spot selection by males that adopt a territorial perching behaviour in spring and summer by comparing the thermal profiles and other structural characteristics of occupied and unoccupied sunlit spots (cf. Vande Velde et al. 2011a, Chapter III). Environmental characteristics of perch sites that may influence thermoregulation - such as size, thermal value, substrate type and height, relative humidity, - and visual perception of butterflies - such as size, UV and light intensity - are expected to change with the season. While the morphological traits depend on developmental decision in function of environmental cues during the larval stage, behavioural decisions may be more anticipative to the current conditions at the adult stage. Behavioural plasticity may, however, be constrained by the morphological design.
IV.2. Material and methods

Study species

The speckled wood (Pararge aegeria L.) is a satyrine butterfly with a wide European distribution range; the species primarily occurs in woodlands. Males may adopt one of two behavioural tactics to locate mates: perching and patrolling. Perching represents a sit-and-wait tactic on a sunlit patch at the forest floor which is aggressively defended by the resident male. Patrolling males fly through the forest searching for females in larger areas of the woodland. Both tactics require different types of flight performance and perching and patrolling co-occur at the population-level (Van Dyck 2003 and references therein). This multivoltine butterfly can be
seen in fly from March to October with successive generations that differ in developmental history: (1) development with a pupal winter diapause resulting in early spring adults; (2) development with a larval winter diapause resulting in late-spring adults; (3) direct development resulting in summer or second generation adults (Wiklund et al. 1983; Van Dyck & Wiklund 2002). In our study, spring males were mainly individuals of the late-spring cohorts, and hence individuals that hibernated as larva.

Study area

The study was conducted the spring of 2010 (from April 27 to June 3) and the summer of 2010 (from August 3 to August 20) in a mixed deciduous-coniferous woodland (220 ha) in central Belgium (Bois de Lauzelle, Ottignies/Louvain-la-Neuve; 50°6’7.74”N, 4°59’7.89”E). In this woodland, we recorded whether a selected sample of sunlit patches were occupied or not by territorial *P. aegeria* males.

Morphology

Samples of perching males were caught on sunlit spots (38 spring males and 19 summer males). Males were dried to constant weight at 70°C in a drying oven and weighed after removal of the wings (Mettler Toledo-MT5 balance; accuracy: ± 1 µg). For each male, we took a digital picture of the right forewing (Canon D450 camera) under standardized light conditions (i.e., cold light, optical fiber ring lamp). Basal wing melanisation, wing area and length were quantified using an image analysis system (Image J software 1.42). Wing area was measured on the four wings while the other
measurements were done on the right forewing. Degree of basal melanisation of the dorsal forewing was measured as grey values (from 0, black to 255, white). Forewing shape was measured as forewing aspect ratio being $4*\text{forewing length}^2/\text{forewing area}$ using untransformed data. Wing loading was calculated as male dry body mass/total wing area. Right forewing length was measured from the connection to the thorax to the apex of the wing. Dry body mass, wing area, wing loading, wing length, basal melanisation and aspect ratio were compared between males caught in the spring and in the summer.

**Characteristics of sunlit patches**

During days with fine weather allowing butterfly activity (>16°C, low degree of cloudiness), we recorded a number of environmental characteristics of a total in 138 (79 unoccupied vs 59 occupied) and 80 (50 unoccupied vs 30 occupied) sunlit patches in the spring and the summer, respectively. In case there was a male sitting on the patch, we tested his willingness to return to the patch after some disturbance by throwing a small stick in the patch (see Van Dyck et al. 1997b). This allows distinguishing between patrolling males that briefly visit a patch and territorial perching males. For each focal sunlit patch, we recorded: (i) the length $L$ and the width $W$ of the sunspot (using a yardstick, precision: ± 0.01 m) to calculate the area of the sunlit patch assuming an ellipsoid area (i.e. $\text{Area} = L.W.\pi/4$); (ii) the main substrate type within the sunlit patch; (iii) the height of the main substrate type (using a yardstick, precision: ± 0.01 m); (iv) the air temperature at 1 m height within the sunlit patch (Physitemp
thermometer type BT-1, accuracy: ± 0.1 °C); (v) the light intensity within the centre of the sunlit spot (764 Environmental Monitor, ELSEC, accuracy: 5% ± 1 displayed digit); (vi) the UV intensity (764 Environmental Monitor, ELSEC, accuracy: 15% ± 1 displayed digit) and; (vii) the relative humidity in the perch site (Data logger TINYTAG view2, accuracy: ± 3.5%).

**Thermal properties of sunlit patch**

To estimate the thermal significance of each of the 218 sunlit patches, we recorded the time needed to raise thoracic temperature from suboptimal (i.e. 25°C) to optimal (i.e. 33°C) using dummy *P. aegeria* butterflies that were placed at a relevant substrate in the sunlit patch (for details: see Vande Velde et al. 2011a *(Chapter III)*).

**Statistical analyses**

For comparisons of morphological traits between seasons we used student t-tests (SAS software, version 9.1). Data on wing melanisation and wing loading were transformed (logarithm) to improve normality and equality of variances, respectively. For forewing aspect ratio, we used a non-parametric Wilcoxon Two-Sample Test because equality of variance was not respected after transforming the data.

Because many environmental variables were interrelated, we used a multi-model inference approach (R software, package written by Gilles San Martin y Gomez, unpublished), based on corrected Akaike’s Information Criterion (AICc) (Burnham & Anderson 2002) to analyze sunlit patch selection. First, we fitted generalized linear
models corresponding to all possible combinations of explanatory variables (i.e. 16384 combinations). Then, the relative importance of each explanatory variable was estimated by its AICc weight. Due to the presence of interaction terms in the models, we used a permutation test (N. Schtickzelle and G. San Martin y Gomez, unpublished) to help the interpretation of the weight of the variables. A *P*-value was computed for each explanatory variable, representing the probability to obtain a value equivalent or higher to the observed AICc weight of the variable when it would have no predictive value for the response (see Chapter II for more details). Variables with a *P*-value < 0.05 were considered to be significant. Generalised linear regression models (with a binomial error distribution and logit link function) were used to analyze the sunlit patch occupancy relative to the characteristics of the sunlit patch (i.e. area, substrate type, substrate height, air temperature, light intensity, UV intensity, and relative humidity) and interaction effects with season. The factor ‘season’ was not included as a main factor as number of sunlit spots measured in each season resulted from experimental design rather than from random measurements. Generalised linear regression models (with a normal error distribution and identity link function) were used to analyze heating time in a dummy butterfly relative to the same set of sunlit patch characteristics previously listed and the interaction effects with season. UV intensity was log-transformed prior to the analysis to improve normality.
IV.3. Results

*Morphology*

Spring and summer males had the same dry body mass (Fig1a), but summer males had larger (Fig1b) and longer (Fig1c) wings than spring males and thus a lower wing loading (Fig1d). Spring males had more narrow wings (i.e. higher aspect ratio) (Fig1e) and the basal part of their dorsal wings was darker (Fig1f) than summer males.
Chapter IV

a. 

Dry body mass (mg)

N = 39, t = 0.16, P = 0.87

b. 

Wing area (mm²)

N = 39, t = -3.81, P = 0.001

c. 

Wing length (mm)

N = 57, t = -3.81, P = 0.01

Season

Spring  Summer
Wing Loading (mg/mm²)

N = 39, t = 2.39, P = 0.02

Aspect ratio

N = 57, z = -2.58, P = 0.01

Melanisation (grey value)

dark to pale

N = 57, t = -5.67, P < 0.0001

Season

Spring                         Summer
Characteristics of sunlit patches

Multi-model inference showed a strong influence of the UV intensity and air ambient temperature as well as of the interactions between season and both these factors on sunlit patch occupancy (Table 2). Patch occupancy was positively correlated with UV intensity in the spring, but slightly negatively in the summer. Sunlit patch occupancy by territorial males was positively correlated with ambient temperature in the summer while slightly negatively in the spring.
Table 2. Multi-model inference gave for each variable the frequency of the variable in the models, the AICc weight expressing the probability that the variable influences the response (here sunlit spot occupancy), the p-value evaluating the significance of the difference between the AICc weight and the a priori weight, and model-averaged parameter estimates and standard error. The significant factors influencing the response are in bold.

**Thermal properties of sunlit patch territories**

Multi-model inference for heating time of a dummy butterfly showed a strong effect of the light intensity and the interaction between season and this factor, as well as of ambient temperature and the interaction between season and this factor (Table 3). Heating was faster under high light intensity, but this effect was more pronounced.
in spring than in summer. Heating was faster with increasing air
temperature, but this effect was more pronounced in summer than in
spring. To a lesser extent, heating tended to be faster with increase UV
intensity in summer while not in spring. Heating also tended to be
faster with higher vegetation.

Table 3 Multi-model inference gave for each variable the frequency of the
variable in the models, the AICc weight expressing the probability that the
variable influences the response (here the heating time of the dummy), the p-
value evaluating the significance of the difference between the AICc weight
and the a priori weight, and model-averaged parameter estimates and
standard error. The significant factors influencing the response are in bold
(in italics when marginally significant).
IV.4. Discussion

In accordance with our prediction based on photoperiod-related cues of morphological development, males captured under abnormal seasonal weather conditions (cf. Belgian meteorological rapport 2010; Table 1) showed the same seasonal morphological variation than males observed under classical seasonal conditions (see Van Dyck & Wiklund 2002); spring males showed a more typical ‘perching design’ compared to summer males. By contrast, territory selection behaviour deviated from what was expected under normal seasonal conditions; spring perching males avoided warmer sites but defended sunlit spots that may facilitate visual perception while summer males defended sunlit spots that decreases their thoracic heating time. We discuss these observations relative to male morphological and behavioural plasticity in the context of more frequent extreme weather conditions with the global warming.

Van Dyck & Wiklund (2002) demonstrated that wild and reared adult speckled wood butterflies from a Swedish population that followed different developmental pathways were found to differ in flight and thermoregulation-related morphology. Males of the first generation had larger body mass, wing area, wing loading and aspect ratio than summer males; which corresponds to a more ‘perching’ type of morphology. Moreover, in line with the photoperiod and the lower temperature in the early season, individuals were darker in spring than in summer. We observed similar seasonal variation in morphology in our study under abnormal conditions. Only male dry body mass and total wing area did not correspond to the general pattern; as we found
smaller males of lower body mass in spring in our study. The dry period of early spring may have reduced host plant quality forcing females to lay eggs on drought-stress plants (Gibbs et al. 2011). Feeding on drought-stressed host plants has been shown to result into smaller adults as we found (Talloen et al. 2004; Pellegroms et al. 2009). However, for the moment we know very little about oviposition behaviour under abnormal thermal conditions in *P. aegeria*. Spring males with darker wings and territorial design have had to deal with warmer, sunny conditions that permitted continuous flight and searching behaviour while summer males have had to be active under wetter, cooler conditions with morphological traits less optimal for thermoregulation (i.e. larger and paler males).

While adult morphological design is determined during the pre-emergence stages (Danilevski 1965; Beck 1990) and could result in phenotypes at the adult stage that do not match well with unexpected weather disturbances, behavioural adjustments are expected to be easier to express under abnormal weather conditions. Our results showed that light and warmth are inextricably linked, heating time being faster under higher light intensity in both seasons while more pronounced in spring. As the spring period was exceptionally dry and sunny and that most parts of the woodland were still open and well illuminated, the thermal value of sunlit spots was probably reduced. On the contrary, sunlit spots should have made flight activity possible during the relatively cloudy and cool summer. Our results suggest that males have been able to adapt their behaviour of territory selection under the aberrant climatic conditions studied here. Despite the fact that heating time of the dummy was faster in
warmer sunlit spot in both seasons, spring males avoided these warmer sites, whereas sunlit spots were selected based on their thermal properties in summer. On the other hand, sunlit spots with high level of UV intensity were preferentially selected by spring males rather than by summer males. A high level of UV intensity within a sunlit spot indicates an open canopy above the territory that permits direct solar radiation. These conditions maximize the part of the visual field occupied by open sky, which facilitates visual perception (Rutowski et al. 1991). Light conditions are important to visually detect flying females (Bergman et al. 2007). A brighter illuminated environment increases the contrast of a moving object against the background, which probably increases the ability of the perching male to detect and pursue females and intruders (Rutowski et al. 1991; Rutowski 2003). So, contrary to the patterns based on the literature relevant for the normal seasonal conditions (e.g. Wickman & Wiklund 1983; Dennis & Sparks 2005), our results suggest that males selected sunlit spots for their properties that facilitate visual perception in spring and that facilitate thermoregulation in summer. It tends to show that males were able to adjust their behaviour to current weather conditions, adapting the function of the sunlit spot to their current needs (i.e. selecting sunlit spots for thermoregulation under cold conditions versus selecting sunlit spots for visual perception under warmer conditions).

Behavioural adjustment may permit males to optimise their flight activity and hence maximize their encounter rate with receptive females and ultimately their fitness relative to seasonal weather conditions. Nevertheless, they have to deal with their morphological
design that could have been suboptimal relative to the experienced weather conditions. For instance, spring males may have avoided warmer sunlit spots to prevent overheating risks due to their darker wings and smaller size (Willmer & Unwin 1981). In addition, under colder and cloudy conditions during summer, females were probably more attracted to sunlit spots to heat up, which, in turn, would make a territorial tactic defending such a warm spot more beneficial than in a typical summer. However, summer males had a less pronounced perching design than spring males (Van Dyck & Wiklund 2002) which may have reduced their fight ability and manoeuvrability to intercept females. Holding a territory is particularly energy demanding (Chapter V) and could result in increasing costs for males with a patrolling design. It would be interesting to compare mate-location tactics frequencies through the season and the relation with morphological design of males to evaluate the impact of extreme seasonal conditions on the efficiency to adopt mating behaviour and on the costs/benefits ratio of the alternative behavioural tactics.

Our results suggest flexible behavioural responses in male *P. aegeria* to abnormal weather conditions, but also important inertia with respect to functional morphology. To what extent suboptimal morphologies have an impact on survival and reproductive success (fitness) is not yet known. Although *P. aegeria* is a widespread and common species, similar effects may be worth analyzing in species of conservation concern in this era of climate change and increased frequency of extreme weather conditions.
V. Lipid economy, flight activity and mate-locating behaviour in the Speckled wood butterfly: on the energetic cost of holding a territory

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[Chapter V is an unpublished manuscript]

In Chapter III and IV, we discussed the significance of defending a sunlit spot for perching males to increase their fitness as it facilitates female detection and male flight activity. However, holding a territory is thought to result in high energetic costs. In this chapter, we compared lipid consumption and metabolic rate of males expressing alternative behavioural tactics in order to better understand the energy-related costs of the ARTs, at least under our controlled conditions.

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Abstract

Active flight is a highly energy demanding way of locomotion. In butterflies, males may locate females by different behavioural tactics that are associated with different flight types. Tactics may differ in energetic costs. This needs to be considered to fully understand the pay-offs of co-existing alternative tactics relative to the environmental context and the phenotypes of the individuals. In the speckled wood butterfly (*Pararge aegeria*) males either adopt i) a territorial wait-and-fight tactic (i.e. territorial perching) in a sunlit patch on the forest floor, or ii) a fly-and-search tactic (i.e. patrolling) in a wider area of the forest to locate females. Perching corresponds to high frequency of take-off flights and aerial combats with high levels of manoeuvrability; such a flight performance is known to be among the most energy-demanding types of aerial locomotion. We tested the impact of different activity levels associated with the behavioural tactics on lipid reserves in males of a recently established breeding stock taking into account the age of the males. Tests were done in the laboratory and in outdoor flight cages. Low-active males that had free access to honey water were capable to synthesize lipids; their lipid reserves increased with age. However, if males had higher flight activity, the effect disappeared. Lipid reserves decreased significantly faster in perching males compared to non-perching males, but resting metabolic rate did not differ between males adopting the alternative behavioural tactics. Territorial perching males had larger investment in flight muscle ratio that favours acceleration and manoeuvrability than non-perching males. Whether perching and patrolling are really
alternative tactics, or whether patrolling rather is a suboptimal tactic (“making the best of a bad job”) is discussed.
V.1. Introduction

In several animal taxa, males may adopt alternative behavioural tactics to locate mates including territorial and non-territorial behaviours (e.g. Gross 1996; Cade 1980; Ide & Kondoh 2000; Plaistow et al. 2004). The ability to acquire and defend a territory through aggressive behaviour can be a principal determinant of a male’s lifetime reproductive success (Andersson 1994). At the proximate level, energetic costs may affect the way males defend territories and compete for mates (Davies & Houston 1984). Hence, to fully understand the pay-off of alternative behaviours, we need to integrate costs and benefits (Cuthill & Houston 1997). In insects, aggressive territory holding males often have higher mating success compared to non-territorial males (Davies 1978; Wickman & Wiklund 1983; Crespi 1988), but the costs and risks may be associated with reduced longevity (Houston 1970) and/or reduced performance of other behaviours like dispersal (Houston 1970; Crespi 1988).

Butterflies should be an interesting group in this context as different mate-locating tactics co-exist in several species, including aggressive territoriality (Wiklund 2003; Wickman 2009). Predictions on male-male territorial competition have been based on asymmetries among the males (e.g. body size – Rosenberg & Enquist (1991), body temperature – Stutt & Willmer (1998), flight morphology – Wickman (1992), residency status – Davies (1978), Wickman & Wiklund (1983), Waage (1988), Takeuchi & Honda (2009)). Male age may also be associated with territory holding capacity in butterflies (Kemp 2002a,b, 2003). Studies on odonates showed that a male’s ability to
hold a territory declines with age (e.g. Forsyth & Montgomerie 1987). Marden & Waage (1990) suggested that any correlation between age and the likelihood of winning male-male conflicts in odonates would reflect a decrease in lipid reserves with age in territorial males. Territorial males may have little opportunity to feed. So, they should burn their lipid reserves to be active over longer periods of time. It is interpreted as the cost of territoriality. Several studies on territorial odonates confirmed that energy reserves are important, if not the principal factor, determining territorial success (Marden & Waage 1990; Marden & Rollins 1994; Plaistow & Siva-Jothy 1996; Plaistow & Tsubaki 2000; Serrano-Meneses et al. 2007; Raihani et al. 2008). Intensive territorial behaviour with repeated escalated fights and high levels of acceleration and aerial manoeuvrability may reduce the energy reserves of a male by 40-50% compared with a pre-territorial male (e.g. Plaistow & Siva-Jothy 1996).

Can we transfer these conclusions on odonates to butterflies? There are differences in male-male combats among both groups (no biting and less physical contacts in butterflies; Kemp & Wiklund 2001), but also significant differences in thermoregulation that may confound simple generalisations (Heinrich 1986; Heinrich & Casey 1978). Hence, experimental work on butterflies is warranted knowing that they are frequently used as study systems to understand the ecology and evolution of reproductive behaviour (Rutowski 1991; Wiklund 2003). To the best of our knowledge, only four studies in this field have analysed energy reserves in butterflies (Kemp 2002a; Takeuchi 2006; Martinez-Lendech et al. 2007; Peixoto & Benson 2011); there is no a clear relationship between energy reserves and the
outcome of male-male conflicts. However, Takeuchi (2006) showed that residents of *Chrysozephyrus smaragdinus* had lower lipid reserves than intruders suggesting that territorial defence should be energetically costly. Similar results were obtained in the tropical butterfly *Eumaeus toxea* (Martinez-Lendeche et al. 2007).

Here we study the relationships between male behaviour and physiological costs in the Speckled wood butterfly (*Pararge aegeria* L.). It is a popular study model for behaviour, including male mate-locating tactics (Van Dyck 2003 and references therein). Males either adopt perching or patrolling behaviour (*sensu* Scott 1974) corresponding to an aggressive territorial versus a non-territorial tactic in this system, respectively. The tactics represent very different flight performances, which in turn may result in different physiological costs (Chown & Nicolson 2004). Perching males monopolize a sunlit patch on the forest floor by a sit-and-wait tactic with short inspection flights and several aggressive interactions with potential intruders, whereas patrolling males show a searching tactic that involves flying over larger areas in the forest (including sunlit patches and shady parts) (Wickman & Wiklund 1983; Shreeve 1987; Van Dyck et al. 1997a). Typically, *P. aegeria* territories or sunlit patches do not contain feeding resources offering little opportunities for territorial males to replenish their energetic reserves (e.g. Kemp & Wiklund 2001; Kemp & Alcock 2003). As take-off is energetically more costly than sustained flight (Dudley 2000), we predict higher costs in terms of lipid use in perching males compared to patrollers.

Other parameters such as ability to up-regulate metabolic flux and produce ATP at a high rate (Harrison & Hall 1993) and contractile
performance of muscles (Marden et al. 2001) should enhance success in territorial defence that depends on high manoeuvrability and powerful flight. Butterflies store fat in extensive abdominal fat bodies (Pullin 1987) and they are known to use lipids as a fuel for extended flight (Crabtree & Newsholme 1975; Downer & Matthews 1976; Candy et al. 1997). The respiratory metabolism of Lepidoptera depends on lipids (Domroese & Gilbert 1964). Active insect flight muscle maintains the highest metabolic rate of any known animal tissue (Beenakkers et al. 1984). Insect species that spend more energy to their activity have higher resting metabolic rates (Reinhold 1999). As the different mate-locating behaviours are related to very different flight performances in *P. aegeria* (and other butterflies), it is interesting to explore the eco-physiology of the tactics to better understand the co-existence under particular conditions. Although mate-locating has been studied intensively in butterflies, eco-physiology has been largely neglected in this field.

In this paper, we analysed the energetic cost of territory holding in *P. aegeria* males by investigating changes in lipid reserves among different treatment groups. As butterflies use lipids for maintenance and for flight activity, lipid resources are expected to decrease with age (e.g. Karlsson 1994; May 1992; Kemp 2002b). However, we are mainly interested in how this decrease may be altered by the level or type of flight activity. Therefore, we compared as a first step lipid reserves of active males that had flown for a few days in large outdoor cages with lipid reserves in low-activity males that had stayed in small containers that allowed fluttering but no continuous flight. As a reference, we also analysed the proportion of
lipids in newly emerged adult males. As a next step, we explored lipid-use among the active males in the outdoor cages in more detail. Males either adopt a territorial perching tactic on a sunlit patch, or a non-perching tactic flying through the cage and resting outside territories. So, we tested lipid reserves in the males relative to their behavioural tactic to test the hypothesis of a high physiological cost of territorial perching. Some studies have shown that the ratio of thorax mass to body mass (i.e. flight muscle ratio) was higher in perching compared to patrolling males (Wickman 1992; Chai & Srygley 1990). This ratio was demonstrated to be the most relevant physiological feature with respect to acceleration and manoeuvrability (Marden 1989; Berwaerts et al. 2002). Therefore, we also tested for differences in flight muscle ratio among the males that adopted different behavioural tactics. We also explored metabolic differences among males engaging in different behavioural tactics by testing their resting metabolic rates. Resting metabolic rate represents the energetic cost of simple subsistence for a poikilotherm, determining an individual’s minimal energy requirements under a standardized set of conditions (Hack 1997b). We predicted higher resting metabolic rates in territorial perching males compared to non-territorial males. Finally, in an additional experiment in the laboratory, we simulated intensive interactions by forcing males to take-off repeatedly and to manoeuvre in a small cage during repeated sessions. Frequent interactions and take-offs are characteristics of perching behaviour in the field (e.g. Van Dyck et al. 1997a). Their use of lipids was compared to that of control males.
V.2. Materials and methods

Study species

The Speckled wood butterfly (*Pararge aegeria*) is a temperate-zone satyrine butterfly that primarily occurs in different types of woodland. Males locate females by perching or by patrolling behaviour corresponding to different types of flight (Van Dyck 2003). Perching requires multiple short, explosive flights with high levels of acceleration and manoeuvrability to approach a passer-by rapidly, whereas patrolling requires longer, but less powerful flights at constant and lower speed (Van Dyck et al. 1997b; Berwaerts et al. 2006). Fast take-off with high acceleration performance is one of the most energetically demanding types of aerial locomotion (Dudley 2000).

Laboratory breeding

All *P. aegeria* used in the experiments were reared in the laboratory from eggs laid by wild caught females of a woodland population in central Belgium (Bois de Lauzelle, 50°67’74”N, 4°59’78”E). The standardized environmental conditions applied in the climate room (Photoperiod (L-D): 16h-8h; Temperature (day-night): 24°C-16°C) induced a direct development without larval diapause (Wiklund et al. 1983). We used the grass species *Poa trivialis* as host plant. Hatched larvae were placed on similarly sized tufts of grass (N = 6 per tuft) covered with a fine-meshed netting. Pupae were placed individually in transparent jars (125 ml) until eclosion. After a fixed
time in the pupal stage (9 days), each pupa was weighed (Ohaus Explorer balance; accuracy: ± 0.1 mg). Pupal mass correlates strongly with adult mass at eclosion (R = 0.92, P < 0.001, N = 156), and was used as an index of adult body mass.

Experiments

Experiment 1: Active versus low-active males

Males eclosed individually in the transparent jars (125ml) and had full access to a feeding source (10% sugar-solution on cotton). Date of eclosion was recorded to track individual adult age. Males were individually marked by writing a unique number on the ventral wing with a permanent, non-toxic marker. Next, males were assigned to one of two treatments: i) active flight in a large outdoor cage, and ii) a control treatment with limited flight activity (males stayed in the small jars). An additional group of males from the same breeding stock were frozen soon after emergence. This sample allowed us to make an estimation of the lipid reserves at the beginning of the adult stage.

For the active flight treatment, we introduced per session simultaneously 12 males of 11 different families (i.e. from 11 different wild caught females) into two semi-cylindrical outdoor cages (6 males per cage of 9.0 x 3.7 x 1.8 m). Camouflage netting with some holes on the cages created dappled light conditions inside the cages like in a forest with sunlit patches. The floors of the cages were covered by wood chips and there were green artificial trees. Similar cages have been used to study _P. aegeria_ behaviour before (Leimar et al. 2003;
Merckx et al. 2003; Kemp et al. 2006a). In the cages, males also had full access to a 10% sugar solution on cotton. Males stayed for 2-3 days in the cage. We did repeated observation sessions to score the behaviour of each individual male (degree of fidelity to a sunlit patch, level of aggressiveness, types of flight). Observations were done when climatic conditions permitted optimal flights (i.e. sunny days, mean temperature in the cages: 30 ± 5°C). This allowed us to assign each male to one of three categories: i) PE2: a strong and permanently perching male (i.e., high fidelity to a sunlit patch, high level of aggression, high frequency of short inspection flights); ii) PE1: a male defending a sunlit patch during one period, but showing non-perching behaviour at several other times; and iii) PE0: a non-perching male (i.e., males that were rarely or never observed to defend a sunlit patch and they regularly flew through the cage, eventually alternated by resting on the netting).

Experiment 2: Forced flight

We also did an experiment in the laboratory with other males from the breeding stock. One-day old males were transferred individually to small cages (0.3 m$^3$) under standardized climate room conditions (see above). They were assigned to one of two treatments: i) a forced flight treatment and ii) a control treatment. Males were forced to fly in their small cage during 5 min by gently touching the legs with a fine-bristled paintbrush each time the male alighted (cf. Gibbs et al. 2010a). Based on preliminary trials, we chose a forced flight time of 5 min so that flight was sustained for long enough to cause fatigue but not total exhaustion or death. These flights
corresponded to repeated take-off flights adopted by perching males in nature to chase intruders or intercept mates. The treatment was repeated three times a day, until a male had flown for 10 sessions of 5 min over 4 days. Control males were not forced to fly. Both experimental groups of males had access to a 10% sugar solution on cotton. Both the males that were forced to fly and the control group were at an adult age of 5 days.

Resting metabolic rate

We measured the resting metabolic rate of all active and low-active individuals of experiment 1. Butterflies were placed individually in a hard plastic chamber (volume: 25 cl) connected to a flow-through respirometer (Sable Systems International). The same system has been used with butterflies before (Niitepõld et al. 2009). Resting metabolic rates were calculated by analyzing the CO₂ production of the butterfly at rest (without active flight or walking). We used a flow-through system with a dual-sensor oxygen analyzer (Sable Systems International Oxzilla II) connected to a CO₂ analyzer (Sable Systems CA-10a) to measure the CO₂. The closed recipient with the butterfly was flushed with CO₂-free and H₂O-free air. Water and CO₂ were removed from the recipient by air going through three successive columns containing silicagel, drierite and ascarite, respectively. After 10 min, the recipient was flushed again at a flow rate of 630 ml/min (Sable Systems International subsample TR-SS3 pump) and the CO₂ gas accumulated during this period in the chamber was measured. Ambient temperature was kept stable at 30 ± 0.1°C (Physitemp Model BAT-12 digital thermometer). Dry body mass was
included as a covariate in the analyses of metabolic rate (mlCO$_2$ h$^{-1}$), but in order to compare the results with the literature, we reported the metabolic rate corrected by the mean dry body mass (i.e. mlCO$_2$ g$^{-1}$ h$^{-1}$). At the end of the measurement in the respirometer, individuals were frozen at -20°C for later lipid extraction (see below). We did not test for differences in flight metabolic rate as this treatment would exhaust the individuals and it may obscure eventual differences in lipids reserves relative to the behavioural tactics expressed in the outdoor cages.

**Lipid extraction**

Lipids were extracted from the body based on the method of Marden (1989). After removing the wings, the body mass was dried to constant weight at 70°C (Mettler Toledo-MT5 high precision balance; accuracy: ± 1 µg). Butterfly bodies were placed individually in paper filter bags. The bags were placed in refluxing diethyl ether of a Soxhlet apparatus for 8 h to extract all lipids. Next, bodies were dried and weighed again with the same balance. The difference of the body mass before and after extraction reflected the total mass of lipids. Male body mass was included as a covariate in the analyses but, in order to compare the results with the literature, we reported the results as the proportion of lipids on dry body mass.

**Flight muscle investment**

For the active males in the outdoor cages, we measured the ratio of the dry thorax mass on the dry body mass after lipids extraction and used it as an index of the flight muscles ratio.
Statistical analyses

The effect of flight activity (experiment 1 and 2) and of behavioural tactics (experiment 1) on lipid reserves, on resting metabolic rate and on flight muscle ratio was analysed using the Multi-Model Inference Approach based on the corrected Akaike’s Information Criterion (AICc) (Burnham & Anderson 2002) (using R software). Male age and male body mass were covariates in all analyses. Generalised linear regression models with a normal residual distribution were used to test the effect of flight activity (experiment 1 and 2). For the analyses of the subsample of active males in the outdoor cages (i.e. perching and non-perching males), the identity of the family was included as a covariate in the model. Data on lipid mass and on metabolic rate were log transformed to improve normality. Models with all possible combinations of the carefully chosen explanatory variables were fitted and compared using Akaike Information Criterion (AIC) corrected for small sample size, AICc. The model selection uncertainty was estimated by calculating the probability for a given model to be selected as the best model (model weight) if new data were re-sampled. A weight is also calculated for each explanatory variable as the sum of the model weights of the models in which each explanatory variable is present. This variable weight is an estimate of the relative importance of each explanatory variable in terms of predictive power. Due to the presence of interaction terms in the models, we used a permutation test (N. Schtickzelle and G. San Martin y Gomez, unpublished) to help the interpretation of the weight of the variables. A P-value was computed for each explanatory variable, representing the probability to obtain a
value equivalent or higher to the observed AICc weight of the variable if it has no predictive value on the response (see Chapter II for more details). Variables with a $P$-value $< 0.05$ were considered as significant. Model averaged coefficients were estimated as the mean of each parameter from each model, weighted by the model weight. A similar approach was used to estimate the standard errors of the model coefficients that takes into account model selection uncertainty and was not conditional to one model. This approach has many advantages over more classical stepwise approaches that end up with a unique "best model" with an unrealistic dichotomous distinction between important and non important explanatory variables (while several other models could be comparable) and in which the standard errors and model coefficients are biased.

For the experiment 1, One-way ANOVAs (SAS version 9.1) were additionally used to compare lipid reserves between the groups of males that flew in the cages (i.e. active males), that rested in the laboratory (i.e. low-active males) and that were killed at adult eclosion (males taken as reference). Means are given ± S.E.

**V.3. Results**

*Experiment 1: Active vs low-active males*

At the beginning of their adult life, the directly developed *P. aegeria* males had on average 21.42 ± 0.57 % of lipids. The proportion of lipids between the three group of activity differed significantly (One-way ANOVA: $F_{2,336} = 18.99, P < 0.0001$). Low-active males had a higher proportion of lipids (+1.34%) compared to
males after emergence, whereas the proportion was lower (-3.82%) in the group of active males (Fig. 1).

![Graph](image-url)

**Figure 1.** Proportion of lipids (i.e. Percentage of lipid mass to dry body mass) in males of different activity level.

Male age strongly affected the mass of lipids, but the effect varied with the level of activity (Table 1). The mass of lipids increased with age in low-active males, while not in active males (Table 1; Fig. 2). Older and larger males had more lipids, but lipid increase with age was stronger in heavy males (Table 1). The increase of lipid mass with male body mass was less strong for active males, but some incertitude exists on the parameter estimate.
Table 1. Multi-model inference gave for each variable the frequency of the variable in the models, the AICc weight expressing the probability that the variable influences the response \((Y = \text{ln-transformed lipid mass})\), the \(p\)-value evaluating the significance of the difference between the AICc weight and the a priori weight and the model-averaged parameter estimates and standard error. For the categorical variables, the estimate expresses the difference with the reference level fixed to zero (i.e. Low-active males; Activity 0). The complete model was: \(\text{log(Lipid volume)} = \text{Male age} (\text{MA}) + \text{Male body mass} (\text{MB}) + \text{Activity} (\text{ACT}) + \text{MA}\*\text{MB} + \text{MA}\*\text{ACT} + \text{MB}\*\text{ACT}\). The significant factors influencing the response are given in bold (in italics when marginally significant).

![Figure 2. The effect of age on lipid reserves of males relative to activity level.](image-url)

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The resting metabolic rate (RMR) was influenced by the activity treatment group, the male age, the male dry body mass and the interaction between both latter factors (Table 2). The RMR was of 7.29 ± 0.34 and 8.57 ± 0.33 ml CO₂ h⁻¹ g⁻¹ for low-active and active males, respectively. Heavier and younger males had higher RMR, but the increase of RMR with dry body mass was lower for older males (Table 2). To a lesser extent, the increase of RMR with dry body mass was higher in active males, but there is some incertitude on the parameter estimate.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Freq (%)</th>
<th>AICc weight (%)</th>
<th>p-value</th>
<th>Parameter estimate</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log(RMR) (n=172)</td>
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<td>100.00</td>
<td>.</td>
<td>-2.8402</td>
<td>0.0204</td>
</tr>
<tr>
<td>Male dry body mass</td>
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<td>100.00</td>
<td>&lt;0.0001</td>
<td>0.0316</td>
<td>0.0073</td>
</tr>
<tr>
<td>Male age</td>
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<td>95.24</td>
<td>0.011</td>
<td>-0.0007</td>
<td>0.0043</td>
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<tr>
<td>Activity 1</td>
<td>72.2</td>
<td>94.04</td>
<td>0.018</td>
<td>0.0601</td>
<td>0.0247</td>
</tr>
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<td>0</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MA*MB</td>
<td>27.8</td>
<td>92.84</td>
<td>0.001</td>
<td>-0.0052</td>
<td>0.0017</td>
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<tr>
<td>MB*Activity 1</td>
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<td>36.15</td>
<td>0.041</td>
<td>0.0047</td>
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<td>MA*Activity 1</td>
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<td>22.66</td>
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<td>0.0019</td>
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</table>

Table 2. Multi-model inference gave for each variable the frequency of the variable in the models, the AICc weight, expressing the probability that the variable influences the response (Y = ln-transformed RMR) the p-value evaluating the significance of the difference between the AICc weight and the a priori weight and the model-averaged parameter estimates and standard error. For the categorical variables, the estimate expresses the difference with the reference level fixed to zero (i.e. Low-active males; Activity 0). The complete model was: log(RMR) = Male age (MA) + Male dry body mass (MDB) + Activity (ACT) + MA*MDB + MA*ACT + MDB*ACT.
Experiment 1: Perching and non-perching males

The behavioural types of the active males in the outdoor cages corresponded to different mass of lipids (Table 3). The proportion of lipids on dry body mass after the experiment was highest in the non-perching males and decreased gradually with the intensity of the territorial perching behaviour (Fig. 3). So, within a time window of 2-3 days intensive perchers (PE2) used 2.97% more of their lipids than non-perching males (PE0). The proportion of lipids used compared to newly emerged males was 5.28% in the case of intensive perchers. The mass of lipids also varied with male age, male body mass and the interaction between age and body mass. The mass of lipids decreases with age and increases with male body mass but the decreases of lipids with age was less pronounced among heavier males. The family of the males also influenced their lipid mass.
Table 3. Multi-model inference gave for each variable the frequency of the variable in the models, the AICc weight, expressing the probability that the variable influences the response ($Y = \ln$-transformed lipid mass for the subsample of active males), the p-value evaluating the significance of the difference between the AICc weight and the a priori weight and model-averaged parameter estimates and standard error. For the categorical variable ‘behavioural tactic’ and ‘family’ the parameter estimate expresses the difference with the reference level fixed to zero (i.e., PE0 and F1 were fixed at zero, respectively). The complete model was: $\log(\text{Lipid mass}) = \text{Male age} \times (\text{MA}) + \text{Male body mass} \times (\text{MB}) + \text{Tactics} + \text{Family} + \text{MA*MB}$. 

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Freq (%)</th>
<th>AICc weight (%)</th>
<th>p-value</th>
<th>Parameter estimate</th>
<th>S.E.</th>
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<td>Intercept</td>
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<td>F3</td>
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<td></td>
<td></td>
<td>-0.1429</td>
<td>0.0582</td>
</tr>
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<td>F6</td>
<td></td>
<td></td>
<td></td>
<td>-0.0198</td>
<td>0.0581</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>-0.1062</td>
<td>0.0599</td>
</tr>
<tr>
<td>F1</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>/</td>
</tr>
<tr>
<td>Tactic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE1</td>
<td>50</td>
<td>99.98</td>
<td>&lt;0.0001</td>
<td>-0.0670</td>
<td>0.0336</td>
</tr>
<tr>
<td>PE2</td>
<td></td>
<td></td>
<td></td>
<td>-0.1577</td>
<td>0.0347</td>
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<tr>
<td>PE0</td>
<td></td>
<td></td>
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<td>0</td>
<td>/</td>
</tr>
<tr>
<td>Male body mass</td>
<td>60</td>
<td>99.94</td>
<td>&lt;0.0001</td>
<td>0.0043</td>
<td>0.0012</td>
</tr>
<tr>
<td>Male age</td>
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<td>0.003</td>
<td>-0.0031</td>
<td>0.0032</td>
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<tr>
<td>MA*MB</td>
<td>20</td>
<td>97.28</td>
<td>&lt;0.0001</td>
<td>0.0010</td>
<td>0.0003</td>
</tr>
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</table>
Flight muscle ratio (FMR, i.e. the ratio of the thorax mass on the dry body mass after lipid extraction) was only affected by male behaviour (Final model: AICc weight for the variable behavioural tactics = 86.4 %, $P = 0.029$, $n = 127$; Parameter estimates with PE0 fixed to zero: PE1: $0.017 \pm 0.011$ and PE2: $0.034 \pm 0.013$). As predicted, flight muscle ratio was the highest in the group PE2 males ($58.79 \pm 1.22 \%$, $60.72 \pm 0.70 \%$ and $62.82 \pm 0.90 \%$ in males adopting PE0, PE1 and PE2 behaviour, respectively). However, one potential pitfall when comparing flight muscle ratio between territorial and non-territorial males is that the higher FMR for territorial males may reflect greater thorax mass but alternatively may indicate that territorial males have expended more energy and eating less during territorial defense resulting in lower abdomen mass and relatively higher FMR. Consequently, we additionally analyzed the thorax mass
and the dry body mass as dependent variables. Thorax mass did not differ between the behavioural tactics (Final model: AICc weight for the variable behavioural tactics = 13.4 \%, \(P = 0.7\), n = 127), while the dry body mass did so with heavier non-perching males (Final model: AICc weight for the variable behavioural tactics = 78.3 \%, \(P = 0.034\), n = 127; Parameter estimates with PE0 fixed to zero: PE1: -0.40 \pm 0.24 and PE2: -0.58 \pm 0.26). The initial male body mass (i.e. pupal mass) also tended to be lower for territorial males compared to non-territorial males (AICc weight for the behavioural tactics = 63.2 \%, \(P = 0.07\), n = 127; Parameter estimates with PE0 fixed to zero: PE1: -3.35 \pm 2.02 and PE2: -3.16 \pm 2.00).

The resting metabolic rate did not differ among males adopting different behavioural types in the outdoor cages (Final model: AICc weight for the variable behavioural tactics = 11.8 \%, \(P = 0.9\), n = 103), even if we only compared the extreme types (PE2 against PE0; details not shown). RMR was only influenced by male dry body mass, with higher value of RMR for heavier males (Final model: AICc weight for the variable male dry body mass = 99.9 \%, \(P < 0.0001\), n = 103; Parameter estimates: 0.044 \pm 0.008).

Experiment 2: Forced flight versus resting

The proportion of lipids was significantly smaller in males that had been forced to fly (15.04 \pm 0.01\%) compared to males of the control group (20.28 \pm 0.01\%). Heavier males also possessed larger mass of lipids (Final model: Log(lipid mass) = flight treatment + Male body mass; AICc weight for the variable flight treatment = 97.6 \%, n
= 49; Parameter estimates with forced flight males fixed to zero: 0.18 ± 0.06; AICc weight for the variable male body mass = 80.3 %, n = 49; Parameter estimates: 0.005 ± 0.002).

V.4. Discussion

Our study showed an effect of the type of flight activity on lipid reserves and how this was altered with age in the speckled wood butterfly (*P. aegeria* L.), a model study species in ecology and evolution (Watt & Boggs 2003). Both the results on free flight in the outdoor cages and the forced flight in the laboratory showed higher lipid metabolism compared to control males. Without active flight, males could even increase their lipid reserves with aging. Our cage experiment provided support for the significant cost of territorial perching behaviour. Although territory holders used more of their lipids than did non-territorial males, there was no difference in resting metabolic rate between the males adopting different tactics. We will discuss our results relative to the pay-off of each behavioural tactic (perching vs. patrolling) and its importance to understand the coexistence of the tactics within a population.

Adult Lepidopterans typically acquire most nutritional resources (including essential amino acids but also most lipids) during larval feeding (Karlsson 1994; Arrese et al. 2001; O’Brien et al. 2005; Geister et al. 2008). Consequently, energetic reserves and physical condition are expected to decline with age (e.g. Kemp 2002b). Interestingly, our results showed a significant increase in the proportion of lipids with age in low-active males that had access to a
sugar solution on cotton. Although we did not focus on the specific lipids here, our results suggest lipogenesis in adult males of *P. aegeria*. The capacity to synthesize lipids at the adult stage has also been demonstrated in carbohydrate-fed Mediterranean fruit flies (Warburg & Yuval 1996, 1997). So, even if the adult lipid budget is mainly the result of larval feeding, lipid storage can result from *de novo* lipid synthesis from carbohydrates at the adult stage (Downer & Mathews 1976; Beenakkers et al. 1984, 1985; Canavoso et al. 2001). However, we did not observe significant increase of lipid reserves with age in active males in the outdoor cages. Based on wild-caught individuals, Karlsson (1994) observed a decrease of the abdomen mass (used as a proxy of lipid reserves) with age in *P. aegeria* and the butterfly *Speyeria mormonia* that only feed on nectar in the adult stage. Hence, active males metabolise more lipids than they are able to synthesize. Males will rest in the canopy during cloudy or rainy days (or even periods of the day) and obviously also at night. So, if they had access to food before such a period of resting or if they were able to alternate between resting and feeding, they may compensate to some extent the loss of energy. There are, however, still considerable gaps in our knowledge of the feeding ecology of *P. aegeria*. Perching males do not feed in their sunlit patch; territories typically do not contain food resources (e.g. Kemp & Wiklund 2001; Kemp & Alcock 2003). *P. aegeria* may have access to diffuse sugar resources including nectar but surely also honeydew, rotten fruits and tree saps (H. Van Dyck, pers. observ.). Whether patrolling males have more feeding opportunities during their flights through the forest compared to territorial perching males is a matter of future field studies.
Males that had flown in the outdoor cages for only 2-3 days (with full access to sugar solution) showed a reduction of c. 4 to 5% of their lipid content compared to resting males or freshly emerged males. In the same way, males that were forced to fly showed a significant reduction of their lipid resources compared to males of the same age maintained at rest under the same conditions. Overall, these results are in line with the idea that flight is highly energy-demanding in *P. aegeria*, as in other insects (e.g. Beenakkers et al. 1984; Candy et al. 1997). But we were more particularly interested in the relative differences with the different behavioural tactics. Our results on the cost of territorial behaviour are consistent with Takeuchi (2006) showing that resident males of the butterfly *Chrysozephyrus smaragdinus* had less lipid reserves than passing or intruding males. The energy cost of territorial defence has received only little attention in butterflies so far, but there is evidence in other insects (e.g. flies: Yuval et al. 1998; odonates: Plaistow & Siva-Jothy 1996; Plaistow & Tsubaki 2000; orthoptera: Hack 1997a).

Forced flight males of our second experiment consumed slightly more lipids than did active territorial males in the outdoor cages. This suggests that intensive periods with a high frequency of take-offs are particularly energy demanding. Under natural conditions, the frequency of interventions (and hence the number of take-offs from a resting position that a territorial perching male undertakes), will be influenced by male density and weather conditions (Wickman 2009). However, at this stage we cannot exclude an additional effect of stress induced by the forced flight treatment. As the time window of the behavioural performances in our experiments was relatively
short (2-3 days) compared to the expected average longevity of a male in the field, we should correct the estimates of the cost of territoriality accordingly. Wickman & Wiklund (1983) estimated the average male lifespan (or at least resident time) to be about 6 days. So, assuming a simple, linear relationship with time, we should multiply the lifetime energetic cost of territorial perchers at least by a factor of 2 to 3. Then, we would reach a reduction of lipid volumes between 8 to 15%. Lease & Wolf (2011) showed a mean lipid content of 12.57% in several species of Lepidoptera based on field caught individuals, but this does not allow getting any more precise figures relative to male behavioural tactics.

Independently of male age and size, several studies showed evidence of a higher resting metabolic rate (RMR) in actively flying insects (May 1979; Zera & Mole 1994; Reinhold 1999). Opposite to our prediction, males adopting different behavioural tactics did not differ in RMR. Knowing that perching and patrolling represent different flight types, there may be differences in their flight metabolic rate. Perchers need a large supply of energy for multiple short periods, while patrollers require a constant provision of energy probably at a lower level. So, independent of RMR, we may expect physiological divergence in flight metabolism between the behavioural tactics. After take-off carbohydrate consumption may prevail, but after some time there is a progressive shift to fatty acid oxidation in the flight muscles and hence a principally lipid-fuelled metabolism for longer and intensive flight performances (Beenakkers et al. 1984). It is now warranted to study the active flight metabolism relative to behavioural tactics in P. aegeria.
Chapter V

The territorial behaviour observed in the outdoor cages corresponded well to the territorial defence observed under natural conditions. Perching males occupied a sunlit spot and reacted to any passing intruder or moving object (see also Kemp & Wiklund 2004; Bergman et al. 2007). However, the situation is somewhat different for patrolling. Most males in this category alternated flights through the cage with periods of resting. Despite the presence of (vacant) sunlit patches, those males did not show any willingness to occupy a territory; even when aggressive territory holders were removed from the cage (L. Vande Velde, pers. observ.). If the non-perching males in our experiment were less active than typical patrollers in the field, we may have underestimated their use of lipids compared to field conditions. Therefore, it would be useful to test lipid use in males of known behaviour in the field and also test lipid use relative to flight performances other than frequent take-offs (e.g. flight endurance) in the laboratory.

The ability of a male to monopolize a territory can be constrained by energy reserves used during aerial flights and manoeuvres (e.g. Marden & Waage 1990; Plaistow & Siva-Jothy 1996). Therefore, one could expect males of lower nutritional status to adopt non-territorial, patrolling behaviour. However, there are different opinions in the literature. Patrolling is sometimes viewed as a real alternative tactic to territorial perching that may even be the dominant tactic in the population under particular environmental conditions (Wickman & Wiklund 1983). Other studies tend to view patrolling as a suboptimal tactic (“making the best of a bad job”) with perching on a sunlit patch as a preferred and dominant tactic (e.g.
Bergman & Wiklund 2009b). So, one should test if the different tactics are condition dependent (Maynard Smith 1982; Gross 1996). Our further research will focus on the impact of initial differences in lipids reserves (i.e. capital breeding) between males relative to their mate-locating behaviour. As territoriality is energetically costly, we could expect only males with the highest fat reserves to afford successful perching. However, we may expect perching males to have to deal with trade-offs between optimal lipid content that increase their ability to sustain aerial fights (i.e. large abdomen) and that increase acceleration and manoeuvrability during aerial competition (i.e. large thorax) (Marden 1989).

Interestingly, and similar to what has been observed in the field (reviewed in Van Dyck 2003), we found morphological differences between territorial perching males and non-perching males in our outdoor cage set-up. Males with intensive territorial behaviour had larger flight muscle ratio (FMR) than non-perching males. This is in accordance with the different types of flight performance associated with perching and patrolling. Berwaerts et al. (2002) showed a positive relation between thoracic mass allocation (i.e. flight muscle ratio) and acceleration capacity during take-off in *P. aegeria*. Although the FMR did not explain the outcome of male-male conflicts among perchers in an outdoor cage (Kemp et al. 2006a), our result under semi-natural conditions confirms the relationship between functional morphology and mate-locating tactic. However, our results showed that the higher FMR of territorial males in our experiment was the result of a lower total body mass rather than a higher thorax mass of perching males. This probably resulted from higher energy
consumption and lower nutritional opportunities of territorial perching males. We do not know the values of FMR at emergence; the pupal mass tended, however, to be still slightly lower in territorial versus non-territorial males. This is a relevant result in the context of the co-existence of both tactics as such a relationship is likely to influence the probability to adopt perching or patrolling and the ability to easily switch (or not) between tactics (e.g. Raihani et al. 2008). Future experiments are required to assess body mass and FMR as factors that may condition the adoption of the behavioural tactics. Thorax mass and body mass have a heritable basis in *P. aegeria* (Berwaerts et al. 2008). Here we found a significant family effect for the proportion of lipids in males, and we have also found evidence in another dataset that brothers were more likely to adopt a similar behavioural tactic (Chapter VII). These observations are in agreement with the relative consistency in adopting one of the behavioural tactics as it was observed in the field (Van Dyck et al. 1997a) and in the outdoor cages (L. Vande Velde, pers. obs).

We tested males that developed directly as a larva corresponding to the summer adult cohort (Wiklund et al. 1983). But the life cycle of *P. aegeria* can be more complicated and the different developmental pathways are associated with different adult seasonal phenotypes that correspond to adaptive differences in functional flight morphology (Van Dyck & Wiklund 2002). So, there is a further perspective to test for adaptive physiological differences among the seasonal cohorts and their behaviour in *P. aegeria*. So, environmental conditions vary with season, but also *P. aegeria* densities (Wiklund & Friberg 2011) which may influence success rates and costs of different
behaviours and hence the variation in pay-offs of the behavioural tactics.

We might expect a trade-off between reproduction and mate-location tactics in nature, perhaps as a consequence of differences in resource allocation to flight versus reproduction – particularly if one mate-location tactic results in a higher number of matings than the other (e.g. Langellotto et al. 2000; Guerra & Pollack 2007; Åhman & Karlsson 2009). Perching males in *P. aegeria* were shown to present higher mating frequency than patrolling males (Davies 1978; Wickman & Wiklund 1983; Bergman et al. 2007). Butterflies can breakdown their flight muscles with age and use these nutrients for reproduction (Stjernholm et al. 2005; Stjernholm & Karlsson 2006). We may expect the rate of perchers and patrollers that recycle part of their flight muscles might be different. Future experiments will focus on the trade-offs between flight and reproduction in *P. aegeria* by analysing the impact of the expression of the behavioural tactics on male reproductive investment and success.
VI. Larval food stress and adult mating
behaviour in the butterfly *Pararge aegeria*: the
energetic cost of territory holding

Lesley Vande Velde, Nicolas Schtickzelle and Hans Van Dyck

[Chapter VI is a manuscript submitted to Animal Behaviour]

In Chapter V, we showed evidence of higher energy costs for
territorial behaviour in the outdoor cages compared to non-territorial
behaviour. Consequently, we predicted that physiological status of a
male may condition the mate-locating behaviour and efficiency of the
tactic. In this chapter, we tested the impact of reduced larval lipid
accumulation on the adult mate-locating behaviour.

Author contribution: Study design: L.V.V. and H.V.D.;
Field/Labwork: L.V.V.; performed research; Data analysis: L.V.V.
and N.S.; Writing: L.V.V. and H.V.D.
Thanks are due to Christophe Pels and Gaëlle Rigaux for practical
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Abstract

In several organisms, males increase their reproductive success by territorial behaviour. Also in some butterfly species, males may defend a territory (i.e., territorial perching tactic), but this is assumed to be an energetically costly way to locate mates. Limitations of the energy budget may affect a male’s aerial flight and fight performance, and may, consequently, force males to adopt an alternative non-territorial searching behaviour (i.e., patrolling tactic) to maximize reproductive success. In this study, we tested to what extent behavioural tactics adopted by adult males of the butterfly *Pararge aegeria* (L.) were constrained by the nutritional conditions during the larval stage. We compared the performance (flight ability, lipid mass, metabolic rate and spermatophore production) of males that were reared on drought-stressed host plants and control males. Perching males tended to metabolise more lipids than non-territorial males. Males reared on a drought-stressed host plant were less likely to adopt the territorial perching tactic. Males of the stress treatment emerged as adults with lower lipid mass than control males, but they were able to restore their lipid mass through adult feeding (and perhaps the breakdown of flight muscles). Host plant quality also tended to affect spermatophore size. Independent of the larval food treatment, territorial perching males produced larger spermatophores and copulated for longer than males adopting non-territorial behaviour. We discuss the results relative to the co-existence of the behavioural tactics (perching and patrolling).
VI.1. Introduction

In territorial species, the ability of males to monopolize a suitable territory is usually linked to mating success (Andersson 1994). But not all individuals are equally capable of obtaining and holding a territory. Individuals may adopt alternative behavioural tactics to enhance mating opportunities (see Gross 1996 for review). Alternative behaviours may arise in response to frequency- and condition-dependent selection (Maynard-Smith 1979). Alternative behaviours may not be fixed traits, but result from genotype x environment interactions (Gross 1996; Gross and Repka 1998a,b).

Competition among males is an important component of sexual selection that may shape phenotypic traits facilitating high levels of contest-related exertion and flight performances (Marden & Cobb 2004). Males of several insects (Lepidoptera, Odonptera, Hymenoptera, Diptera) compete for access to females, for example, by aerial male-male interactions. These interactions have been considered wars of attrition; both contestants continue fighting or displaying until one male retreats as the loser (Maynard Smith 1979). The outcome of such physical performance is influenced by the resource-holding potential (RHP, i.e., the contestant’s capacity for inflicting fitness costs on his opponent and/or minimizing his own costs; Parker 1974). Contests are often settled on the basis of RHP asymmetries between opponents (reviewed by Andersson 1994). Energy available for flight may restrict male mating success; this is particularly the case in species that do not replenish their nutritional and energetic resources at the adult stage (Petersson 1989). Therefore, resource constraints may
function as the basis of asymmetries in RHP (Kemp & Alcock 2003; Marden & Cobb 2004). Males with greater energy reserves, and thus greater RHP, are predicted to win more contests (Leimar & Enquist 1984).

RHP can be influenced by genes (G), the environment (E) and G x E interactions. In holometabolous organisms, like butterflies, males can be affected by the environment at the adult stage, but also by the conditions during the larval stages. Butterflies have access to sources of nutrients at the larval stage that are unavailable (or at least more limited) at the adult stage (e.g. Baker & Baker 1973; Boggs & Ross 1993; O’Brien et al. 2005). Hence, larval host plant quality is a key determinant of adult phenotype and performance (Moreau et al. 2007). Variation in larval and adult food availability and quality is likely to be common in the wild, and may affect resource allocation to life-history traits (e.g. Delisle & Hardy 1997; Moreau et al. 2007). Plaistow & Tsubaki (2000) showed that flight muscles and body lipid content was much reduced in males and females of the damselfly *Mnais costalis* that were reared under low nutritional conditions.

Male flight physiology has been shown to relate to the outcome of male-male competition in species that engage in aerial contests, particularly in Odonata (Marden & Waage 1990; Marden & Rollins 1994; Plaistow & Siva-Jothy 1996). Depleted energy reserves may force males to adopt alternative behaviours other than territorial behaviour. It is, however, unclear whether the same mechanism would apply to butterflies. Four studies investigated aspects of energy reserves in this RHP-context of male-male competition in butterflies (Kemp 2002; Takeutchi 2006; Martínez-Lendech et al. 2007; Peixoto
& Benson 2011). Resident males of *Hermeuptychia fallax* had larger lipid reserves than intruders (Peixoto & Benson 2011), but there was no such an effect in the three others (*Hypolimnas bolina* Kemp 2002; *Chrysozephyrus smaragdinus* Takeuchi 2006; *Eumaeus toxea* Martínez-Lendech et al. 2007; *Moneuptychia soter* Peixoto & Benson 2011). Hence, whether lipid reserves can be used as a reliable indicator of fighting ability in butterflies is unclear. Aerial interactions are relatively short in butterflies compared to odonates for which lipid content is a good predictor of the ability to win a territorial contest (e.g. Marden & Waage 1990; Marden & Rollins 1994; Plaistow & Siva-Jothy 1996; Raihani et al. 2008). However, here we are not only interested in the issue of territorial male-male disputes *per se*, but particularly in the co-occurrence of alternative mate-locating tactics. In butterflies, alternatives may include territorial perching and non-territorial patrolling (Scott 1974). Few studies have addressed the issue of energy budgets and physiological limits to male flight performance in the context of mate locating behaviours (e.g. Gwynne 1990; Gwynne & Simmons 1990; Proctor 1992). Warburg & Yuval (1997) showed, for example, in Mediterranean fruit flies that nutritional status affected the reproductive tactic a male adopted.

Besides physiological measures, functional morphology may also be of significance to RHP and male mate-locating behaviour. One of the most studied morphological traits with respect to acceleration and manoeuvrability is the ratio of thoracic mass to total body mass (i.e., flight muscle ratio (FMR); Marden 1989; Berwaerts et al. 2002). Differences in functional morphology were related to contest ability in some cases (Convey 1989; Marden 1989; Coelho & Holliday 2001),
but not in others (e.g., *P. aegeria*; Kemp et al. 2006a). However, males adopting alternative mate locating tactics may differ in functional morphology. Territorial perching requires a design that favours high acceleration and manoeuvrability, which corresponds to high trait values of wing loading, wing aspect ratio and relative thoracic mass compared to patrolling designs that require different flight performance like flight endurance (Wickman 1992; Van Dyck 2003).

A reduction in larval food quality or quantity generally results in reduced adult body size and lipid content with negative effects on female fecundity (for review, see: Awmack & Leather 2002; for example, see: Fischer & Fiedler 2001), and on male reproductive success (e.g., Otronen 1995; Delisle & Hardy 1997). Butterfly reproduction is a nutrient-limited process (Boggs 1997), and because of the physiological costs, male mating activities may be limited by energy reserves (Proctor 1992). Ejaculates transferred by male lepidopterans contain proteins and lipids that can be significant to females and can therefore be considered as parental investment (Boggs 1981b; Wiklund et al. 1993).

In this paper, we tested to what extent behavioural tactics adopted by adult males of the butterfly *Pararge aegeria* (L.) are constrained by the nutritional conditions during the larval stage. Gibbs et al. (2004) suggested that males reared on stressed host plants are able to compensate for being small by adopting the perching tactic to maximize female encounter rate. However, the energetic cost of obtaining and maintaining a territory is typically high (e.g. Yuval et al. 1998; Plaistow & Siva-Jothy 1996; Plaistow & Tsubaki 2000).
Therefore, males should only be able to fight and defend a territory if they have a sufficient amount of resources to do so. Flight performances differ between perching and patrolling tactics; perching males show a high frequency of take-off flights and aerial combats with high levels of manoeuvrability, whereas patrolling males show longer flight periods. Powerful take-offs are known to be the most energy demanding type of locomotion (Dudley 2000). By varying host plant quality through a drought-stress treatment, we have manipulated male energy level to determine the impact on male mating behaviour, flight ability, metabolic rate and spermatophore production. Adult males strongly depend on their larval-derived resources for reproduction (i.e. mate location, courtship, spermatophore production). Hence, we predict males that were fed as a larva on drought-stress host plants of lower nutritional quality, to have a poorer condition to defend a territory compared to males of a control group. We also predict males reared on poor host plant quality to be restricted in their reproductive investment measured by assessing the spermatophore mass, fertile sperm number and copulation duration. Finally, we also explored effects of the treatment on resting metabolic rate.

VI.2. Material and methods

Laboratory breeding and larval food stress

All butterflies used in the experiments were reared from eggs laid by wild caught females from the Lauzelle woodland population in central Belgium (50°67′74″N, 4°59′78″E). Breeding took place under
standardized environmental conditions in a climate room (Light-Dark: 16h-8h; 24°C during the day and 16°C at night). *P. aegeria* has different seasonal cohorts that experience different environmental conditions, with adults of different cohorts varying in flight-related morphology (Van Dyck & Wiklund 2002). In our breeding stock, larvae were allowed to develop directly without diapause (Wiklund et al. 1983). Drought-stress treatment was similar to Talloen et al. (2004), with the grass *Poa trivialis* used as the host plant. Potted host plants of the control group had full access to water, whereas the drought-stressed host plants had been deprived of water for 10 days in the climate room before first instar caterpillars from the breeding program were transferred to them. During the period of larval feeding, the stressed host plants received a 1 day water/7 days drought cycle. Talloen et al. (2004) showed that leaf nitrogen, carbon and water concentrations were lower in drought stressed-plants compared to the control group. Hatched larvae were randomly transferred to similarly sized tufts of grass (N = 6 per tuft) of one of the two treatments. Pupae were placed individually in transparent jars (125 ml) until adult eclosion. After nine days of development, the pupae were weighed (Ohaus Explorer balance; accuracy: ± 0.1 mg). Pupal mass correlates strongly with adult mass at eclosion (R = 0.92, P < 0.001, N = 156), and was used as an index of adult body mass. Butterflies eclosed in the transparent jars and had access to a 10% sugar-solution on cotton. Date of eclosion was recorded so that the adult age of every individual was known. Males were individually marked and maintained in a cold room (Light-Dark: 10h-14h; 11 ± 2 °C) until experimental trials.
Experimental design

Males of both treatment groups were released in two identical outdoor cages (9.0 x 3.7 x 1.8 m). 3 males of the host plant stress treatment group and 3 control males were introduced per cage (i.e. 12 males in total per session with two cages). Camouflage netting with some holes on top of the cages created dappled light conditions like in a forest with sunlit patches. The floors of the cages were covered by wood chips and there were small, green artificial trees that were used as perches by *P. aegeria*. Similar cages have been used before to study male behaviour in this species (Leimar et al. 2003; Merckx et al. 2003; Kemp et al. 2006a). Males stayed for 2-3 days in the cage and had access to a 10% sugar solution on cotton and in artificial flowers. We did repeated observation sessions to score the behaviour of each male. This allowed assigning each male to one of two types: 1) a permanent percher (i.e., high fidelity to a sunlit patch, high level of aggression, high frequency of short inspection flights); and 2) a non-percher (i.e., males that were rarely or never observed to defend a sunlit patch and that regularly flew through the cage, eventually alternated by resting on the netting). Males that showed mixed behaviour (i.e., males that defended a sunlit patch during one period in their stay in the cage, but behaved as a non-percher for most of the time in the cage) were not considered, and hence excluded from the analyses, in this study as we were interested in the impact of male status on the probability to adopt the real territorial versus non-territorial tactics and that control males and males reared on stressed plants adopted the mixed behaviour in the same proportion (results not shown). After their stay in the outdoor
cage, males were transferred again to the climate room for mating with a virgin female.

**Copulations and spermatophore measurements**

Males were placed individually in a small cage (0.3 m³) under standardized climate room conditions (see above). A virgin female of known age and pupal mass from the breeding stock was introduced in the cage. All females were also reared on poor quality host plants. Twelve cages (and thus pairs) were observed at the same time (i.e. 6 males of the stress treatment group and 6 control males) in order to record copulation events and duration. Immediately after the copulation, females were killed by freezing (-20°C) to recover the received spermatophore. Males were kept for one day in the cold room before measuring resting metabolic rate.

The bursa copulatrix was carefully dissected from the abdomen of the frozen females. We carefully removed the spermatophore from the bursa and measured its mass (Mettler Toledo-MT5 balance; accuracy: ± 0.001 mg). Next, the spermatophore was placed in a drop of modified Barth Saline on a slide (Gurdon 1991). The spermatophore wall was ruptured by forceps to release the sperm that was gently stirred with a fine needle. Males transfer the eupyrene sperm in bundles which later break apart (Cook & Wedell 1996). In order to count the number of bundles, we fixed the drop with 70% ethanol and coloured the DNA of the spermatozoids with 4’,6’-diamidino-2-phenylindole. After drying, the sperm bundles can be easily counted under microscope (x 100; Epifluorescence Polyvar). In
butterflies, each bundle contains 256 euphyrene sperm cells (Cook & Wedell 1996).

Metabolic rate

Resting metabolic rates (RMR) were measured by respirometry analysing the CO₂ production of a male at rest (Hack 1997b). A flow-through system using a dual-sensor oxygen analyser (Sable Systems International Oxzilla II) connected to a CO₂ analyser (Sable Systems CA-10a) was used to measure the CO₂ emission. After flushing the metabolic chamber with CO₂-free and water-free room air, each butterfly was maintained in the metabolic chamber which consisted of hard plastic box with a total volume 25 cl at 30°C. Water and CO₂ were removed of the chamber by air going through three successive columns containing silica gel, drierite and ascarite, respectively placed before the chamber. After 10 min, the chamber was flushed again at a flow rate of 630 ml/min (Sable Systems International subsample TR-SS3 pump) and CO₂ gas accumulated during this period in the chamber was measured. Chamber temperature varied less than 1°C during any single trial (Physitemp Model BAT-12 digital thermocouple thermometer). Dry body mass was included as a covariate in the analyses of metabolic rate (mlCO₂ h⁻¹), but in order to compare the results with the literature, we reported the metabolic rate corrected by the mean dry body mass (i.e. mlCO₂ g⁻¹ h⁻¹). At the end of the experiment, males were frozen at -20°C for lipid extraction.
Lipids extraction and flight muscle ratio

Lipid reserves were compared between males of the stress treatment group and the control group that had flown in the cages and also between males adopting territorial perching and non-territorial patrolling behaviour. We included here two additional groups of individuals: 1) a sample of males from the treatment and control group directly frozen at emergence to evaluate the impact of drought-stress on the accumulation of lipid reserves, and 2) a sample of males from both treatment groups that were maintained at rest under laboratory conditions during the same number of days to compare lipid consumption for somatic maintenance when males had access to sugar solution.

Lipids were extracted from the body using the method of Marden (1989). After removal of the wings, dry body mass was measured for each individual male. Body parts were dried to a constant weight at 70°C. All individuals were individually placed into a paper filter and placed in refluxing diethyl ether in a Soxhlet apparatus for 8 h before being dried and weighed again (Mettler Toledo-MT5 balance; accuracy: ±1 µg). Lipid mass (mg) corresponds to the difference in weight before and after the extraction. Male body mass was included as a covariate in the analyses but, in order to compare the results with the literature, we also reported the results as the proportion of lipids on dry body mass (%).

In order to evaluate the FMR, we measured the ratio of the dry thorax mass of males after lipid extraction divided by the total dry body mass after lipid extraction (Mettler Toledo balance). Note that
FMR-measures are not directly comparable with values in the literature and in our earlier papers as the prior lipid extraction will affect the absolute values.

Statistical analyses

We used a multi-model inference approach (using R software) based on the corrected Akaike’s Information Criterion (AICc) (Burnham & Anderson 2002). Generalised linear regression models with a normal residual distribution were used to analyse the effect of the drought-stress treatment and of the behavioural tactic relative to physiological (i.e. lipid reserves and resting metabolic rates), reproductive and morphological traits of males. Male age and male body mass (i.e. pupal mass and dry male body mass) were included as covariates. Data on lipid reserves and metabolic rate were log transformed to improve normality. Models with all possible combinations of the carefully chosen explanatory variables were fitted and compared using Akaike Information Criterion (AIC) corrected for small sample size, AICc. The model selection uncertainty was estimated by calculating the probability of a given model to be selected as the best model (model weight) if new data were re-sampled. A weight is also calculated for each explanatory variable as the sum of the weights for each model where the explanatory variable is present. This variable weight is an estimate of the relative importance of each explanatory variable in terms of predictive power. Due to the presence of interaction terms in the models, we used a permutation test (N. Schtickzelle and G. San Martin y Gomez, unpublished) to help the interpretation of the weight of the variables.
A *P*-value was computed for each explanatory variable, representing the probability to obtain a value equivalent or higher to the observed AICc weight of the variable if it has no predictive value on the response (see Chapter II for more details). Variables with a *P*-value < 0.05 were considered as significant. Model averaged coefficients were estimated as the mean of each parameter from each model, weighted by the overall model weight. A similar approach is used to estimate the standard errors of the models coefficients that takes into account model selection uncertainty and are not conditional to one model. This approach has many advantages over more classical stepwise approaches that result in a unique "best model" with an unrealistic dichotomous distinction between important and non important explanatory variables (while several other model could be almost as good) and in which the standard errors and model coefficients are biased.

Two-way ANOVAs (SAS version 9.1) were used to compare lipid reserves between the groups of males that flew in the cages, that rested in the laboratory and that were killed at adult eclosion relative to drought-stress treatment. Average values are reported ± S.E.

**VI.3. Results**

*Host-plant stress and behavioural tactics*

Males of the host plant stress treatment group and of the control group were able to defend a territory, but frequencies differed significantly in line with our prediction. Males of the larval stress group adopted the territorial perching behaviour significantly less
frequently than males of the control group (Territorial tactic: 26 control males versus 16 males of the host plant stress treatment group; Non-territorial tactic: 10 control males versus 18 males of the host plant stress treatment group; $\chi^2 = 4.15, P = 0.04$).

*Metabolic rate*

There was no evidence either of an effect of larval stress treatment ($10.97 \pm 0.83 \text{ mlCO}_2 \text{ g}^{-1}\text{h}^{-1}$ and $9.97 \pm 0.48 \text{ mlCO}_2 \text{ g}^{-1}\text{h}^{-1}$ for active versus low-active males, respectively) nor of an effect of the behavioural tactics ($11.16 \pm 0.66 \text{ mlCO}_2 \text{ g}^{-1}\text{h}^{-1}$ and $9.37 \pm 0.61 \text{ mlCO}_2 \text{ g}^{-1}\text{h}^{-1}$ for territorial versus non-territorial males, respectively) on adult resting metabolic rate (Table 1). However, the resting metabolic rate increased with male body mass (Table 1).
Table 1. Analysis of resting metabolic rate. Multi-model inference based on AICc weight of each factor expressing the probability of importance in explaining the variation in the response variable ($Y = \ln$-transformed RMR). The p-value, evaluating the significance of the difference between the AICc weight and the a priori weight, helps to interpret AICc weight value despite differences in the frequency of the variable in the models (Freq). Factors with high support are indicated in bold. Model-averaged parameter estimate and standard error is given for each factor. For the categorical trait ‘behavioural tactic’, the value of non-territorial males was set at zero as a reference. For the categorical trait ‘food stress treatment’, the value of the control males group was set at zero as a reference. The complete model was: \( \log(\text{RMR}) = \text{Male age (MA)} + \text{Male dry body mass (MBD)} + \text{Behavioural tactic (Behav)} + \text{Food stress treatment (TR)} + \text{MA*MBD} + \text{MA*behav} + \text{MA*TR} + \text{MB*behav} + \text{MB*TR} + \text{Behav*TR}. \)
Male reproductive investment

Territorial males produced larger spermatophores than non-territorial males (0.328 ± 0.007 and 0.297 ± 0.008 mg, respectively; Table 2). To a lesser extent, the larval stress treatment tended to affect spermatophore production with males of this group producing slightly smaller spermatophores compared to control males (0.332 ± 0.006 mg and 0.309 ± 0.006 mg for the control and the stress treatment group, respectively). Female traits (age and body mass) also tended to be associate with spermatophore mass; larger and older females tended to receive on average larger spermatophores while the increase of spermatophore mass with female age was weaker for older females (Table 2).

Neither the larval food stress treatment, nor the behavioural tactic affected sperm production. Sperm number was principally affected by male age, with older males transferring more sperm bundles. The impact of male age on sperm production was, however, influenced by female and male body mass. The interaction terms suggested that the increase of sperm number was lower in larger females and lighter males (Table 2).

The larval food stress treatment had no effect on the copulation duration, but territorial males copulated for longer than non-territorial males (22.34 ± 0.66 and 21.14 ± 0.94 min, respectively; Table 2). Copulations lasted for longer with young compared to older females. To a lesser extent, males of lower body mass copulated for longer than heavy males, but this relationship decreased with female age (Table 2).
<table>
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<th>p-value</th>
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Table 2. Analysis of reproductive investment. Multi-model inference based on AICc weight of each factor expressing the probability of importance in explaining the variation in the response variable (Y = Spermatophore mass, bundles number and copulation duration, respectively). The p-value, evaluating the significance of the difference between the AICc weight and the a priori weight, helps to interpret AICc weight value despite differences in the frequency of the variable in the models (Freq). Factors with high support are indicated in bold; those with intermediate support are in italics and bold. Model-averaged parameter estimate and standard error is given for each factor. For the categorical trait ‘behavioural tactic’, the value of non-territorial males was set at zero as a reference. For the categorical trait ‘food stress treatment’, the control males group was set at zero as a reference. The complete model was: Y = Male age (MA) + Male body mass (MB) + Female age (FA) + Female body mass (FB) + Food stress treatment (TR) + Behavioural tactic (behav) + MA*MB + MA*FB + MB*FB + MA*FA + MB*FA + FB*FA + Behav*TR.
Lipid reserves

Males of the larval food stress treatment eclosed as adults with a significantly lower relative lipid mass than control males (mean lipid mass for males of the larval food stress versus control males: 4.30 ± 0.14 mg and 4.63 ± 0.10 mg, respectively; mean dry body mass for males of the larval food stress versus control males: 15.88 ± 0.27 mg and 15.51 ± 0.35 mg; Fig. 1). There was no longer such a difference between the two treatments when males were kept for 2-3 days in the laboratory in small jars (i.e. a low activity treatment) or when they had flown in the outdoor flight cages (Fig. 1). Both in the jars and the outdoor cage, males have access to a honey water solution. Lipid reserves were larger in freshly emerged males compared to males that were kept in jars and in the outdoor cages (Two-way ANOVA: Log(Lipid mass) = Groups + Treatment + Groups x Treatment; $F_{5,333} = 26.24, P < 0.0001$; Fig. 1).
Figure 1. Mean (± S.E.) relative lipid volume (i.e., ratio of lipids/ dry body mass) of adult males that were reared on drought-stressed host plants (S) or on control plants of high quality (C) when they eclosed as adult butterfly (I), after 2-3 days under laboratory conditions with only low flight activity and access to honey water (II), and after 2-3 days in an outdoor flight cage with access to honey water (III).

Lipid mass in males that had flown in the outdoor cage was not more different between males of the larval food stress treatment and control males (mean lipid mass for males of the larval food stress versus control males: 2.76 ± 0.13 mg and 2.40 ± 0.10 mg, respectively; mean dry body mass for males of the larval food stress versus control males: 13.24 ± 0.28 mg and 12.57 ± 0.23 mg; Table 3). However, behavioural tactic tended to affect lipid mass; males adopting the territorial perching tactic had a lower lipid mass than males adopting a non-territorial behaviour (Proportion of lipids on dry
body mass: 19.00 ± 0.05% and 23.00 ± 0.05%, respectively; Table 3). Other factors did not significantly affected the lipid mass (Table 3).

<table>
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Table 3. Analysis of lipids reserves. Multi-model inference based on AICc weight of each factor expressing the probability of importance in explaining the variation in the response variable (Y = In-transformed lipid mass). The p-value, evaluating the significance of the difference between the AICc weight and the a priori weight, helps to interpret AICc weight value despite differences in the frequency of the variable in the models (Freq). Factors with high support are indicated in bold. Model-averaged parameter estimate and standard error is given for each factor. For the categorical trait ‘behavioural tactic’, the value of non-territorial males was set at zero as a reference. For the categorical trait ‘food stress treatment’, the value of the control males group was set at zero as a reference. The complete model was: log(Lipid mass) = Male age (MA) + Male body mass (MB) + Behavioural tactic (Behav) + Food stress treatment (TR) + MA*MB + MA*Behav + MA*TR + MB*Behav + MB*TR + Behav*TR.
Chapter VI

Flight muscle ratio

Males of the larval food stress group had slightly lower FMR ratios than control males (Table 4; FMR: 56.79 ± 1.37 % and 60.04 ± 0.93 %, respectively). To a lesser extent, young males tended to have higher FMR ratios than old males, but the estimation of this relation is uncertain. However, this relationship was stronger among males of the larval food stress (Table 4). FMR ratios were not significantly different between the behavioural tactic (Table 4; 58.97 ± 1.17 % and 57.62 ± 1.14 % for territorial and non-territorial males, respectively).

One potential pitfall when comparing flight muscle allocation between two groups of males is that the higher FMR may reflects greater thorax mass or alternatively lower abdomen mass. Consequently, we additionally analyzed the thorax mass and the dry body mass as dependent variables. Our results indicate that lower FMR for males of the larval food stress males results from a higher total dry body mass (Final model: AICc weight for the variable larval food treatment = 86.38 %, \( P = 0.05 \), \( N = 71 \); Parameter estimates with control males fixed to zero: \( 0.74 ± 0.42 \)) rather than a difference in the thorax mass per se (Final model: AICc weight for the variable larval food treatment = 38.24, \( P = 0.74 \), \( N = 71 \)).
Table 4. Analysis of flight muscles ratio (FMR). Multi-model inference based on AICc weight of each factor expressing the probability of importance in explaining the variation in the response variable \(Y = FMR\). The p-value, evaluating the significance of the difference between the AICc weight and the a priori weight, helps to interpret AICc weight value despite differences in the frequency of the variable in the models (Freq). Factors with high support are indicated in bold; those with intermediate support are in italics and bold. Model-averaged parameter estimate and standard error is given for each factor. For the categorical trait ‘behavioural tactic’, the value of non-territorial males was set at zero as a reference. For the categorical trait ‘food stress treatment’, the value of control males group was set at zero as a reference. The complete model was: \(FMR = \text{Male age (MA)} + \text{Male body mass (MB)} + \text{Behavioural tactic (Behav)} + \text{Food stress treatment (TR)} + \text{MA*MB} + \text{MA*Behav} + \text{MA*TR} + \text{MB*behav} + \text{MB*TR} + \text{Behav*TR}\).
VI.4. Discussion

In line with our predictions, we showed that males that fed as larvae on drought-stressed host plants were less likely to adopt territorial perching behaviour as an adult. Lower nutrient levels also tended to result into the production of smaller spermatophores, and lower adult initial lipid mass as well as smaller investment in the flight muscle ratio. Independent of the larval feeding treatment, we found several physiological differences relative to the mate locating tactic adopted by a male; Perchers tended to metabolise more of their lipids. Males adopting territorial perching produced larger spermatophores and copulated for longer than males adopting non-territorial behaviour.

The host plant stress treatment had a clear effect on the relative lipid mass at the beginning of the adult life, but things were different after several days of activity. There was no longer such a treatment-dependent difference in males that were kept in small jars in the laboratory or in males that had flown in outdoor cages. Hence, males were able to compensate for their lower lipid content, probably by compensatory feeding. Males (both in the laboratory and the outdoor cages) had full access to honey water allowing them to synthesize lipids from adult feeding sources (Canavoso et al. 2001; Chapter V). We have, however, no observational data to confirm whether the males of the larval feeding stress treatment fed more or more frequently than control males. This can be tested in future experiments using cameras. So, the larval feeding treatment may have shifted
males from being largely capital breeders to partly income breeders (e.g. May 1992). High-nutritional status may be a prerequisite to adopt energy-demanding behaviours like territorial perching (e.g. Warburg & Yuval 1997). Energy-depleted males may first invest in foraging to complete their reserves instead of starting immediately to locate mates (Proctor 1992). Males that defend non-resource-based territories like in *P. aegeria* have little opportunity to forage (e.g. Kemp & Wiklund 2001; Kemp & Alcock 2003). Depletion of energy reserves can exclude males from costly territory holding (Otronen 1995; May 1992) making males more likely to adopt less costly, non-territorial tactics to find mates. Moreover, this may offer better opportunities to forage as well. However, little is actually known about their feeding ecology in the field. *P. aegeria* are rarely seen on flowers but feed on honey dew and rotten berries (Shreeve 1986). An interesting extension of this work would be to test whether typical perching and patrolling males differ in adult food intake. However, adult food intake in butterflies is largely limited to carbohydrates, and much less to other components like proteins for which they depend on larval intake (Boggs 2003).

We also observed that males of the larval feeding stress group tended to have, after a few days of activity, lower FMR than control males. Our result suggested that this lower FMR in males of the larval feeding stress group resulted from higher abdomen mass rather than lower thorax mass. Nevertheless, we cannot exclude initial differences in thorax mass (i.e. at emergence) between the groups. In butterflies (including *P. aegeria*), there is evidence for compensating nutrient depletion via the breakdown of thoracic flight muscles (Stjernholm et
al. 2005 and references therein). Males of the larval feeding stress group could have reallocated nutrients from their flight muscles into their reproductive system. Indeed, males of the stress group tended to produce smaller spermatophores, but there was no significant difference for the number of sperm bundles. Moreover, a decrease of thoracic mass was observed with age in our experiment and in a more pronounced way in the males of the larval feeding stress group. Degenerative processes in flight muscles with age are common in insects (e.g. Collatz & Wilps 1986; Karlsson 1994; Stjernholm et al. 2005). However, muscle histolysis is expected to be more pronounced under conditions of low food quality (Boggs 2009).

Large values of the ratio of flight muscle mass in territorial perching individuals may correspond to frequent take-off flight performance that requires high acceleration and manoeuvrability capacities (Wickman 1992). Berwaerts et al. (2002) showed experimental evidence of the link between this flight morphological trait and this type of flight performance in *P. aegeria*. In our experiment, males with a larval development on a high quality host plant tended to have better opportunities to develop high FMR-values and to adopt the territorial behaviour. However, there was variation among males as some of the poor larval food treatment group became successful territory holders. It is possible that some genotypes are better able to deal with poor quality food than others in building strong designs that allow aggressive perching, however, further work on this system with a full quantitative genetic design would be necessary to test such G x E interaction effects on the adoption of alternative mate locating tactics.
After a few days of adult activity, we observed similar resting metabolic rates among males of the two experimental groups. We have already discussed the issue of compensation, but another possibility is that larval feeding stress would result in reduced survival relative to the males of the control group (cf. Geluso & Hayes 1999). It is also possible that there could be differences in flight metabolic rate between larval feeding stress and control males as well as between perching and patrolling males. We did not test for differences in survival or flight metabolic rate trade-offs here for logistic reasons but will in future work.

Due to the high physiological cost of spermatophore production in insects (e.g. Oberhauser 1992; Svärd & Wiklund 1989; Lauwers & Van Dyck 2006), the size of the spermatophore has often been used as a measure of male quality (e.g. in the Lepidoptera; reviewed by Marshall & McNeil 1989). Our results showed that *P. aegeria* males reared on low-quality plants produced 1.07 times smaller spermatophore than control males. Lauwers & Van Dyck (2006) showed that females receiving a small spermatophore (from a recently mated male) produced fewer offspring than females that received a normally-sized spermatophore. Females that received a small spermatophore also showed a greater propensity to remate with another male (e.g. Vande Velde et al. 2011 (Chapter VIII); Torres-Vila et al. 1997). Hence, larval diet may affect male reproductive success. The number of sperm bundles did not differ between the treatment groups, but other qualitative aspects may, however, differ among spermatophores of differently treated males. For example, Delisle & Bouchard (1995) observed in *Choristoneura rosaceana* that
whereas spermatophore mass of males reared on low quality food was only 1.5 times smaller than of males reared on high quality food, female fecundity of females that mated with males reared on low quality food was 40% lower. In the same vein, Delisle & Hardy (1997) showed that males reared on an artificial diet produced larger spermatophores than males reared on high quality natural food, but their reproductive success was actually lower. Therefore, the quality of the spermatophore can be reduced independently of its mass (reviewed by Marshall & McNeil 1989). Another indicator of male quality may relate to the chemical signals that males release during courtship (Birch et al. 1990). As plants may provide chemical precursors of the insect pheromones, larval food quality may affect the production and quality of male pheromones (Landolt & Phillips 1997). Whether larval food quality affects the quantity and quality of accessory gland secretions in *P. aegeria* needs further study. Delisle & Hardy (1997) showed that females mated with males fed on low quality food produced fewer fertilized eggs, although they received sufficient quantities of sperm. One possible mechanism can be the deficiency of accessory gland secretions that affect sperm mobility (Khan & Musgrave 1969).

Given the significant cost to produce spermatophores, one may predict males to modulate their reproductive investment relative to female quality. In line with previous studies (e.g. Rutowski et al. 1983), males tended to transfer larger spermatophores to large females. But, we also found some surprising results like the tendency to transmit larger spermatophores to older females. Nevertheless, these results must be taken with caution as the females offered to the
males were all reared on the drought-stress host plants, which may have altered male perception of female quality. Larval food quality is clearly able to affect various life history traits (Boggs & Ross 1993; Talloen et al. 2004; Pellegroms et al. 2009; Gibbs et al. 2011) and may also have consequences for male attractiveness (Kemp et al. 2006b). However, this needs further experimental work.
VII. Male reproductive investment relative to age and flight behaviour in the monandrous butterfly *Pararge aegeria*

Lesley Vande Velde, Pauline Silvestre, David Damiens and Hans Van Dyck

*The content of Chapter VII has been accepted for publication in Behavioural Ecology and Sociobiology*

In the two previous chapters, we demonstrated that territorial males consumed more lipid resources than non-territorial males. Spermatophore production represents a non-negligible cost for *Pararge aegeria* males.

From a flight-reproduction trade-off viewpoint, we tested in this chapter whether territorial males will be more restricted in their reproductive investment and how flight activity levels (and also other life-history traits) may impact the reproductive output of males.

**Author contribution:** Study design: L.V.V., D.D. and H.V.D.; Field/Lab work: L.V.V. and P.S.; Data analysis: L.V.V.; Writing: L.V.V., D.D. and H.V.D.

Thanks are due to Hubert Baltus and Melanie Gibbs for practical assistance, to Gilles San Martin and Eric Le Boulengé for statistical assistance and to Ellyn Bitume for language editing.
Abstract

Male reproductive investment may signify a considerable cost to male insects that produce sperm packages or spermatophores. Male butterflies allocate much of their active time budget to mate location, and they may adopt different behavioural tactics to do so. In the Speckled wood butterfly (Pararge aegeria L.) males adopt either a territorial wait-and-fight tactic (territorial perching) or a fly-and-search tactic in wider areas (patrolling). In this study, we analysed the impact of male age, male size, and male behaviour (i.e., behavioural tactics and levels of activity) on spermatophore investment (i.e. spermatophore mass, number of eupyrene sperm bundles). As predicted, reproductive investment increased with male age and size. Nevertheless, the increase of spermatophore mass and of number of eupyrene sperm bundles (i.e. fertile sperm) with age was stronger in low-activity males compared to active flying males. This suggests that flight activity has a negative impact on male reproductive investment. However, males that were forced to fly in the laboratory produced more eupyrene sperm bundles than resting males. We discuss the potential effects of male-male competition and predation risk on current versus future male reproduction. Males adopting different mate-locating tactics (perching and patrolling) in outdoor cages did not differ in spermatophore traits as was predicted from their very different flight performances. Copulations of territorial perching males took somewhat longer than copulations with non-perching males. There was a significant family effect of spermatophore size and of the expression of male mate-locating tactics suggesting heritable
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variation. Female traits (i.e., age and size) did not affect spermatophore production. We discuss the results relative to both ultimate and proximate explanations of the complex relationships between butterfly activity, behavioural tactics, age, and spermatophore production.
VII.1. Introduction

Males invest little in individual sperm cells, but rather increase fitness by maximizing the number of sperm produced and the number of matings (Parker 1970, 1984; Trivers 1972). However, energetic allocation to total sperm production may vary considerably by mating system both within and among taxonomic groups (Andersson & Iwasa 1996). Contrary to vertebrates, in several insect species sperm production signifies a considerable cost. This is particularly the case in species that transfer a spermatophore containing both sperm and other nutrient-rich substances as a nuptial gift, such as crickets and butterflies (Svärd 1985; Simmons et al. 1993; Bissoondath & Wiklund 1996). In Lepidoptera, it takes a recently mated male much longer to transfer a spermatophore during the second copulation compared to a virgin male, and the spermatophore will be much smaller as well (e.g., Boggs 1981b; Rutowski et al. 1983; Svärd 1985; Svärd & Wiklund 1986; Oberhauser 1989). Svärd (1985) showed evidence for this in the Speckled wood butterfly (*Pararge aegeria*); it took a male up to 7 days after the first copulation before the second spermatophore had a size comparable with the first spermatophore. Male mating history affects spermatophore size and copulation duration in the monandrous *P. aegeria*, which also affects female reproductive output (Lauwers & Van Dyck 2006). The quality of the spermatophore is known to affect male fitness (Marshall 1982; Karlsson 1994; Bissoondath & Wiklund 1996) directly through sperm transfer and indirectly through the transfer of carbohydrates and proteins in the spermatophore affecting female reproduction and survival (Wiklund et al. 1993; Boggs 1981b,
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2003). Besides mating status, several other variables may also affect the quality of the transferred spermatophore including age, size, activity level, reproductive tactic, life expectancy, competition intensity, and heritability. Additionally, males may in principle also adjust their reproductive investment relative to the quality of the female (e.g., age or size).

The quality of the spermatophore may change with age (e.g., LaChance et al. 1977; Boggs 1981b; Oberhauser 1988; Gage 1994). In the field, adult males of *P. aegeria* live for 10 days or even less (e.g., Wickman & Wiklund 1983), but for their flight activity they are sensitive to weather conditions. This heliothermous insect requires high body temperatures to be fully active in such a thermally heterogeneous forested environment, (Shreeve 1984; Van Dyck & Matthysen 1998) and sunlit patches on the forest floor play an essential role as rendezvous sites, including favourable conditions for thermoregulation (Vande Velde et al. 2011a (Chapter III)). On cool or cloudy days, males will be inactive and stay in the canopy. Therefore, age at first mating may vary considerably.

Male body mass may also be of significance to spermatophore production. In many Lepidoptera for example, larger males have been found to transfer larger spermatophores at the first mating (Boggs 1981b; Rutowski et al. 1983; Svärd & Wiklund 1986; Rutowski et al. 1987; Oberhauser 1988; Gage 1994; Bissoondath & Wiklund 1995, 1996). Hence, resources accumulated during the larval stage will influence the potential size of the ejaculate that the adult male butterfly is able to produce (Boggs 1981b). Consequently, the proportional increase of reserves with body size should be paralleled
by an increase in reproductive effort, which in turn explains the positive relationship between body size and ejaculate size (Wickman & Karlsson 1989). Large ejaculates have been shown to increase paternity (Rutowski et al. 1987; Bissoondath & Wiklund 1996). In *P. aegeria*, larger spermatophores of virgin males resulted in higher numbers of hatching offspring compared to small spermatophores of non-virgin males (Lauwers & Van Dyck 2006).

Male butterflies allocate much of their active time budget to mate location, but there is variation in behavioural tactics to find females (Shreeve 1992). Male butterflies use different behavioural tactics that correspond to different activity levels and flight types (Rutowski 1991; Wickman 1992); flight is known to be physiologically costly (Chown & Nicolson 2004). *P. aegeria* males either adopt perching or patrolling behaviour to locate females (*sensu* Scott 1974). Perching males monopolize a sunlit patch on the forest floor where they adopt a sit-and-wait tactic with short inspection flights and several aggressive interactions with potential intruders, whereas patrolling males show a searching tactic covering larger areas of the forest (including sunlit patches and shady parts) by flight (Wickman & Wiklund 1983; Shreeve 1987; Van Dyck et al. 1997a). Although *P. aegeria* has attracted much interest in behavioural ecology (Van Dyck 2003 and references therein), the relationship between male behaviour and resource allocation to spermatophores, with respect to body size and age, has not yet been fully explored. This relationship is, however, a key aspect to ultimately understand the pay-offs of the different behavioural tactics under particular environmental and population conditions (Van Dyck 2003).
Butterflies strongly depend on the reserves accumulated during their larval stage to allocate energy to somatic maintenance, flight activity, and reproduction, since adult resources (like nectar) are only rich in carbohydrates and water, although the balance between capital and income breeding may vary considerably among species (Jervis et al. 2005; Erhardt & Mevi-Schütz 2009). Territorial males of _P. aegeria_ defend sunlit patches on the forest floor and they rarely have adult feeding sources available in their sunlit patches (Kemp & Wiklund 2001) and territorial defence is assumed to be particularly costly in terms of energy (Candy 1997). Therefore, energy needs to be traded-off between flight activity, body maintenance (longevity), and reproduction. Moreover, intensive male-male competition and predation risks may further impact the balance between current and future reproduction, but to the best of our knowledge, little is known about these aspects in our study system.

In this paper, we analyzed the impact of male age, male size, activity level, mate-locating tactic, and heritability (i.e., family effect) on copulation duration and on male reproductive investment estimated by the spermatophore mass and the number of eupyrene sperm (i.e., fertile sperm). We predict older and larger males will produce larger first spermatophores. We compared spermatophores of active males that had flown in large outdoor cages for a few days with spermatophores of low-activity males that had stayed in small containers that allowed fluttering but no continuous flight. Next, we compared spermatophore traits among the active males that had engaged in different behavioural mate-locating tactics in the outdoor cages. In another experiment, we simulated intensive interactions by
forcing males to take-off repeatedly and to manoeuvre in a small cage during repeated sessions. The spermatophores of these males were compared with spermatophores of resting males. From a flight-reproduction trade-off viewpoint, we predict i) low-activity males to be able to produce larger spermatophores than actively flying males in the experiment in the outdoor cages and in the forced flight experiment in small cages in the laboratory. In line with recent results showing that territorial *P. aegeria* males in the outdoor cages metabolised more lipids through their flight performance compared to non-territorial males (Chapter V), we also predict ii) territory holding, perching males to produce smaller spermatophores than patrolling males. The experimental design of our experiments with butterflies of a recently established breeding stock allowed taking into account family effects of the studied traits. Hence, we also tested whether brothers had a higher tendency to adopt the same mate-locating behaviour than what would be predicted by chance. We also studied the impact of female quality on male reproductive investment. Although there is no evidence for pre-copulatory male mate choice in *P. aegeria*, males may show cryptic mate choice by varying the amount of resources allocated to mating on the basis of female quality (Bonduriansky 2001). Therefore, we also included female traits (size and age) in the analyses.
VII.2. Materials and methods

Study species

The Speckled wood butterfly (*Pararge aegeria*) is a temperate-zone satyrine butterfly that primarily occurs in different types of woodland. Eggs are laid singly on different grass species (Shreeve 1986). Females mate soon after eclosion and usually mate only once in their life, unless the first spermatophore was non-substantial (Wickman & Wiklund 1983; Vande Velde et al. 2011b (*Chapter VIII*)). Males locate females by perching or by patrolling behaviour, which represent different types of flight (Van Dyck 2003). Perching requires multiple short, explosive flights with high levels of acceleration and manoeuvrability in order to approach a passer-by in a fast way, whereas patrolling requires longer, but less powerful flights at constant and lower speed (Van Dyck et al. 1997b; Berwaerts et al. 2006). Fast take-off with high acceleration performance is one of the most energetically demanding types of aerial locomotion (Dudley 2000).

Laboratory breeding

Experiments were done with individuals from a recently established laboratory breeding stock. Founding females were caught in a woodland population in central Belgium (Bois de Lauzelle in Louvain-la-Neuve, 50°67'74"N, 4°59'78"E). Breeding took place under standardized environmental conditions in a climate room (Light-Dark: 16h-8h; 24°C during the day and 16 °C at night). These conditions induce a direct larval development without diapause.
(Wiklund et al. 1983). We used potted *Poa trivialis* reared in a greenhouse as the host plant. Hatched larvae were placed on similarly sized tufts of grass (N = 6 per tuft) covered with a fine-meshed netting. For further details on the breeding protocol, we also refer to Lauwers & Van Dyck (2006). Pupae were placed individually in transparent jars (125 ml) until eclosion. Pupal mass was weighed on day 9 after pupation (Ohaus Explorer balance; accuracy: ± 0.1 mg). Pupal mass correlates strongly with adult mass at eclosion (R = 0.92, P < 0.001, N = 156), and was used as an index of adult body mass.

*Active flight versus low-activity treatment in males*

Males eclosed in the transparent jars. They had access to a 10% sugar-solution on cotton. Date of eclosion was recorded (to track the age of each individual). Next, males were assigned to one of two treatments: active flight in a large outdoor cage versus a control with limited flight activity. The latter males were kept in their small jar in the climate room. For the active flight treatment, we introduced the individually marked males into two identical semi-cylindrical outdoor cages (9.0 x 3.7 x 1.8 m). Camouflage netting with some holes on the cages created dappled light conditions as in a forest with sunlit patches. The floors of the cages were covered with wood chips and there were small, green artificial trees that were used as perches by the butterflies. Similar cages have been used to study *P. aegeria* behaviour before (Leimar et al. 2003; Merckx et al. 2003; Kemp et al. 2006). There were 6 males per outdoor cage per session. All males were issued from 11 different families in total. Males stayed for 2-3 days in the outdoor cages and they had access to a 10% sugar solution.
on cotton. We did multiple observation sessions per group of males to score the behaviour of each male. This information allowed classifying each male into one of three types: 1) PE2: a strong and permanent percher (i.e., high fidelity to a sunlit patch, high level of aggression, high frequency of short inspection flights); 2) PE1: an occasional percher (i.e., males that defended a sunlit patch during one period, but behaved as a non-percher for another and typically longer period of time in the cage), and 3) PE0: a non-percher (i.e., males that were rarely or never observed to defend a sunlit patch and that regularly flew through the cage, eventually alternated by resting on the netting).

**Forced flight treatment**

In order to simulate intensive male-male interactions, and therefore, frequent take-offs flights, we did an additional experiment in the laboratory. One-day old males from the breeding stock that were not involved in the other experiments were transferred individually to small cages (0.3 m$^3$) under standardized climate room conditions (see above). They were assigned to one of two treatments: forced flight treatment or control treatment. Males were forced to fly in their small cage (at 25°C in the climate room) during 5 min by gently touching their legs with a fine-bristled paintbrush each time they alighted (cf. Gibbs et al. 2010a). The treatment was repeated three times a day, until a male had flown for 10 sessions of 5 min (i.e., 4 days). One hour after the final forced flight session, males were allowed to mate with a virgin female. Control males were not forced to fly and were also offered a female to mate at the same age as the
males in the forced flight treatment. Both groups had access to a 10% sugar solution on cotton.

*Matings*

After the flight or control treatment, males were placed individually in a small cage (0.3 m$^3$) under standardized climate room conditions (see above). In the first experiment, males varied in age from 2 to 20 days. In the forced flight treatment, all males mated at the age of 5 days. A virgin female of known age and pupal mass from the breeding stock was introduced in the cage. Several cages (and thus pairs) were observed at the same time in order to record copulation events and their duration. Immediately after the copulation, the female was killed by freezing (-20°C).

*Spermatophore traits*

The bursa copulatrix was carefully dissected from the abdomen of the frozen females. The mass of the bursa was measured (Mettler Toledo-MT5 balance; accuracy: ± 0.001 mg). Next, we carefully removed the spermatophore from the bursa and measured its mass. The difference between both mass values is assumed to reflect the mass of the accessory substances since the mass of the thin wall of the bursa is negligible (Marshall 1982). The spermatophore was placed in a drop of modified Barth Saline on a slide (Gurdon 1991). Next, the spermatophore wall was ruptured by forceps to release the sperm that was gently stirred with a fine needle. Males transfer eupyrene sperm in bundles which will later break apart (Cook and Wedell 1996). In
order to count the number of bundles, we fixed the drop with 70% ethanol and coloured the DNA of the spermatozoids with 4',6'-diamidino-2-phénylindole. After drying, the bundles can be easily counted under a microscope (x 100; Epifluorescence Polyvar). As bundles contain 256 eupyrene sperm cells per bundle (Cook and Wedell 1996), we multiplied the number of bundles by 256 to obtain the total number of eupyrene sperm cells.

Statistical analyses

We analyzed spermatophore mass, number of eupyrene sperm bundles and length of the copulation relative to the level of activity or the behavioural tactic. The analyses took into account the covariates male age and body mass, as well as female age and body mass, and all biological relevant interaction terms. For model selection, we used a multi-model inference approach (using R software), based on corrected Akaike’s Information Criterion (AICc) (Burnham & Anderson 2002). Generalised linear regression models with a normal residual distribution were used. Models with all possible combinations of the carefully chosen explanatory variables were fitted and compared using Akaike Information Criterion (AIC) corrected for small sample size, AICc (The values of AIC and AICc tend to converge when sample size increases). The model selection uncertainty was estimated by calculating the probability for a given model to be selected as the best model (model weight) if new data were re-sampled. A weight is also calculated for each explanatory variable as the sum of the model weights of the models in which each explanatory variable is present. This variable weight is an estimate of
the relative importance of each explanatory variable in terms of predictive power. Due to the presence of interaction terms in the models, we used a permutation test (N. Schtickzelle and G. San Martín y Gomez, unpublished) to help the interpretation of the weight of the variables. A \( P \)-value was computed for each explanatory variable, representing the probability to obtain a value equivalent or higher to the observed AICc weight of the variable if it has no predictive value on the response (see Chapter II for more details). Variables with a \( P \)-value < 0.05 were considered as significant. Model averaged coefficients were estimated as the mean of each parameter from each model, weighted by the model weight. A similar approach was used to estimate the standard errors of the model coefficients that takes into account model selection uncertainty and was not conditional to one model. This approach has many advantages over more classical stepwise approaches that end up with a unique "best model" with an unrealistic dichotomous distinction between important and non important explanatory variables (while several other models could be comparable) and in which the standard errors and model coefficients are biased. For the active males in the outdoor cages, family was also included as a covariate in the model.

The association between the family effect and the behaviour adopted by males was evaluated using \( \chi^2 \)-test (SAS, version 9.1). All mean values are given ± SE.
Chapter VII

VII.3. Results

Active and low-active males

Spermatophore mass was positively correlated with the mass of the bursa copulatrix (R = 0.79, F_{1,189} = 313.38, P < 0.0001). Heavier males produced heavier spermatophores, but there was also an additive effect of the age of the male, the activity level, and the interaction effect between age x activity (Table 1). Older males produced larger spermatophores than young males but the increase of spermatophore size with age was more pronounced in low-active males (Fig. 1a). Very young males transfer very small spermatophores. As there was some bias with more very young males in the low-activity group compared to the active group and the reverse for old males, we repeated the analysis excluding the youngest males (< 6 days) and oldest males (> 16 days) (interval indicated by dashed lines on Fig. 1a). The interaction effect between age x activity also remains significant with this age-adjusted dataset (Table 2). Spermatophore mass was 0.336 ± 0.004 mg and 0.365 ± 0.007 mg for active and low-active males, respectively.

The number of bundles was higher in older males, but this increase with age was much more pronounced in low-activity males compared to active males (Table 1; Fig. 1b). The number of bundles was positively correlated with spermatophore mass (R = 0.57, F_{1,189} = 89.91, P = 0.001). When we redid the analysis with the age-adjusted data as explained before, there was no longer a strong support for the interaction effect between age x activity (Table 2). Independently of male age, active males produced less eupyrene bundles than low-
activity males (Table 2; see dashed lines Fig 1b). A spermatophore contained on average $53.47 \pm 1.6$ and $77.18 \pm 2.8$ eupyrene bundles in active and low-activity males, respectively.

![Figure 1](image1.png)

**Figure 1.** The effect of age on reproductive investment of males relative to the level of activity ($A0 =$ Low-activity males; $A1 =$ active males) in terms of a) spermatophore mass, and b) the number of eupyrene bundles per spermatophore. The dashed lines represent the range of ages used in the age-adjusted analyses (see text).
Copulations lasted on average 23.5 ± 0.3 min. Any factors influenced copulation duration in a strong way (Table 1 and 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Freq (%)</th>
<th>ATC weight (%)</th>
<th>p-value</th>
<th>Parameter estimate</th>
<th>S.E.</th>
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<td>Spermatophore mass (n=178)</td>
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<tr>
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<td>0.0001</td>
<td>0.0001</td>
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Table 1. Results on reproductive investment in active compared to low-activity males by multi-model inference of the support of each variable to explain spermatophore mass, number of sperm bundles per spermatophore.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Freq (%)</th>
<th>AICc weight (%)</th>
<th>p-value</th>
<th>Parameter estimate</th>
<th>S.E.</th>
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<td>Number of sperm Bundles (n=178)</td>
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and length of copulation, respectively. The statistical support of each variable is expressed by the AICc weight expressing the probability that the variable affects the response (strong support is indicated in bold). The p-value, evaluating the significance of the difference between the AICc weight and the a priori weight, helps to interpret AICc weight value despite differences in the frequency of the variable in the models (Freq). Model-averaged parameter estimates and standard errors are given. For the categorical variable ‘activity’, the parameter estimate expresses the difference with the reference level fixed to zero (i.e., low-activity males: Activity 0). The full model was: $Y = \text{Male age (MA)} + \text{Male body mass (MB)} + \text{Female age (FA)} + \text{Female body mass (FB)} + \text{Activity (ACT)} + \text{MA*MB} + \text{MA*FB} + \text{MB*FB} + \text{MA*FA} + \text{MB*FA} + \text{MA*ACT} + \text{MB*ACT}$.

<table>
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<th>p-value</th>
<th>Parameter estimate</th>
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### Table 2. Same analysis of the table 1 but on the age-adjusted data set (see Fig. 1).

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| **Length of copulation (n=145)** |          |                 |          |                    |       |
| Intercept           | 100      | 100.00          | .        | 23.9836            | 0.7189|
| Activity 1          | 77.3     | 73.39           | 0.108    | **-1.1912**        | 0.7262|
| Male body mass      | 90.2     | 59.87           | 0.439    | -0.0216            | 0.0220|
| Male age            | 90.2     | 47.24           | 0.771    | 0.0061             | 0.0630|
| Female body mass    | 85.5     | 45.86           | 0.671    | -0.0086            | 0.0105|
| Female age          | 85.5     | 43.60           | 0.748    | -0.0265            | 0.0574|
| MA*MB               | 41.2     | 15.03           | 0.345    | **-0.0018**        | 0.0020|
| MB*Activity 1       | 35.5     | 11.42           | 0.391    | 0.0036             | 0.0081|
| MA*Activity 1       | 35.5     | 10.85           | 0.441    | **-0.0186**        | 0.0314|
| MB*FA               | 39.2     | 9.73            | 0.578    | 0.0007             | 0.0010|
| MB*FB               | 39.2     | 7.19            | 0.762    | 0.0002             | 0.0013|
| MA*FA               | 39.2     | 6.26            | 0.844    | **-0.0010**        | 0.0021|
| MA*FB               | 39.2     | 5.79            | 0.899    | 0.0003             | 0.0003|
| FB*FA               | 37.3     | 5.61            | 0.868    | -0.0001            | 0.0003|
Perching and non-perching males

Under our controlled conditions, males of the different behavioural types did not differ in age ($F_{2,109} = 1.94, P = 0.149$), nor in body mass ($F_{2,109} = 1.55, P = 0.216$). Families differed in the frequencies of the male behavioural tactics ($\chi^2 = 36.98$, ddf = 20, $P = 0.0118$).

The analysis showed that there was no significant difference between the three behavioural types neither in spermatophore mass nor in number of eupyrene sperm cells. This was still valid if we only compared the strong perchers (PE2) against the non-perchers (PE0), hence by excluding the intermediate group (details not shown). Copulation length tended, however, to be weakly influenced by the behavioural tactic (Table 3): copulations with territorial PE2 males tended to be on average longer (average copulation length: PE2 males: $24.2 \pm 0.7$ min; PE1 males: $22.7 \pm 0.7$ min and PE0 males: $21.6 \pm 0.8$ min). Spermatophore mass was highly affected by family, male body mass, male age and by the interaction between both the last two factors (Table 3). Older and heavier males produced larger spermatophores, but the increase of spermatophore size with age was lower in heavier compared to lighter males. The number of eupyrene sperm was only strongly affected by male age and tended to be influenced by the family effect (Table 3). The copulation duration was influenced by the family effect and by the interaction between male age and male body mass (Table 3).
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### Chapter VII

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Table 3. Results on reproductive investment in males that differ in mate-locating strategy in outdoor cages by multi-model inference of the support of each variable to explain spermatophore mass, number of sperm bundles per spermatophore and length of copulation, respectively. The statistical support of each variable is expressed by the AICc weight expressing the probability that the variable affects the response (strong support is indicated in bold; intermediate support is indicated in italics and bold). The p-value, evaluating the significance of the difference between the AICc weight and the a priori weight, helps to interpret AICc weight value despite differences in the frequency of the variable in the models (Freq). Model-averaged parameter

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<th>AICc weight (%)</th>
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estimates and standard errors are given. For the categorical variable ‘family’ and ‘behavioural tactic’, the parameter estimate expresses the difference with the reference level fixed to zero (i.e., F01 and PE0 were fixed at zero, respectively). The complete model was: $Y = \text{Male age (MA)} + \text{Male body mass (MB)} + \text{Female age (FA)} + \text{female body mass (FB)} + \text{tactic (Tactic)} + \text{Family} + \text{MA*MB} + \text{MA*FB} + \text{MB*FB} + \text{MB*FA} + \text{MA*FA} + \text{FB*FA}$.

Forced flight and resting males

Spermatophore mass was also positively correlated with the mass of the bursa copulatrix in this sample ($R = 0.79$, $F_{1,46} = 77.32$, $P < 0.0001$, $N = 48$). Spermatophore mass was only influenced by male body mass, but not by flight treatment (Table 4, Fig.2a). The number of euphyrene bundles was higher in males that were forced to fly compared to the males in the control group (Table 4, Fig. 2b). Copulations lasted longer in heavy compared to light males (Table 4).
Table 4. Results on reproductive investment in males of the forced-flight experiment by multi-model inference of the support of each variable to explain spermatophore mass, number of sperm bundles per spermatophore and length of copulation, respectively. The statistical support of each variable is expressed by the AICc weight expressing the probability that the variable affects the response (strong support is indicated in bold). Model-averaged parameter estimates and standard errors are given. For the categorical variable ‘treatment’, the parameter estimate expresses the difference with the reference level fixed to zero (i.e., forced flight males). The complete model was: \[ Y = \text{Male body mass (MB)} + \text{Female age (FA)} + \text{female body mass (FB)} + \text{Forced Flight Treatment (FFT)}. \]
Figure 2. The effect of forced flight on reproductive investment of males in terms of a) spermatophore mass, and b) the number of sperm bundles. FFT: males under forced-flight treatment. RT: control group of resting males.
VII.4. Discussion

As predicted based on the literature (e.g., Boggs 1981b; Oberhauser 1988; Gage 1994), we observed an increase of *P. aegeria* spermatophore mass with male body mass and with male age in our experiments. For Lepidoptera, it has been proposed that males require a maturation period of several days after emergence to accumulate sufficient sperm and accessory fluids for successful copulation (LaChance et al. 1977). The relationship with age has been confirmed in several other species (e.g., Oberhauser 1988; Bissoondath & Wiklund 1996; Delisle & Bouchard 1995), but the novel aspect of our study is the interaction effect with the level of flight activity. The increase of male spermatophore mass with age was stronger in low-activity males compared to active males, which suggests that male flight activity has a negative impact on seminal accumulation. However, the effect of flight activity on fertile sperm accumulation was less clear. In the same vein, we argue that male flight activity related to behavioural tactics may impact spermatophore size and sperm number; trade-offs between flight and egg size have been shown in female *P. aegeria* in experiments where they were forced to fly for short periods (Gibbs et al. 2010a). Typical perching behaviour implies repeated short and powerful take-off flights and high levels of acceleration and manoeuvrability which are the most energy demanding types of locomotion (Marden 2000; Chapter V). Hence, a stronger trade-off between flight and reproduction was predicted for perching males. However, in the experiment in the outdoor cages, we did not observe an effect of mate-locating behaviour on
spermatophore traits. We discuss our results relative to both adaptive and mechanistic explanations of the complex relationship between male activity, age and reproductive investment.

In a previous study on *P. aegeria* males, Svärd (1985) did not find any relationship between body mass and spermatophore mass, nor with the mass of accessory gland substances. She suggested that the absence of such a correlation was due to the quantitatively small investment made by the male in terms of spermatophore mass (1.4% of the body weight) compared to several polyandrous species where spermatophore mass was strongly correlated to male body mass. However, in our study, we did observe larger males producing larger spermatophores. As we have shown a strong effect of age on spermatophore mass, the age of the butterflies used in both studies may have been different, and consequently alter the body mass/spermatophore mass relationship. Of course, there could be other intra-population differences that contribute to the differences between the studies as we worked with a population of Belgian origin and Svärd with a Swedish population. Further studies are required to study variation in reproductive investment along a latitudinal gradient.

In the first experiment, we showed a negative impact of flight activity on the increase of spermatophore mass with age. The physiological mechanism behind this flight-spermatogenesis relationship is still unclear at the moment. Low-activity males did not pay the full operational energetic cost of flight activity leaving them with more available energy to allocate to the spermatophore at an older age compared to actively flying males. Moreover, independently of male age, low-activity males had higher fertile sperm accumulation
than males that had actively flown in the outdoor cages. These results highlight an apparent trade-off between the energy invested in flight and in reproduction.

From a flight-reproduction trade-off viewpoint, we predicted similar results for males in the forced flight treatment. However, we obtained contradicting results. Males that had been forced to fly produced spermatophores of equal mass and more fertile sperm than resting males (taking into account age and size effects). Of course, such manipulative experiments may also induce other effects or they may interfere with the perception and motivation of individuals to respond in a particular way. Hence, we remain cautious when interpreting these conflicting results in terms of flight performance only. Actively flying males in the cages may, for example, have metabolised more energy in flight activity than forced flight males. This, in turn, may have resulted in a steeper flight-reproduction trade-off during the first experiment. However, recent results showed that forced flight males lose as much as lipid reserves as do flying males in the outdoor cages (Chapter V). Still, factors other than energy use could also intervene. The frequent contacts during the forced flight experiment could simulate high intensity of male competition with conflicts that may alter spermatophore production even independent of flight performance per se. Such subjective effects may alter the male’s strategy to maximize current investment at the expense of future reproduction. Several studies on sperm competition in insects have shown that in the presence of sexual rivals, males increase the number of sperm in the ejaculate compared to conspecific males that were kept alone (Lepidoptera: Gage 1991; Gage & Baker 1991; Orthoptera:
Gage & Barnard 1996). However, all these studies concern polyandrous species with high levels of sperm competition. We may expect a similar pattern in monandrous species since the operational sex ratio is virtually always male-biased as females are no longer receptive after their first mating. We cannot exclude the possibility that there were other differences between the outdoor cage experiment and the forced flight experiment that would interfere with male reproductive decisions. One may think about differential effects in terms of environmental stress and perception of predation risks. The forced flight treatment, as we applied it here, may have generated a stimulus of high predation risk as males were repeatedly touched to make them fly. Such a stimulus may also increase current investment at the expense of longevity. Of course, it remains unknown (and somewhat speculative at this stage) how this type of manipulative experiments would interfere with stress responses independent of flight performance.

Trade-offs between flight and reproduction have been particularly studied in females (e.g., Gibbs et al. 2010a), but much less so in males. As the behavioural tactics adopted by P. aegeria males to locate females represent different flight performances, it is an interesting case to test for differences in such trade-offs between males adopting different mate-locating tactics. However, while the type of flight of a territorial perching male, as the behaviours were expressed in our outdoor cages, was energetically more costly than a patrolling male (Chapter V), we did not find evidence for differences in spermatophores between the three behavioural types. Perching males tended to take longer to transfer their spermatophore compared
to non-perching males. At this stage, we cannot yet definitely exclude
a link between mate-locating behaviour and reproductive investment
for two reasons. First, there can be qualitative differences between the
spermatophores of the different behavioural types that are independent
of spermatophore size. For example, in the Armyworm moth
_Pseudaletia unipuncta_ there was no relationship between lipid and
hydrocarbon content and the mass of the spermatophore (Marshall &
McNeil 1989). Secondly, the way territorial perching and patrolling
behaviours were expressed in the outdoor cages may need further
consideration relative to their potential impact on spermatophore
production. The territorial perching behaviour strongly resembled this
behavioural tactic as it can be observed under field conditions (Van
Dyck et al. 1997a), as has also been noticed in other studies on male
behaviour of _P. aegeria_ in cages (e.g., Kemp et al. 2006a). The
expression of patrolling behaviour may be somewhat different in the
outdoor cages compared to real field conditions. Several males were
classified as ‘non-perchers’ but they were not necessarily in flight
most of the time and several of them were frequently sitting on the
netting of the cage. Then, energy consumption differences would in
principle have been larger in our cages compared to field conditions.
However, there are also other factors that differ as patrollers in the
outdoor cages are more frequently chased and attacked by perchers
than the average patroller under field conditions. In the outdoor cages,
patrollers have little opportunity to escape from aggressive territory
holders, whereas it has been documented that males may avoid
crossing certain territories in the field (Wickman & Wiklund 1983).
Therefore, patrolling males in the outdoor cages may have
experienced relatively high competition pressure which may result in a higher current reproductive investment (cf. forced flight experiment as discussed above). It would be worth doing similar experiments in much larger cages or with marked individuals in a natural population.

The gradual increase of spermatophore size with age may have different consequences for perching and patrolling males as males of the two main tactics could differ on average in their age at the first mating. Patrolling males have been reported to locate females at a faster rate than perching males, but they were more restricted by thermal conditions (i.e., convective cooling during flight; Shreeve 1984). Therefore, there may be differences for the age at first mating between typical perchers and patrollers. This needs further work, such as releasing virgin females in a field study with several marked males of known history and of different mate-locating tactic.

In our experiments, males of all ages were willing to mate. We observed that very young males produced very small spermatophores. Dewsbury (1982) suggested that selection would favour males waiting between matings until the amount of material to transfer to the female ensured a certain paternity level. This is not the case in P. aegeria and in other butterflies (e.g., Oberhauser 1988; Bissoondath & Wiklund 1996). In temperate-zone regions with variable weather conditions, these short-living flying heliotherms are likely to be time-stressed, particularly if operational sex ratio is strongly male biased. Hence, there will be selection for mating at the first opportunity, rather than taking into account potential future matings. It would be interesting to study variation in time-stress and the consequences for reproductive
investment in *P. aegeria* along a latitudinal and hence climatic gradient (Gotthard et al. 1999).

Although our study was not designed to study quantitative genetics, the highly significant family effect of spermatophore mass is in line with heritable variation underlying this trait. Several adult traits, including body mass, are known to have a heritable basis in *P. aegeria* (Van Dyck et al. 1998; Berwaerts et al. 2008), but these analyses have not yet included traits relating to male reproductive investment. We found a significant family effect of the mate-locating tactic. In other words, brothers tended to adopt similar mate-locating behaviours. This result is in accordance with the field study by Van Dyck et al (1997b) showing that males were more likely to be consistent in either perching or patrolling rather than switching between both behavioural tactics. Nevertheless, we also observed families with males adopting both tactics.

Rutowski et al. (1983) showed for seven butterfly species that males transferred larger spermatophores to large females. Males may also discriminate between spermatophores on the basis of age, providing more sperm to younger females (e.g., Johnson & Hubbell 1984; Proshold 1996). However, in our experiments, we have not found evidence of any effect of female body mass and age on male reproductive investment. Forsberg’s (1987) model showed that mate choice by males is only profitable under high densities of receptive females and under low mortality rate. As the sex-ratio is typically heavily male-biased, these conditions appear rarely, if ever, in populations of *P. aegeria*. At this stage, we cannot completely exclude the role of female quality for spermatophore quality. Bissoondath & Wiklund
(1996) showed, for example, that males of *Pieris napi* provided more proteins to larger females, but spermatophore mass was not affected. It would be very relevant to focus further work on the composition of spermatophores. Choice experiments to force a male into making a choice between two or multiple females would be valuable as well. In our experiments, males did not have a choice between females as only one female was offered. In summary, there is clearly scope for additional experimental work on male reproductive investment and tests of male choice to improve our understanding of the reproductive biology of *P. aegeria*, and particularly to better understand the relative pay-offs of perching and patrolling mate-locating tactics.
Chapter VIII

VIII. Spermatophore and sperm allocation in males of the monandrous butterfly Pararge aegeria: the female’s perspective

Lesley Vande Velde, David Damiens and Hans Van Dyck

[The content of Chapter VIII has been published in Ethology 117 (2011) 645–654]

Recently mated males produced smaller spermatophores. Receiving a small spermatophore from a recently mated male represents a fitness cost for the female. In this chapter, we tested whether females are able to discriminate between mating status of males and whether they are able to compensate for receiving a small spermatophore by remating.

Author contribution: Study design: L.V.V., D.D. and H.V.D.; Field/Lab work: L.V.V.; Data analysis: L.V.V.; Writing: L.V.V., D.D. and H.V.D.

Thanks are due to Hubert Baltus, Christophe Pels and Melanie Gibbs for practical assistance, and to Ellyn Bitume for language editing.
Abstract

In insects, spermatophore production represents a non-trivial cost to a male. Non-virgin males have been shown to produce small spermatophores at subsequent matings. Particularly in monandrous species, it may be an issue to receive a sufficiently large spermatophore at the first and typically only mating. Females of the monandrous Speckled wood butterfly *Pararge aegeria* (L.) produce fewer offspring after mating with a non-virgin male. After mating, females spend all their active time selecting oviposition sites and typically ignore other males. Here we show that females did not discriminate between a virgin male and a recently mated male in our laboratory experiments. We demonstrate that the number of eupyrene sperm bundles relative to spermatophore mass differed with subsequent male matings. Males transferred a significantly smaller spermatophore after the first copulation, but the spermatophore mass did not decrease further with subsequent matings. However, the number of eupyrene sperm bundles decreased linearly. Therefore, there was proportionally more eupyrene sperm in the male’s second spermatophore compared to the first and the later spermatophores. Such a pattern has been shown in polyandrous species. Hence, it suggests that differences in sperm allocation strategy between polyandrous and monandrous butterflies may be quantitative rather than qualitative. There was also a tendency for females that had mated with a recently mated male to have higher propensity to re-mate than did females that had mated with a virgin male. We discuss the results
relative to the mating system in *P. aegeria*, including female remating opportunities in the field and male mate-locating behaviour.
Males invest little in individual sperm cells, but rather increase fitness by maximizing sperm numbers and the number of matings (Parker 1970, 1984; Trivers 1972). However, in some insect species, sperm cells are delivered to the female in a package or spermatophore; the resources needed to produce such a spermatophore (i.e., sperm and additional substances) can be a limiting factor to male fitness (e.g., Van Voorhies 1992; Wedell et al. 2002; Sella & Lorenzi 2003). Sperm production itself can also be costly and some species have limited amounts of sperm at emergence (Boivin et al. 2005); sperm depletion has been demonstrated in several species (Gordh & DeBach 1976; Nadel & Luck 1985; King 2000). However, males are known to mate independently of sperm availability. In some species, males continue to mate even if sperm is exhausted (Damiens & Boivin 2006; Steiner et al. 2008). Small spermatophores may have negative fitness consequences. Effects of male mating history on female fitness have been studied in several Lepidoptera (e.g., Svärd & Wiklund 1986, 1989; Kaitala & Wiklund 1994; Wedell & Cook 1999; Jiménez-Pérez & Wang 2004a; Torres-Vila & Jennions 2005; Marcotte et al. 2007), but also in a few other insect groups (e.g., Montrose et al. 2004; Wedell & Ritchie 2004; Rönn et al. 2008).

Female butterflies that copulated with a recently mated male received a smaller spermatophore with fewer resources than in the case of a spermatophore from a virgin male (e.g., Rutowski et al. 1987; Lauwers & Van Dyck 2006). Hence, from an ultimate viewpoint a female may adopt one of two behavioural strategies:
discriminate between males based on their mating history, or mate more than once to replenish her sperm stock. Polyanndrous species typically follow the latter option, although the driving force may also be genetic diversity, nutritional benefits, or reducing time loss from male harassment, rather than sufficient sperm supply per se (Thornhill & Alcock 1983, Ridley 1988). In monandrous species, receiving a sufficiently large spermatophore at the first and only mating is likely to be a significant life history issue for a female.

In insects, females have been shown to choose a male based on several male characteristics including size, age, or mating status (Rutowski 1984; Morris et al. 1989; Iyengar et al. 2001). Females of the cockroach *Nauphoeta cinerea* discriminate, for example, against multiply mated males (Harris & Moore 2005). It was suggested that females are able to detect the cues of males from previous mates and use this information to avoid mating with sperm-exhausted males. In several Lepidoptera, females mate preferentially with virgin males (Jiménez-Pérez & Wang 2004b, but see review in Torres-Vila & Jennions 2005).

By mating several times, females can reduce the cost of mating with a sperm-depleted male and so they can maintain or reach the critical load of sperm and other male-derived resources of the spermatophore (Ridley 1988). Hence, females may copulate several times at the beginning of their life in order to have sufficient viable sperm to fertilize all the eggs laid throughout their reproductive life (Chevrier & Bressac 2002; Damiens & Boivin 2006), or they re-mate later in life to replenish the spermatheca to maintain fecundity (Hsu & Wu 2000; Mery & Joly 2002). Among the Lepidoptera, there are
several examples of females that re-mate much faster after an initial mating with a non-virgin male than with a virgin male (Kaitala & Wiklund 1994; Foster & Ayers 1996; Marcotte et al. 2007, Elzinga et al. 2011). This has also been shown in other insects (e.g., Savalli & Fox 1999; Bailey & Nuhardiyati 2005; Perez-Staples et al. 2008). In Lepidoptera, the factors that trigger remating may be either physical or chemical (i.e., physiological). The size of a spermatophore may, for example, stimulate the stretch receptors in the female’s spermatheca (Obara et al. 1975; Sugawara 1979). Seminal fluid components or male accessory gland components may provide a physiological signal to the female (Giebultowicz et al. 1991; Proshold 1995; Torres-Vila et al. 1997, Marcotte et al. 2006).

Here we address spermatophore production in the monandrous Speckled wood butterfly (*Pararge aegeria* L.). Spermatophore counts in wild-caught females of a Swedish population showed that the majority of females mated only once, and some females mated more than once (number of spermatophores in wild: 1.04, Wickman & Wiklund 1983). In this species, the production of a spermatophore generates non-trivial costs for male reproduction (Svärd 1985); it takes up to seven days after the first copulation before the male is able to produce a similarly sized spermatophore. Moreover, when a male mates twice on the same day, the second spermatophore will reach only about a third of the mass of the first (Lauwers & Van Dyck 2006). Male mating status did not affect female life span or the number of eggs laid, but Lauwers and Van Dyck (2006) showed an effect on the number of caterpillars the female produced. Observations in the laboratory and in outdoor cages indicate that *P. aegeria* males
are willing to mate with another female soon after copulation (Svärd 1985; Van Dyck, pers. observ.). Therefore, females that mate only once with a recently mated male may face a reproductive cost, unless they have the opportunity to avoid bad insemination or to re-mate.

Within this framework, the aims of our paper were more specifically: (1) to test how male spermatophore size and sperm number change in successive matings, (2) to determine whether females discriminate between virgin males and recently mated males, (3) to explore whether females that had been inseminated by a recently mated male have an increased willingness to re-mate in order to compensate for the disadvantages of the small first spermatophore.

VIII.2. Material and methods

Study species

The speckled wood (*Pararge aegeria* L.) is a temperate-zone satyrine butterfly of the western Palearctic. It mainly occurs in different types of woodlands where sunlight can penetrate to the forest floor and also along woodland edges (Tolman & Lewington 1997). The species has a complex life cycle with different developmental pathways corresponding to different seasonal cohorts (Wiklund et al. 1983; Nylin et al. 1995). Females lay their eggs singly on different host grass species (Shreeve 1986). Males may adopt a territorial sit-and-wait or perching tactic on a sunlit patch at the forest floor, or alternatively, they may adopt a searching patrolling tactic to locate females (Wickman & Wiklund 1983; Shreeve 1984, Van Dyck 2003).
Laboratory breeding

All butterflies used in the experiments were from a recently established laboratory breeding stock; eggs came from wild caught females of a mixed forest population in central Belgium (Bois de Lauzelle, 50°67’N, 4°59’E). Females were allowed to oviposit on potted tufts of the host grass *Poa trivialis* (Shreeve 1986) in small cages (0.30 m³) in the laboratory. *P. trivialis* was grown on a standard soil mixture in a greenhouse. Eggs were collected and transferred to Petri dishes. Hatched larvae were placed on the potted host grass (five larvae per potted plant) covered with a fine-meshed netting. They were allowed to develop directly without any diapause under standardized environmental conditions in a climate room (Light-Dark: 16h-8h; Day: 25 ± 2 °C and night: 15 ± 2 °C). Pupae were individually placed in small transparent jars (125 ml) until adult eclosion. Since pupal mass is strongly correlated with adult mass after eclosion (*r* = 0.92, *P* < 0.001, *n* = 156), we used in the analyses pupal mass as a measure of adult body mass. Pupae were weighed nine days after pupation (Ohaus Explorer balance; accuracy: ± 0.1 mg). Females were transferred to a cold room (Light-Dark: 10h-14h; 11 ± 2 °C) until they were used for the experiments. Males were maintained in transparent jars under the same laboratory conditions as during their larval development (see above) and they had access to a sponge soaked with a 10% sucrose water solution.
Experiments

Multiple matings and spermatophore production

Males were allowed to mate with a virgin female in small cages (0.30 m$^3$) under standardized laboratory conditions (see above). When the pair was separated after copulation, females were immediately killed by freezing (-20 °C). Males were transferred to the cold room (see above). After two days, males were moved again to the cages to repeat the mating experiment with a new virgin female. This procedure was repeated four times for each male. The bursa copulatrix was dissected from the female abdomen and the spermatophore was carefully removed and weighed (Mettler Toledo-MT5 balance; accuracy: ± 0.001 mg). The spermatophore was then placed in a drop on a slide (modified Barth Saline Gurdon 1991). The spermatophore wall was broken with forceps in order to release the sperm that was then gently stirred with a fine needle. During copulation, eupyrene sperm is transferred to the female in bundles that later break apart (Cook & Wedell 1996). To count the number of eupyrene sperm bundles, we fixed the drop with ethanol 70% and coloured the DNA in the spermatozoids with 4',6'-diamidino-2-phenylindole. After drying, the bundles were easily counted under a fluorescence microscope (x100; Reichert Polyvar Optical Microscope). In butterflies, each bundle contains 256 eupyrene sperm cells (Cook & Wedell 1996).

Virgin versus non-virgin male

To assess whether mating occurred with virgin males over recently mated males, virgin females (N = 69) were individually introduced into small cages (0.30 m$^3$) under the same laboratory
conditions as described above. In each cage, there was one mated male (i.e. mated 24 h before) and one virgin male. Both males were kept under laboratory conditions (see above) from emergence until the mating choice test. Males were individually marked with a fine, non-toxic permanent marker pen. Male size and age were taken into account. Age was standardized to control for such an effect (i.e. virgin and mated males proposed to the females were of similar age; ± 1 d). Male and female behaviours were observed to determine whether a female accepted a male. We used a sample of males within a limited range of body mass (no significant difference between virgin and mated males). After the copulation, females were immediately killed by freezing at -20°C and dissected as explained before.

Female remating propensity

To explore the probability of remating in females that had mated with a male of different mating status, we compared 15 females that had mated with a virgin male before and 15 females that had mated with a recently mated male. 24 h after their first mating, each female was placed for eight hours in a cage with one virgin male. We recorded whether females accepted (or not) mating with this new male. This informed us about female willingness to re-mate relative to the treatment of the first mating (virgin vs non-virgin male). The 30 females were dissected in order to check whether there is a relationship between female propensity to re-mate and the size of the spermatophore transferred during the first mating. However, there is a time lag effect as the females that re-mated did so early in the morning, about 24 h after the first mating. The females that refused to
re-mate were left with the male during the entire second day, so the spermatophore in that case had been collected 10 h later and its degradation was too advanced to estimate its original mass.

**Statistical analyses**

We used mixed regression models to analyse the data on multiple matings and spermatophore production (SAS, version 9.1, mixed procedure, SAS Institute Inc., Cary, North Carolina, USA). We used ‘male identity’ as random effect since each male produced 4 spermatophores. Degrees of freedom were estimated by the Kenward-Roger method. To compare reproductive investment between matings with a virgin and a non-virgin male, we used the nonparametric Wilcoxon-Mann-Whitney test because of reasons of normality and homogeneity of variance. We used a $\chi^2$ goodness of fit test to analyse female choice. Test of independence was used to analyse remating probabilities between the two experimental groups of females. We used G-test of independence adjusted by the Williams correction to prevent overestimation of G due to small sample sizes. All mean values are given ± SE.

**VIII.3. Results**

*Multiple matings and spermatophore production*

Spermatophores were much heavier at the first copulation compared to the male’s later copulations (Fig. 1a; $F_{3,86}= 200.76$, $P < 0.001$ – Average mass of first spermatophore: $0.288 \pm 0.007$ mg; second: $0.133 \pm 0.008$ mg; third: $0.160 \pm 0.007$ mg; fourth: $0.144 \pm$
0.005 mg). The number of eupyrene bundles in the spermatophore also declined significantly with the number of matings (F_{3,86} = 18.27, \( P < 0.001 \)), but interestingly, in a much more linear way (Fig. 1b: first spermatophore: 40.27 ± 2.02 bundles; second: 29.78 ± 2.62 bundles; third: 26.58 ± 2.28 bundles and fourth: 22.12 ± 1.63 bundles). The ratio of the number of eupyrene bundles on the spermatophore mass was significantly higher during the second mating compared to the first one (F_{3,86} = 14.36, \( P < 0.001 \)). For the next matings, the ratio decreased again reaching a level close to the one of the first mating (Fig. 1c).
**Figure 1.** The effect of *P. aegeria* male mating history on male reproductive investment in four successive spermatophores (each interval between matings: 48h): (a) spermatophore mass, (b) the number of eupyrene sperm bundles, and (c) proportion of eupyrene sperm bundles per unit of spermatophore mass (normalized to a spermatophore mass of 0.287 mg, i.e. mean spermatophore mass of the first mating).

**Female choice**

We tested the mate choice of 69 females that had access to a virgin male and a non-virgin, recently mated male. According to the observed behaviours, female choice did not occur. When the female was introduced in the cage with both males, the latter typically showed more active behaviour than did the female. When the female moved, she was rapidly intercepted by the most active male. The male positioned himself in front of the female and beat his wings against those of the female. Next, the male turned round the female trying to connect his abdomen to the genitalia of the female. The most active male in the cage had the highest mating probability. In the case of two highly active males, the mating pair was often disturbed by the other male who continued courtship displaying behaviour and aggressive attempts to take over mating. Females that were not willing to mate showed typical refusal behaviour. 31 out of the 69 females mated with a recently mated male, against 38 with a virgin male ($\chi^2_1 = 0.71, P = 0.47$). So, under the conditions tested, there is no evidence for any discrimination based on the mating history of the male.

In line with the first experiment and the literature (see Introduction), we found a significant difference in reproductive investment between virgin males and recently mated males. Spermatophore mass of virgin males was on average larger than that
of recently mated males (Fig. 2; 0.342 ± 0.011 mg vs. 0.130 ± 0.005 mg; Wilcoxon-Mann-Whitney test, U = -4.9343, \( N_1 = 21 \) \( N_2 = 14 \), \( P < 0.001 \)). Correspondingly, the same was true for the number of sperm bundles in the spermatophores of virgin males against recently mated males (Fig. 2; 65.88 ± 4.11 vs. 41.00 ± 3.30 bundles; Wilcoxon-Mann-Whitney test, U = -3.5577, \( N_1 = 17 \) \( N_2 = 14 \), \( P < 0.001 \)).

**Figure 2.** The effect of male mating status (virgin or not) on the two measures of reproductive investment: spermatophore mass and the number of eupyrene sperm bundles.

**Female remating propensity**

We did not observe any remating in the group of females that had previously mated with a virgin male, whereas 20% (3/15) of the females that had previously mated with a recently mated male re-
mated. So, there was a non-significant tendency to mate again in females that mated with a non-virgin male before (Gadj = 3.507, df = 1, \( P = 0.061 \)). All females (except one) had received a spermatophore from the first mating. Two of the three females that re-mated had a small spermatophore in their bursa copulatrix from their first mating (0.079 mg and 0.088 mg) and the third female had even not received a spermatophore at the first mating, although she copulated during a similar amount of time (c. 20 min). Females that had been inseminated by virgin males typically showed refusal behaviour to the second male, including the behaviour in which they fold their wings and fall down as can be observed in the field (H. Van Dyck, pers. observ.). The 12 females inseminated by mated males that did not re-mate showed similar behaviour. The females that re-mated received at the second mating a spermatophore of similar mass (0.347 ± 0.049 mg) to the spermatophore that the females received of a virgin male at the first mating (see above).

VIII.4. Discussion

As in other Lepidoptera (e.g., Svärd & Wiklund 1986), \textit{P. aegeria} males transferred significantly smaller spermatophores during subsequent matings compared to the first mating. For \textit{P. aegeria}, this is a confirmation of the results by Svärd (1985) and Lauwers & Van Dyck (2006). However, here we also showed the effect on the number of euphyrene sperm bundles. Interestingly, the pattern of spermatophore mass and the number of sperm bundles was different; the number of sperm bundles declined linearly whereas there was a
threshold effect for mass. Hence, there was proportionally more sperm in the second spermatophore compared to the first spermatophore. Such a pattern has been shown in polyandrous butterflies like *Pieris rapae*, and has been interpreted as different allocation strategies under different levels of sperm competition (Cook & Wedell 1996; Wedell & Cook 1999). Our results suggest that there is a quantitative rather than a qualitative difference between polyandrous and monandrous butterflies in sperm allocation strategies. Artificial selection experiments by Torres-Vila et al. (2002) have shown that there is heritable variation for polyandry in the usually monandrous moth *Lobesia botrana*. It would be interesting to study the relationships between mating system (degree of polyandry) with male investments in spermatophore size and content in a phylogenetic framework in order to better appreciate relative differences between the male strategies and their evolution.

It has been suggested that spermatophore mass is not necessarily strongly correlated with the amount of sperm (e.g., Marshall & McNeil 1989; Oberhauser 1992). Hence, spermatophore mass should be used carefully as an indicator of reproductive investment (e.g. Boggs 1981b; Svärd & Wiklund 1989, 1991). Here we showed that this is also the case in *P. aegeria*. Females that typically mate only once would receive a very different spermatophore if their mate was virgin or not. Lauwers and Van Dyck (2006) showed that males that were allowed to mate twice on the same day provided a spermatophore that was only about a third of the mass of the first spermatophore during the second mating. Our study showed that this mass stayed more or less stable at that level for the
next spermatophores, even if there was 48 h between subsequent matings. So there was no recovery in terms of mass within this timeframe, and the number of eupyrene sperm continued to decline. For logistic reasons, we were not able to investigate apyrene sperm in our experiments, but other studies have demonstrated that apyrene number may also change with male mating history (e.g. Cook & Wedell 1996).

There is evidence of a reproductive cost when *P. aegeria* females mate with a recently mated male compared to mating with a virgin male (Lauwers & Van Dyck 2006). In our experiment, females did not discriminate between virgin and non-virgin males. The level of activity of the both males – but independent of their mating history – may play a role for their probability to mate. We cannot exclude that some female choice would occur under natural conditions. Our cages do, for example, not allow long pursuit flights, and perhaps other courtship behaviours, known to occur in some butterfly species (Wiklund 2003; Wickman 2009). On the other hand, the risk of mating with a non-virgin male under natural conditions is difficult to estimate as little is known about mating frequency distributions of males in the field. In flight cages with several females, Svärd (1985) observed that *P. aegeria* males copulated on average 1.88 ± 1.02 times during their lifetime. From observations in the field and in outdoor cages, we know that males are immediately available for other females after a mating (Van Dyck, pers. observ.). It is not unusual to find old males that had several mating opportunities before on a sunlit patch (Van Dyck et al. 1997a).
After the first copulation, *P. aegeria* females did not show any interest anymore in the excited males. The refusal behaviours observed in the laboratory cages corresponded well to the behaviours observed in the field in this species (Van Dyck, pers. observ.) and in other satyrine butterfly species (Wiklund 1982). However, our results suggest that the females that had copulated with a recently mated male showed a tendency, though not significant with the low sample sizes of tested individuals, for a higher propensity to re-mate compared to females that had mated with a virgin male. It is now warranted to repeat this experiment with higher sample sizes. Remating after having received a small spermatophore has been observed in polyandrous Lepidoptera (Kaitala & Wiklund 1994; Marcotte et al. 2007) but also in monandrous Lepidoptera (Danthanarayana & Gu 1991; Foster & Ayers 1996; Elzinga et al. 2011). The small size of the spermatophore transferred by a recently mated male may be insufficient to stimulate the stretch receptors in the bursa copulatrix of the female to induce a long refractory period (Obara et al. 1975; Sugawara 1979). Monandrous females of Naryciinae species (Lepidoptera: Psychidae) were able to re-mate after a non-successful copulation (Elzinga et al. 2011). Females of the moth *Epiphyas postvittana* usually mate only once, but they do mate occasionally two or three times (Danthanarayana & Gu 1991; Foster & Ayers 1996). However, Foster & Ayers (1996) showed that a small proportion of the females that received a normally sized, first spermatophore from a virgin male did also re-mate. They suggested that the propensity to re-mate was not related to the size of the spermatophore but was most probably the result of a deficiency of some substances that elicit the
refractory behaviour in females. In our experiment, 12 out of 15 females received a small spermatophore and did not re-mate. At this stage we can only speculate about the mechanism in *P. aegeria*, and so further work is definitively needed. On the other hand, other factors such as a critical threshold number of eupyrene sperm or apyrene sperm in the spermatheca (Taylor 1967; Raulston et al. 1975; Proshold 1995; Torres-Vila et al. 1997; Marcotte et al. 2007), female weight (Torres-Vila et al. 1997) or variation in the quantity of seminal fluid components or male accessory gland components could also trigger female remating behaviour (Giebultowicz et al. 1991; Proshold 1995; Torres-Vila et al. 1997, Marcotte et al. 2006). We need to be careful with generalisations anyway, even within the Lepidoptera. In *Lariophagus distinguendus* for example, females typically mate only once in their lifetime, but there was no tendency to re-mate after an initial mating with a male whose sperm had been experimentally depleted (Steiner et al 2008).

In the field, mated females are known to leave the sunlit patches in the forest where they were inseminated. After leaving, females select wetter parts of the forest with suitable grasses to oviposit and alternate egg-laying with dispersal flights (Wickman & Wiklund 1983; Shreeve 1986). Consequently, the different distribution patterns of both males and females may generate a cost of lost opportunities for oviposition if females have to leave suitable egg-laying sites to revisit a male in case the first spermatophore was too small, as in the case of a recently mated male. Nevertheless, this cost may be reduced if females meet a male without searching (i.e. a patrolling male). For *P. aegeria* it has been suggested that territorial
perching males would have higher mating probabilities than patrolling males (Davies 1978; Wickman & Wiklund 1983; Bergman et al. 2007). Hence, on average it may be more likely to meet a non-virgin perching male than a non-virgin patrolling male. From a ‘sexy son’ type of reasoning, females would then prefer territory holders, but such a preference may compromise the females’ total fecundity in case of receiving a small spermatophore only. However, patrolling males cover larger areas of the woodland (Shreeve 1987; Van Dyck et al. 1997a; Van Dyck 2003) and may be more likely to meet these poorly inseminated females with a small spermatophore. Hence, this may complicate the reproductive pay-off between perching and patrolling tactics and may help explain the co-existence of both tactics in male *P. aegeria*. There is a further interesting scope for studying male and female reproductive behaviours in monandrous species like *P. aegeria* and its eco-evolutionary dynamics in time and space.

There is a final observation that offers a perspective of further research. In our experiments, *P. aegeria* stayed in copula for about 20 minutes (23.6 ± 1.2 min). There was actually very little variation in copulation duration. Copulation duration at the first mating with individuals of another Belgian population, gave a comparable duration (c. 28 min; Lauwers & Van Dyck 2006). However, Svärd (1985) reported copulation durations twice as long (c. 42 min) at first mating in individuals of a Swedish population. In butterflies, the length of copulations varies considerably at the interspecific level (from 10 min to 102 h; Wickman 2009), but, to the best of our knowledge, very little is known about the significance of intraspecific variation in the duration of copulations. As time budgets of active behaviours vary
considerably among geographic regions that differ in climatic conditions, the costs and benefits of copulation duration may vary as well.
IX. General discussion

According to game theory (Maynard Smith 1982), the coexistence of alternative reproductive tactics (ARTs) may evolve within diverse contexts (see details in Introduction). However, empirical studies have shown that most ARTs are condition-dependent (for examples: see Oliveira et al. 2008). The main objective of this PhD-project was to better understand the coexistence of alternative mate-locating behavioural tactics within *P. aegeria* populations from a conditional strategy viewpoint. To do so, I conducted various experiments to better understand the cost-benefit ratio of each behavioural tactic under particular conditions (Figure 1).

In a first step, we focused on the perching tactic the way it was naturally expressed in the field (Chapter III and IV). The quality of the sunlit spots that are used as territories by perching males has been assumed to play an important role relative to male fitness (Bergman et al. 2007). Therefore, we needed to better understand what makes a sunlit spot a territory for *P. aegeria* by understanding which characteristics of a sunlit patch are associated with male presence (Chapter III). We demonstrated that territory selection was principally based on characteristics of the sunlit spot that facilitate both visual perception and thermoregulation. Sunlit spot characteristics and territory selection are expected to vary with weather conditions, and hence among different seasons. However, we addressed to what extent abnormal weather conditions may disturb the
well-documented seasonal variation in male morphology and sunlit spot selection in the field; we also discussed the consequences for the efficiency of the mate-locating behaviours (Chapter IV). Morphological design is determined during the pre-adult stage which may result in suboptimal phenotypes under unexpected climatic conditions. However, behavioural plasticity has the possibility to temper or compensate for earlier developmental decisions about flight morphology by males adapting their sunlit spot selection relative to current weather conditions.

In a second step, we did laboratory and semi-natural experiments to measure the relative physiological and metabolic costs relative to the behavioural tactics as they were expressed under controlled conditions (Chapter V and VI). We found evidence in line with the idea of territorial perching being energetically costly (Chapter V). By varying the energy reserves of males, we demonstrated that the behavioural tactics may be constrained by the physiological status of the male and by the host plant quality during larval development (Chapter VI).

In a final step, we observed the impacts of the expression of the behavioural tactics on male reproductive output (Chapter VII). Despite a supported trade-off between resources allocated to spermatophore production and flight activity, there was no evidence of an effect of the behavioural tactics on male reproductive investment. Finally, we also investigated whether females were able to discriminate males based on male mating status, or whether they were
able to compensate when they received a very small spermatophore (chapter VIII). Our results showed that females did not avoid matings with recently mated males, but could compensate from receiving a non-substantial spermatophore by remating.

**Conditional strategy**

Figure 1. Scheme of the studied parameters in this PhD-thesis from a conditional strategy viewpoint. In blue: the studied extrinsic or intrinsic factors (and the associated chapters) that may condition the behavioural tactic expression. In green: beneficial consequences of territory holding (and the associated chapters). In red: the parameters (and the associated chapters) studied to evaluate the physiological or reproductive costs inferred by the expression of each tactic under controlled conditions.

In this general discussion, we will discuss the implications of our results in combination with the literature and how it may explain the coexistence of alternative mate-locating tactics within *P. aegeria* populations based on the conditional strategy concept. The conditional strategy implies that: 1) the ARTs are not genetically fixed, but are rather alternatives that individuals adopt to maximise their fitness according to certain environmental or intrinsic conditions; 2) mean
ARTs’ fitness payoffs are unequal (Gross 1996; Taborsky & Brockmann 2010). Therefore, after a clarification of the definitions of key terms, we will discuss the genetic versus environmental influences on alternative reproductive tactics in *P. aegeira*. Next, we will discuss the benefits and costs of each behavioural tactic. We will also explore how male personality may interfere in the context of alternative behaviours. We will end the discussion with several perspectives for future research.

**IX.1. Perching and patrolling: clear definitions**

Although the terms ‘perching’ and ‘patrolling’ have been frequently used in the literature on butterfly mate-locating behaviour since the paper by Scott (1974) there is still some confusion. Therefore, it is important to be very clear about definitions. Among territorial, perching males, there are ‘resident males’ that occupy and defend a territory and ‘intruders, non-resident males’ that will try to settle in an unoccupied territory or they may fight to become the resident of an already occupied territory. During these fights, there are winners and losers. Fights between a resident and an intruding male are often won by the former (Davies 1978), but that is not always the case (Wickman & Wiklund 1983; Stutt & Willmer 1998). Davies (1978) first interpreted the “resident-always-wins” observation based on the bourgeois strategy, i.e. the occupancy was used as a cue for settling territorial contests. However, Wickman & Wiklund (1983) showed that, after voluntary absence, the original owner of a sunlit spot was able to drive away the new male. It was suggested that winners of the
contests possessed better fighting abilities than losers. Consequently, many researches focused on properties related to the outcome of contests and asymmetry between winners and losers (see Kemp & Wiklund 2001 for a review) such as body mass (e.g. Rosenberg & Enquist 1991; Kemp 2000), age (e.g. Kemp 2000, 2002a,b, 2003; Fischer & Fiedler 2001; Kemp et al. 2006a), motivation (Bergman et al. 2010), body temperature (e.g. Stutt & Willmer 1998) or physiological status (e.g. Kemp 2002a; Takeuchi 2006; Martínez-Lendech et al. 2007).

In the literature, the term ‘patrolling, non-territorial males’ is often used for males that have lost a fight with a resident male or territory holder. The former male is often referred to as the intruder or non-resident male. However, due to the difficulties of following non-territorial males for longer periods in a forest, short term behavioural observations do not necessarily allow distinguishing between a loser that is looking for another vacant sunlit patch and a male adopting the alternative patrolling tactic flying in search of females as described by Wickman & Wiklund (1983). Bergman & Wiklund (2009b) examined the behaviour of resident and non-resident males of *P. aegeria* in large outdoor cages and demonstrated that while non-resident males showed a long ‘exodus flight’ in search of another sunlit spot when they lost a fight, theses males did not engage in continuous patrolling flight to encounter receptive females. They proposed that all *P. aegeria* males are perchers and either adopt a stationary resident, perching behaviour on a suitable sunspot or, a less stationary non-resident perching
behaviour in suboptimal smaller sunspots. The latter would be previous losers that are doing the best of a bad job strategy.

When assuming non-territorial males being non-resident perching males rather than males adopting the patrolling tactics (cf. Bergman & Wiklund 2009b), we should not expect morphological differences between territorial and non-territorial males. However, other studies have demonstrated behavioural, morphological as well as flight performance-related differences between perching (i.e. territorial males) and patrolling *P. aegeria* males (i.e. non-territorial males) in the field and in experiments in the laboratory (Van Dyck 2003 and references therein) (Table 1). The perching tactic requires energy demanding short flights, whereas patrolling requires longer, less powerful flights. In addition, Wickman and Wiklund (1983) have shown that patrolling males never engaged in long-lasting spinning-wheel flights and when such two males crossed each other the interaction was typically very short (< 2 sec). These patrolling males have apparently no intention of challenging territorial males in order to take over a territory. Previous studies have demonstrated morphological differences between perching and patrolling *P. aegeria* males (Van Dyck 2003 for a review) in line with similar interspecific morphological differences (Wickman 1992). Perching males share morphological traits that permit high acceleration and manoeuvrability such as a higher ratio of thoracic mass on body mass, a higher aspect ratio and wing loading. On the opposite, patrolling males present a morphological design that allows longer flights through sunlit patches and also through shady zones of the forest; these traits include, for
example, lower wing loading and darker dorsal basal wings (Van Dyck et al. 1997a,b). The latter trait has been shown to affect heating rates (Van Dyck & Matthysen 1998). Others metabolic and/or physiological differences are also expected in relation to the different flight performances of the behavioural tactics. In Chapter V, we showed, for instance, that non-territorial males had a lower flight muscle ratio than territorial males. All these results suggests that some *P. aegeria* males are likely to adopt the patrolling tactic *per se* rather than just be non-resident perching males that look for a new territory.

<table>
<thead>
<tr>
<th>Morphological traits</th>
<th>Behavioural tactics</th>
<th>references</th>
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<tbody>
<tr>
<td>Ratio of thoracic mass/body mass</td>
<td>Percher &gt; Patroller</td>
<td>Van Dyck et al. 1997a,b, Wickman 1992</td>
</tr>
<tr>
<td>Wing loading</td>
<td>Percher &gt; Patroller</td>
<td>Van Dyck et al. 1997b, Wickman 1992</td>
</tr>
<tr>
<td>Degree of melanisation</td>
<td>Percher &lt; Patroller</td>
<td>Van Dyck &amp; Matthysen 1998</td>
</tr>
<tr>
<td>Wing area</td>
<td>Percher ~ Patroller</td>
<td>Van Dyck et al. 1997a</td>
</tr>
<tr>
<td>Wing length</td>
<td>Percher = Patroller</td>
<td>Van Dyck et al. 1997b</td>
</tr>
<tr>
<td>Body length</td>
<td>Percher &lt; Patroller</td>
<td>Van Dyck et al. 1997b</td>
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**Table 1.** Summary of the main morphological differences observed between perching and patrolling males of *P. aegeria*.

During our experiments in the outdoor cages, we observed males showing high aggressiveness in a consistent way (i.e., inducing a lot of male-male interactions) and also high fidelity to a territory (classified as ‘PE2’ males) versus males showing no interest to defend a territory even when in presence of suitable sunlit spots without residents or possible intruders (classified as ‘PE0’ males). We believe that this latter group corresponds to the patrolling males in a strict sense. Nevertheless, as the limits of the outdoor cages do not allow males to show a continuous flight, we have chosen to name these males ‘non-perching’ rather than ‘patrolling’ and considered their behaviour in a gradual intensity of territorial defence. In our study,
'non-perching males’ where rarely or never observed defending a territory.

We had also observed an intermediate group of males (classified as ‘PE1’ males), that behave principally as the ‘non-perching males’ but that were occasionally observed defending a sunlit spot in an aggressive way. We believe that these males may more correspond to the non-resident males described by Bergman & Wiklund (2009b). They probably represent males with a high motivational willingness for territorial perching but were excluded from territories by other territorial males (i.e. PE2). Removing aggressive perching males from the cages could permit testing this hypothesis by looking at the behaviour of intermediate males when sunlit spots are free and potential competitors are absent.

**IX.2. Genotypic or phenotypic alternatives**

Austad (1984) proposed a classification of alternative reproductive behaviours regarding; 1) their degree of genetic determination, 2) whether fitness is equal (or not), and, 3) whether alternative behaviours were reversible (or not). Therefore, he suggested that the first step for studying ART’s on the field is to ascertain the behavioural genetics of the systems in question. However, as with any other biological trait, ARTs are a product of genes and the environment and of interactions between these causal factors (Van Dyck & Windig 2009). As a consequence, it is impossible to remain
the position that ARTs are either ‘genetically’ or ‘environmentally’
determined (Caro & Bateson 1986).

IX.2.1. Environmental factors influencing *P. aegeria* mate-locating
behaviour

Although the mode of male mate-location behaviour can be species-
specific, there are species in which males can shift between perching
and patrolling (for examples: see Shreeve 1992). Factors influencing
the expression of mate location behaviour have thus attracted a lot of
attention. Several ecological factors such as population density, air
temperature and suitable territory availability have been shown to
influence mate location behaviour in several butterflies species
(Wiklund 2003; but see introduction for more details and examples).
The propensity of individuals to adopt a certain type of mate-location
behaviour may also be influenced by phenotype (Wickman 2009).
Until now, various environmental factors have been shown to
influence mate-locating behaviour in *P. aegeria* (Table 2). As the cost
of being territorial increases with the number of intruders, higher
patrolling frequency and less male-male interactions were observed
under increased population density (Wickman & Wiklund 1983).
Ambient temperature and light intensity were also found to impact the
mate-locating behaviour of *P. aegeria*, the timing of flight being
longer under warmer and sunnier conditions, favouring patrolling
behaviour (Wickman & Wiklund 1983; Shreeve 1984; Shreeve 1987;
Stutt & Willmer 1998). In Chapter IV, we observed that the function
of sunlit spots changed with environmental conditions. Under colder
conditions, sunlit spots are principally used as hotspots to increase thoracic temperature and, hence, flight activity whereas under lighter and warmer conditions, they facilitate visual perception and so female detection rather than thermoregulation. We observed that under unexpected changes in seasonal weather pattern (i.e. warm, dry spring versus cold, wet summer), males were able to adapt their sunlit spot selection behaviour (i.e. selecting perch for thermoregulation under colder conditions in summer versus selecting perching sites for visual perception under warmer, lighter conditions in spring) in order to maintain flight activity and hence maximise female interception despite non-appropriate ‘seasonal’ morphology. Consequently, the availability of suitable territories is expected to play an important role for the frequency of the perching tactic. Nevertheless, in our study, we have not compared perching and patrolling frequencies across different environmental conditions (i.e. seasonal variation). However, Wickman & Wiklund (1983) showed that when ambient temperature increases through the season, the thermal value of holding a territory decreases and consequently, a reduction of male-male interactions duration has been noticed (Wickman & Wiklund 1983; Stutt & Willmer 1998). Differences in the frequencies of the behavioural tactics were also highlighted in different habitats (continuous woodland versus highly fragmented agricultural landscape with hedgerows and small patches of woodland; Merckx & Van Dyck 2005). In accordance with higher temperature and light intensity in the agricultural landscape, the frequency of patrolling males was higher in this landscape type (Merckx & Van Dyck 2005). As previously mentioned, functional morphology was showed to differ between
perching and patrolling males within woodland populations (Van Dyck, 2003 for a review). Territorial perching males are on average paler and somewhat smaller males than patrolling males (Van Dyck et al. 1997a,b; Van Dyck & Matthysen 1998). In Chapter VI, we observed that the physiological status of a male may also impact the male-locating behaviour. Males reared on low nutritional host plants emerged with less energy reserves and developed lower flight muscle ratios. These males showed indeed a higher propensity to adopt the non-territorial behaviour.

Table 2. Summary of the environmental factors that influence male mate-location behaviour in *P. aegeria*. * Behavioural expressions are expected to vary with season (Wickman & Wiklund 1983) and habitat fragmentation (Merckx & Van Dyck 2005) according to environmental variation.

<table>
<thead>
<tr>
<th>Factors affecting ARTs</th>
<th>Predictions on behavioural expression</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Population density</td>
<td>High population density: increasing costs of territorial defence → favour patrollers rather than perchers</td>
<td>Wickman &amp; Wiklund (1983)</td>
</tr>
<tr>
<td>Territory quality</td>
<td>Decrease of suitable territory quality → favour patroller rather than perch</td>
<td>Wickman &amp; Wiklund (1983)</td>
</tr>
<tr>
<td>Functional morphology</td>
<td>Link between morphology and thermoregulation</td>
<td>Van Dyck et al. (1997a,b)  Van Dyck &amp; Matthysen (1998)</td>
</tr>
<tr>
<td>Physiological status</td>
<td>Low lipid reserves Low flight muscle ratio → favour patrolling rather than perch</td>
<td>Chapter VI of this thesis</td>
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</table>

Together these results indicate that multiple factors have an effect on mate locating behaviour in *P. aegeria*. At this stage, an approach based on modelling would be interesting to further explore the multiple impacts of these factors and interaction effects on the frequencies of perching and patrolling as alternative mate-locating
tactics and, hence, make significant further steps to the better understanding the evolution of coexistence of ARTs within variable environments in *P. aegeria*.

**IX.2.2. Genetic influence on *P. aegeria* mate-locating behaviour**

In Chapter VII of this PhD thesis, we have compared the behaviour of brothers among and between different families. Our results suggested that brothers tended to adopt similar mate-locating behaviours (Table 3, Figure 2). So, in addition to be condition-dependent, mate-locating behaviour expression in *P. aegeria* may hence be genetically influenced.

<table>
<thead>
<tr>
<th>Contingency Table</th>
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<tr>
<td><strong>F01</strong></td>
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<tr>
<td>PE0</td>
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<tr>
<td>PE1</td>
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<tr>
<td>PE2</td>
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<td>Sum</td>
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</table>

**Table 3.** Contingency table showing the observed number of individuals from different families adopting one of the three behavioural tactics (‘PE2’: a strong and permanent percher; ‘PE1’: an occasional percher; ‘PE0’: a non-percher). Test of independence between the family effect and the behavioural tactics shows that families differed in the frequencies of the male behavioural tactics ($\chi^2 = 36.98$, ddf = 20, $P = 0.0118$; see Chapter VII)
Figure 2. Biplot resulting from the factorial correspondence analysis between the family effect and behavioural tactics (‘PE2’: a strong and permanent percher; ‘PE1’: an occasional percher; ‘PE0’: a non-percher). CFA-axis 1 (Dim1) principally reflected the variation in the category ‘PE0’ among the families (Loadings axis 1: 75.9% PE0, 8.4% PE1 and 15.7% PE2), while CFA-axis 2 (Dim 2) contrasted the importance of the variation of ‘PE1’ and ‘PE2’ among the families (Loadings axis 2: 0.5% PE0, 52.5% PE1 and 47.0% PE2). Particularly family F10 had a strong PE0 profile (highly positive value on axis 1; i.e., high propensity among the brothers of that family to engage in non-perching behaviour) compared to the males of the families F01, F03, F11 and F13 (highly negative value on axis 1). Families F01 and F11 had positive values for axis 2 (i.e., tendency of an aggressive perching ‘PE2’ profile), whereas families F03, F07 and F12 have a more ‘PE1’ profile (negative values on axis 2).

One possibility of the apparent genetic influence is that the alternative behavioural tactics may be correlated with one or several specific alleles (Emlen 2008). During my master thesis (Vande Velde 2007), I used allozymes in order to explore this issue. The efficiency of particular biochemical cycles (e.g. Krebs cycle) for specific flight performances under particular conditions may depend on specific
enzymatic forms (e.g. Watt 1983; Goulson 1993; Haag et al. 2005). Allozymes represent various enzymatic forms coding as different alleles on one locus (Richardson et al. 1986). As perching males require a highly energy demanding, short powerful flight in order to intercept a passer-by very quickly, while patrolling males fly for much longer at a lower speed, we may expect to find allelic differences that reflect functional differences in the energy metabolism associated with the flight (e.g. Krebs cycle). However, I did not find any evidence for the seven allozymes that were tested (phosphoglucoisomerase PGI, phosphoglucomutase PGM, Glutamate oxalate transaminase GOT, glucose-6-phosphate dehydrogenase G6PD, isocitrate dehydrogenase IDH, beta-hydroxybutyrate dehydrogenase HBDH, malate dehydrogenase MDH). But, we need to stay cautious about these preliminary results. Indeed, enzymatic electrophoreses do not reveal all genetic variation (e.g. cryptic variation; Ayala 1982; Wheat et al. 2005). Moreover, we observed very little genetic diversity for most alleles compared to other butterfly species representing similar dispersal capacities and population densities (e.g., Aglais urticae Vandewoestijne et al. 1999; Melanargia galathea Vandewoestijne et al. 2004; Polyommatus icarus Schmitt et al. 2003; Maniola jurtina Schmitt et al. 2005; Polyommatinus coridon Schmitt & Seitz 2002). For each studied locus, only one allele was overrepresented. Consequently, the detection of genetic differentiation between the behavioural tactics was compromised. There may be several potential methodological or biological explanations for such a low genetic diversity. However, none of the biological conditions associated with typically low genetic variation (i.e., low population density, recent
bottleneck or fixation of adapted alleles) are applicable to *P. aegeria* in central Belgium (Vande Velde 2007) and so the observed low genetic diversity stays a puzzle that requires further research.

The genetic influence on behaviour can be direct (i.e. fixed alleles as mentioned above) or indirect (Austad 1984). Suppose that expression of ARTs dependents on a morphological trait such as body mass that itself is heritable. Selection on this morphological trait may have an indirect effect on behaviour and could lead to disruptive patterns. In addition, other extrinsic cues may influence body mass such as temperature, humidity, photoperiod, light conditions, food and population density. This example illustrates how gene x environment interactions are of prime importance for our understanding of the expression of alternative behavioural tactics. Several functional morphological traits that relate to the behaviours are heritable (Van Dyck et al. 1998; Berwaerts et al. 2008), but that does not exclude a significant role of the environmental conditions for the morphological design of *P. aegeria* males and females. Van Dyck & Wiklund (2002) showed seasonal variation in morphology that was mainly induced by different developments as a response to photoperiod. But other environmental factors may also influence larval development and hence, adult morphology. Temperature is used as a cue by developing larvae to decide when and at which size to emerge (Gotthard et al. 1994). Under higher temperatures, individuals emerged earlier resulting in smaller individuals (Sibly et al. 1997). Host plant quality may also impact on morphology (Talloen et al. 2004). This may interfere with habitat fragmentation and other changes in landscape
structures that have the capacity to alter microclimatic conditions such as air ambient temperature, ground temperature, wind speed, solar radiation and other key resources distributions (Matlack 1993; Malcolm 1998; Honnay et al. 2001; Merckx et al. 2008). Butterfly thermoregulation is highly constrained by microclimatic conditions, and butterflies are particularly sensitive to light intensity and air temperature (Clench 1966, Dennis 1993). Consequently, thermoregulation-related morphology may vary between habitats that vary in the degree of fragmentation (Merckx & Van Dyck 2006) with, for instance, darker and more powerful (i.e. with larger thoracic mass) individuals observed in more fragmented landscapes (Berwaerts et al. 1998; Thomas et al. 1998). In addition, Talloen et al. (2009) showed that environmental stress may impact expressed heritable variation on specific morphological traits. In their study, heritability for the degree of melanisation was higher under stress conditions.

An alternative explanation for the observed family effect on the expression of the behavioural tactics in our study can be that a heritable threshold mechanism underlies condition-dependent selection. Based on the conditional strategy viewpoint, the expression of a tactic is the result of the interaction of a condition and of the threshold value of the condition; all individuals with a condition higher than the threshold value will express one phenotype whereas all others will express the alternative phenotype (Gross 1996). Some studies have shown that such a threshold responsible for tactic expression may have significant genetic variation and can hence respond to selection (Hazel et al. 1990; Gross & Repka 1998a,b;
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Emlen 2008). The better match between tactic expression and selective circumstances, the better, on average, the genotypes will perform. Consequently, numerous components of sensory detection processes (e.g. the types of cues detected, sensitivity of individuals to these cues, and the relationship between detected cues and circulating signallers like hormones; see Emlen 2008 for more details) can vary genetically between individuals within or among populations. All genotypes may then present different internal threshold mechanisms and will then vary in the choice of tactics under one particular cue (e.g. male salmon *Salmo salar* Aubin-Horth et al. 2005; scarab dung beetles *Onthophagus acuminatus* Emlen 1996). The thresholds can also be affected by additional environmental conditions.

Finally, as we used the individuals of the first generation in our experiment, behavioural consistency within families may be the results of maternal effects rather than genetic effects. The maternal choice of oviposition site may influence offspring performance (Qvarnström & Price 2001). For instance, in the butterfly *Polygonia c-album*, protein content in spermatophores and cryptic mate choice vary with host plant quality (Wedell 1996). However, as rearing procedures and eggs laying were controlled in our experiments, maternal effects, if they were present, may in principle only result from differences in egg provisioning. Egg provisioning has been shown to greatly affect offspring survival and growth in insects, including *P. aegeria* (Mousseau & Dingle 1991; Uller 2008; Bernardo 1996; Gibbs et al. 2010b). Parents may exert a nongenetic influence on some sexually selected traits (see examples in Qvarnström & Price
2001) that can relate to alternative mating tactics (e.g., horn dimorphism in *Onthophagus Taurus*; Moczek & Emlen 1999; Hunt & Simmons 2000). The impact of parental effects on male condition and the expression of mate-locating behaviour expression require further attention to better understand ARTs evolution in *P. aegeria*.

**IX.3. Relative fitness payoffs of ART’s in *P. aegeria***

The coexistence of alternative male mating tactics depends on the balance between costs and benefits (Gross 1996). Variation in the ratio of benefits and costs of defending a territory are expected relative to environmental conditions and male status. Although several studies have focused on the determination of environmental factors affecting male mate-locating behaviours (see Introduction), none have concretely measured the costs and benefits of adopting the alternative behaviours under particular conditions. In this project, we have initiated this process by evaluating benefits and costs of territorial defence under determined conditions.

**IX.3.1. The benefits of territorial defence***

A large number of studies have demonstrated unequal average fitness payoffs associated with alternative behavioural tactics (insects: Toivanen et al. 2009; crustacean: Brockman et al. 1994; Tsunenori 1998; fish: Elisabeth & Taborsky 1997; Foote et al. 1997; Fuller 1999;
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Moran et al. 1996; mammal: Coltman et al. 1999). In insects, aggressive territory holding often result in higher mating success compared to non-territorial males (Davies 1978; Fisher & Fiedler 2001; Hunt & Simmons 2001; Bergman et al. 2007; Toivanen et al. 2009). In some butterfly species, females have been shown to avoid courtship with non-territorial males and hence delay copulation until they have found a territorial male to mate with (e.g. Coenonympha pamphils Wickman & Jansson 1997; P. aegeria Wickman & Wiklund 1983; Gotthard et al. 1999). Bergman et al. (2007) demonstrated that resident P. aegeria males experience greater mating success than non-resident males, with resident males being almost twice as successful as non-residents. Nevertheless, there was no evidence that females actively preferred to mate with resident males per se, or that they prefer for visiting the large sunspot defended by perching males. It suggests that higher mating frequency for resident males results from a better female detection ability by males within large sunlit spots. In this context, the quality of the sunlit spot and the perch selection are expected to be of significance to increase male fitness in this species. Bergman & Wiklund (2009a) showed evidence for the hypothesis of more efficient visual detection in large sunlit patches and showed that males preferentially defended such large patches in the field. In Chapter III, we confirmed the pattern of perching males that were more likely to occupy large sunlit spots. Nevertheless, we showed that thoracic heating time of the males was reduced in these large sites, which may facilitate flight activity and efficiency to intercept mates and chase intruders. Consequently, our results showed that territory selection was not only based on visual characteristics that increase
female detection but also on thermal influences. Davies (1978), as well as Wickman & Wiklund (1983), have investigated male mating success between perching and patrolling males in the field by releasing and following virgin females. Their results confirmed the higher mating success for resident males. Nevertheless, regarding the difficulties of following individuals on the field, observations were based on small sample sizes. Additional experiments in the field are required to confirm the higher mating success of perching males under a range of environmental conditions. We attempted to do so by releasing virgin males from our laboratory stock in the forest (Bois de Lauzelle) during three days and recaptured them after having determined their mate-locating behaviour and directly mating them with a virgin female from our stock. It takes up to 7 days for a *P. aegeria* male to transmit a spermatophore of the same size of the first one (Svärd 1985). Consequently, by assessing the size of the spermatophore transmitted to a virgin female, we were able to determine the mating status of the males (i.e., still virgin male with large spermatophore versus mated male producing a small spermatophore). However, as the wild population density was exceptionally low during this experiment (due to exceptional weather conditions of May and June 2010), all males tested (79 recapured males out of 445 released males) were still virgin when we let them mate with virgin females in the laboratory (Vande Velde & Van Dyck, unpubl. data). So, we could unfortunately not address this issue.
**IX.3.2. The costs of territorial defence**

The higher mating success of territorial males compared to non-territorial males, may however be related to significant costs and risks of territorial defence, including reduced longevity (Houston 1970; Toivanen et al. 2009), and reduced performance of other behaviours like dispersal (Houston 1970; Crespi 1988). So far, the energetic cost of territorial defence has received only little attention in butterflies (Kemp 2002; Takeutchi 2006; Martínez-Lendech et al. 2007; Peixoto & Benson 2011), but there is evidence in other insects (e.g. flies: Yuval et al. 1998; odonates: Plaistow & Siva-Jothy 1996; Plaistow & Tsubaki 2000; Orthoptera: Hack 1997a). In Chapter V, we showed that intensive perching activity – including frequent take-offs and male-male interactions – was particularly energy demanding, at least within our outdoor cage setting. When comparing lipid reserves after the expression of each behavioural tactic in large outdoor cages for a short period (3 days) under controlled conditions, we noticed that males that had aggressively defended a sunlit spot during all the trials had consumed more lipids than non-territorial males.

Based on life-history theory, the allocation of resources to one trait reduces the resources that can be allocated to other traits, especially when each trait is energetically costly (Stearns 1989; Roff 1992). Butterflies are flying holometabolous insects that collect the major part of their resources as larvae feeding on host plants. Some nutrients, like nitrogen, are not abundantly available for adults (Boggs 2009). Thus, we would expect trade-offs between flight ability and
other nitrogen-demanding traits, since flight muscles drag nitrogenous resources away from potential reproductive investment in egg and spermatophores (Åhman & Karlsson 2009). Generally, the literature provides support for such a trade-off in females, often referred to as the oogenesis-flight syndrome (e.g. Johnson 1963; Edwards 1970; Tanaka 1993, 1994; Zera & Denno 1997; Tanaka & Suzuki 1998; Marden 2000; Hughes et al. 2003; Jervis et al. 2005; Lorenz 2007). Studies that show evidence of a trade-off between flight ability and reproduction focused mainly on wing dimorphic species. When raised on the same resource, flight-capable, winged females have lower fecundity than flightless females in crickets (Tanaka 1976, Roff 1984; Roff 1995; Roff et al. 1997; Zera et al. 1997), aphids (Dixon 1972, Wratten 1977, Walters & Dixon 1983), planthoppers (Denno et al. 1989, Denno 1994), water striders (Anderson 1973, Zera 1984), corixids (Young 1965), and pea weevils (Utida 1972). The relationship between wing form and components of fitness in males has, however, been largely neglected (Denno 1994, Ott 1994; Zera & Denno 1997), although a similar trade-off is likely to exist in males as well (Marden 2000). Even if the production of gametes is less expensive for males relative to females, male reproductive behaviour is nevertheless costly in other respects such as courtship behaviour (e.g. song courtship in crickets Mitra et al. 2011; protein-rich salivary secretions in scorpionfly Engqvist et al. 2011). But the evidence for a trade-off between flight capability and reproductive success in male insects is mixed. While some studies have shown a fitness advantage to flightless males (Fujisaki 1992, Kaitala & Dingle 1993, Crnokrak & Roff 1995, Novotny et al. 1995, Fairbairn & Preziosi 1996; Sakaluk,
Male Lepidoptera transfer a spermatophore to females during copulation. Spermatophores consist of sperm and accessory gland secretions, which are rich in nutrients, particularly proteins and carbon, but which also contain sodium, lipids and hormones (Marshall 1982; Bissoondath & Wiklund 1995; Karlsson 1995). The resources needed to produce a spermatophore can be a limiting factor to male fitness (e.g., Van Voorhies 1992; Wedell et al. 2002; Sella & Lorenzi 2003). The cost of spermatophore production is shown by the fact that copulation durations were longer and ejaculates were smaller for recently mated males (e.g., Boggs 1981b; Rutowski et al. 1983; Svärd 1985; Svärd and Wiklund 1986; Oberhauser 1989). The impact of the degree of polyandry (e.g., Svärd & Wiklund 1989; Bissoondath & Wiklund 1995), the male mating history (e.g., Bissoondath & Wiklund 1996; Lauwers & Van Dyck 2006) and other life-history traits such as male size (e.g., Boggs 1981b, Oberhauser 1988; Svärd and Wiklund 1986; Rutowski et al. 1983, 1987; Gage 1994; Bissoondath and Wiklund 1995, 1996) and age (e.g., LaChance et al. 1977; Boggs 1981b; Oberhauser 1988) on spermatophore production have attracted much attention. The potential influence of different flight activity levels on spermatophore production had been completely neglected. However, as flight performance related to territorial defence was strongly energy demanding compared to non-territorial behaviour (Chapter V), we tested whether this cost may impact male
reproductive investment. However, overall our results did not show evidence for a trade-off between behavioural tactic and reproductive investment in males. In Chapter VII, our results showed, however, a negative impact of flight activity per se (i.e., active versus non-active males) on spermatophore mass and sperm number, suggesting a flight-reproduction trade-off in males (Table 4). But, contrary to our predictions based on the higher energy costs for territorial defence (including male-male interactions and numerous take-off flights), male reproductive investment either did not differ between the behavioural tactics, nor between other experimental groups like with forced flight treatment, or even increased for territorial and forced flight males (Table 4). These surprising results may suggest that territorial and forced flight males may perceive a stress associated to male-male competitions or predation risk, maybe associated to our experimental procedures. Consequently, such subjective perception should have increased current investment at the expense of longevity (Boggs 1981a). We also observed that territorial males copulated longer than non-territorial males (Table 4). Nevertheless, although copulation duration was shown to be correlated to sperm load and the future propensity for the female to mate again (e.g. Prokelisia dolus Langellotto et al. 2000), the impact of longer copulation duration observed in territorial P. aegeeria males remains unclear at this stage.
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Table 4. Summary of the results obtained in this PhD-thesis on the impact of different flight activity levels, induced by different treatments, on the reproductive traits of male and copulation duration.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Spermaphore mass</th>
<th>Euphyrene number</th>
<th>Copulation duration</th>
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<td>Flight activity</td>
<td>Active males &lt; Resting</td>
<td>Active males &lt; Resting</td>
<td>Chapter</td>
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<td>VII</td>
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<td>Behavioural</td>
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<td>treatment</td>
<td>resting males</td>
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<td>VII</td>
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To cover the required energetic costs of reproduction, some species have evolved mechanisms to reallocate somatic resources to reproduction. A common phenomenon is flight muscle histolysis which implies the reallocation of resources from the flight muscles to increase fecundity (e.g., Kaitala 1988; Kaitala & Hulden 1990; Tanaka 1993; Tanaka & Suzuki 1998; Stjernholm & Karlsson 2000; Stjernholm et al. 2005). This process is quite current among females in order to maintain the lipid content in the eggs (e.g. Karlsson 1994, 1998). Several studies on butterflies demonstrated that males may also be able to histolyse their flight muscles (Stjernholm & Karlsson 2000; Norberg & Leimar 2002), and use these resources to increase their reproductive effort (Stjernholm et al. 2005; Stjernholm & Karlsson 2006). The phenomenon of flight muscle histolysis could impact flight ability (Engqvist et al. 2011). A high flight muscle ratio means that body mass is concentrated in the thorax, yielding an advantage in terms of manoeuvrability and take-off capability (e.g. Marden & Chai 1991; Marden 2000; Berwaerts et al. 2002; Almbro & Kullberg 2008). However, although the absolute thorax mass decreases with age, the abdomen mass may decrease even more rapidly, so that the relative
thorax mass instead increases with age. Thus, even with a decreasing thorax mass, flight performance may remain unchanged or even increase with age, as it was shown in *P. aegeria* (Karlsson 1994). However, muscle histolysis is expected to be more pronounced under conditions of low food quality (Boggs 2009). In Chapter VI, we observed that the flight muscle ratio was lower in males reared on low quality plants compared to control males. As the ratio decreased with male age in a more pronounced way in males reared on low quality plants in this experiment, this may be the result of muscle hydrolysis. Nevertheless, the hydrolysis of flight muscles for territorial males that engage in intense flight interactions should be more limited than for males where flight is of less importance for acquiring mates (Stjernholm & Karlsson 2000). That should explain our observation that males reared on low quality host plants and having lower flight muscles ratio preferentially adopted the non-territorial behaviour. A modulation of behaviour to maximise ejaculates was observed in other animals. For instance, Okada et al. (2010) showed that males of the flour beetles *Gnatocerus cornutus*, decreased their level of aggressiveness for some days when they lost a male-male conflict and increased reproductive investment during this period for future sperm competition. Receiving a small spermatophore from a recently mated male affects female fecundity in *P. aegeria* (Lauwers & Van Dyck 2006). Moreover, we demonstrated that females receiving a small spermatophore from a recently mated male had an increased willingness to remate (Chapter VIII). Consequently, males may benefit to reallocate their lipid reserves from flight muscles to reproduction in order to transmit a sufficiently large spermatophore.
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IX.4. Behavioural persistency: link with behavioural syndromes?

A relative consistency to adopt one of the behavioural tactics was observed previously in the field (Van Dyck et al. 1997a) and in our outdoor cages (L. Vande Velde, pers. observ). In our experimental cage settings, we noticed that some males were highly aggressive when defending a territory, some males were non-territorial (even when sunlit spots were available) and other males that were territorial but were rapidly dominated and excluded from spots by more aggressive males. These behaviours were consistent during all observation trials (i.e., 3 days). While the period of observations was relatively short, field studies over longer time frames demonstrated long site fidelity of perching males (> one week; Wickman & Wiklund 1983; Van Dyck et al. 1997b; Vande Velde, personal obs). On 21 recaptured males, Van Dyck et al. (1997b) observed only one individual which had shifted its mate locating tactic (15 males remained consistent perchers and 5 consistent patrollers). This consistency of behaviour within and between males may suggest a link with behavioural syndromes or personalities (Sih et al. 2004; Luttbeg & Sih 2010), although this popular field has – to the best of our knowledge – not yet been linked with mate locating in insects, including butterflies. As suggested by the theoretical framework of the conditional strategy, a behavioural syndrome may imply that individuals perform well in some contexts and poorly in others which helps explaining the maintenance of individual variation in behaviour,
and in particular of alternative mate locating tactics. Sih et al. (2004) suggested that behavioural correlations across situations might be particularly important for ecology and evolution when trade-offs exist such that different behaviours are favoured in different situations and behavioural consistency across situations results in less than optimal plasticity across situations. Nevertheless, despite the observed consistency of behaviour in *P. aegeria*, at this stage we cannot be sure that it corresponds to behavioural syndrome (i.e. male personality). Indeed, by definition, a behavioural syndrome involves both within- and between-individual consistency in behaviour across either different contexts (i.e. a functional behavioural category; e.g. predator avoidance) or situations (e.g. different levels of predation risks) (Sih et al. 2004). Therefore, in order to confirm that males exhibit behavioural syndromes, we need to observe the same individuals across two or more situations or contexts. However, considering the short life of the butterflies, we can assume that the fact that it exhibits the same behavioural tactic during one week may reveal a behavioural consistency through various situations. Further experiments are now warranted in this context like in the semi-natural outdoor cages by placing males of initial known behaviour (i.e., under one particular context or situation) under various environmental conditions (e.g., temperature, male density, etc.) and monitor whether males maintain or shift their initial behaviour.
IX.5. Perspectives

In this PhD project, we studied male mate-location tactics within large outdoor cages. While some studied have observed very natural behaviour within similar cages (Leimar et al. 2003; Merckx et al. 2003; Kemp et al. 2006a), we could expect that natural behavioural expression in the field may face different constraints and, consequently, induce different costs than would be observed in our experiments. This is particularly true for the patrolling tactic. Long flights characterising this behaviour were impossible under our semi-natural conditions which may have biased the relative costs between both behavioural tactics. Measurements based on wild individuals should then complete our current results. However, field data make it difficult to control for several life-history traits (e.g., age or mating history) that could significantly influence the measured costs.

In our study, costs of reproduction were estimated using some male reproductive traits (i.e. spermatophore mass, eupyrène number and copulation duration). However, other parameters such as spermatophore quality (i.e. qualitative rather than quantitative measurements), female fecundity and offspring success after mating with males of different tactics should be evaluated. Costs on survival are also important to be measured as it may directly influence fitness. On the other hand, we investigated the benefits and costs of ARTs based on measurements largely done on male traits, assuming low impacts of females. However, the evolution of ARTs should be also and explicitly considered in response to female choice and behaviour.
(cf. Bergman et al. 2011). Therefore, a larger focus on female behaviour is required for future work in the field and in the laboratory.

Our measurements for evaluating the physiological and reproductive costs related to male-location tactics expression in P. aegeria is only a start to the better understanding of ARTs in butterflies. There is still a lot of work that needs to be done to fully understand the fitness payoffs of each behavioural tactic under various conditions. The distribution of P. aegeria includes different forest types and also fragmented agricultural landscapes (Merckx & Van Dyck 2005). Consequently, different populations face different ecological resource settings including thermal conditions. As discussed above, morphology (Merckx & Van Dyck 2006) and mate-locating behaviour (Merckx & Van Dyck 2005) have been shown to differ among these habitat types. Therefore, it would now be interesting to analyze physiological and reproductive costs in P. aegeria populations originating from different habitat types. Overall, benefits and costs of ARTs should be measured and compared under different environmental and intrinsic conditions relative to ARTs expression.

In addition to the different habitat types, P. aegeria also occurs at a relatively large geographic scale across Europe representing different climatic conditions (Hill et al. 1999). Therefore, it would be interesting to repeat our experiments with different populations along a latitudinal gradient. Morphology and life cycle regulation have been studied intensively along latitudinal gradients, but that is less the case for behavioural variation. We often base the characterization of the
reproductive behaviour of a species on a single of a limited number of field studies – and hence a limited number of ecological settings – which may underestimate intraspecific variation in these traits. Due to climatic differences along a latitudinal gradient, populations of *P.aegeria* show significant variation in the pattern of adult phenology which has consequences for the time spent searching for a male and the female’s choosiness (e.g. Gotthard et al. 1999). In South Sweden, generations are discrete and non-overlapping resulting in highly synchronized adult flight periods with protandry, whereas in southern parts of the distribution (e.g. Madeira) several generations overlap with no clear peak for the availability of virgin males. Hence, selection pressures could be different for the *P. aegeria* mating system across a latitudinal gradient with variable preferences for territorial males over non-territorial males relative to the costs of searching (Gotthard et al. 1999). A general prediction from models of mate choice is that as the costs of searching increase, choosiness should decrease (Real 1990; Reynolds and Gross 1990). There is a further interesting scope for studying male and female reproductive behaviours in monandrous species like *P. aegeria* and its eco-evolutionary dynamics in time and space.
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