

Dissertation  
Tobias Kehl



Old male mating advantage in *Bicyclus anynana*  
butterflies - sexual conflict or cooperation?

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Tobias Kehl  
geboren am 16.02.1985  
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Dekan: Prof. Dr. Klaus Fesser

1. Gutachter: Prof. Dr. Klaus Fischer

2. Gutachter: Prof. Dr. Christer Wiklund

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# **INTRODUCTION**



# 1 Introduction

## Sexual conflict and cooperation between sexes

For sexually reproducing organisms, it is of pivotal importance that males and females cooperate in order to produce offspring. For a long time it has been assumed that both sexes should have identical interests regarding reproduction. Hence, mating has been considered as a largely “harmonious” event. During the last decades, this view has changed dramatically as more and more evidence accumulated, suggesting that sexual conflict is all pervasive (Arnqvist & Rowe, 2005). Except for the rare case of genetic monogamy, in which partners sire offspring only with each other, the reproductive interests of males and females are typically not the same (Arnqvist & Rowe, 2005). In short, the reproductive interests of both sexes are not identical. Such novel insights prompted much research interest and over recent decades, sexual conflict became one of the main fields of research accompanied with an exponential increase of publications in this field (Arnqvist & Rowe, 2005).

Besides an impressive variety of examples for sexual conflict throughout the animal kingdom, a striking example is sexual conflict over the optimal number of mating. Males benefit from higher mating numbers, because contrary to their female counterparts, they invest fewer resources into their offspring (Trivers, 1972; Janowitz & Fischer, 2012). As reproduction raises non-trivial costs for both, males and females, selection should maximise the own genetic reward regardless of the potential fitness consequences for the sexual partner (Arnqvist & Rowe, 2005; Bergman *et al.*, 2011). For males, sexual selection is expected to favour traits increasing the number of matings and sperm competitive ability, even if these adaptations have deleterious effects for the female partner (Rice, 1996; Kemp & Rutowski, 2004). An impressive example are some components of the seminal-fluid in *Drosophila melanogaster*, which up-regulate the females’ fecundity and at the same time, by reducing the remating propensity and females’ lifespan, reflecting a tremendous loss of fitness (Chap-

man *et al.*, 1995; Chapman, 2001; Wigby & Chapman, 2005). Furthermore, the females' reproductive output can be manipulated by males beyond their optimum (Fischer, 2007).

As a consequence, selection enables females to interact with males to the effect that their individual fitness will be maximized. This can be mediated through cryptic female mate choice. The resulting post-copulatory sexual selection may bias paternity towards the preferred males (Eberhard, 1996; Edvardsson & Arnqvist, 2000). Males and females underlie differential selective pressures which provoke an evolutionary arms race between the sexes. This so called sexually antagonistic coevolution empowers the individuals to counteract the evolved sex traits of their counterparts and as a result gain the maximum reproductive success (Rowe & Arnqvist, 2002; Arnqvist & Rowe, 2005; Wedell *et al.*, 2006). Thus, the “cooperation-conflict balance” between the sexes is often shifted towards conflict.

## Old male mating advantage

Given that in animals with finite growth, such as holometabolic insects, senescence basically starts at sexual maturation and condition diminishes with age (Karlsson, 1994; Fischer *et al.*, 2008), young virgin males have been predicted to gain the highest mating success (Vahed, 1998; Wedell & Karlsson, 2003; Ferkau & Fischer, 2006). Although even sperm quality and quantity typically decrease with age (Kaitala & Wiklund, 1995; Cook & Wedell, 1996; Wedell & Ritchie, 2004; Marcotte *et al.*, 2005; Damiens & Boivin, 2006; Sasson *et al.*, 2012), older males have been shown to have a higher mating success in a variety of insect taxa. These are, for example, the forked fungus beetle *Bolitotherus cornutus* (Conner, 1989), the potato tuber moth *Phthorimaea operculella* (Cameron *et al.*, 2005), the field cricket *Gryllus bimaculatus* (Simmons & Zuk, 1992), the Mexican fruit fly *Anastrepha ludens* (Perez-Staplez *et al.*, 2010) and the butterfly *Bicyclus anynana* (Fischer *et al.*, 2008; Karl *et al.*, 2013; Kehl *et al.*, 2014). Note in this context that older *B. anynana* males gain up to a 4× higher mating success despite a clearly poorer condition

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compared to younger individuals, which is strikingly evidenced by a decrease in body mass, fat content, and wing wear with increasing age (Fischer *et al.*, 2008). In contrast, throughout the animal kingdom male mating success is typically positively related to such traits, *i.e.* the resource holding potential (Marden & Waage, 1990; Kemp & Wiklund, 2001; Lappin *et al.*, 2006).

Nevertheless, old male mating advantage has been predicted for two reasons. First, in groups lacking physical means, usually unable to inflict harm upon a combatant, such as butterflies, simple asymmetries in resource holding potential are unlikely to (fully) determine male mating success (Kemp & Rutowski, 2004). Rather, mating success seems to depend on a male's aggressiveness and his willingness to persist during courtship (Fischer, 2006; Geister & Fischer, 2007; Fischer *et al.*, 2008; Kehl *et al.*, 2014). The second reason is that life-history theory predicts such risky behaviour, like an increased aggressiveness or persistence during courtship, should generally increase with age, because the residual reproductive value, *i.e.* the opportunity for future reproduction, decreases. As a consequence, younger males generally pay greater costs than older ones when being killed or injured during mating or courtship (Parker, 1974; Enquist & Leimar, 1990; Kemp, 2002, 2006).

Old male mating advantage has been convincingly shown in the tropical butterfly *B. anynana*, despite of a clearly poorer condition compared to younger males (Fischer *et al.*, 2008; Karl *et al.*, 2013). However, the underlying proximate and ultimate reasons are under debate. On the one hand, older males have been found to court more often and for longer time bouts, suggesting a higher intrinsic motivation as predicted by the residual reproductive value hypothesis (Karl *et al.*, 2013; Karl & Fischer, 2013; Kehl *et al.*, 2014). The latter comprises a lower residual reproductive value with increasing age, resulting in a higher aggressiveness and willingness to persist during courtship (Fischer, 2006; Geister & Fischer, 2007; Fischer *et al.*, 2008; Karl & Fischer, 2013). Additionally, females having mated with an older male have shown reduced egg hatching success and a higher remating propensity, suggesting that mating with an older male is disadvantageous to females, thus challenging the female choice hypothesis (Karl & Fischer, 2013). On the other

hand, there is firm evidence that male pheromone profiles play an important role in male mating success (Costanzo & Monteiro, 2007; Nieberding *et al.*, 2008; Karl *et al.*, 2013). As the composition of male sex pheromones (MSP) shows age-specific variation in *B. anynana*, females may use this information to choose specific males (Nieberding *et al.*, 2012). In particular, the male sex pheromone hexadecanal shows large variation across male ages and inbreeding levels and seems to play a crucial role in female choice (Nieberding *et al.*, 2012; Van Bergen *et al.*, 2013). Regarding old male mating advantage, females may prefer males which have proven a high fitness in terms of survival, thus essentially reflecting a “good genes” hypothesis (Trivers, 1971; Brooks & Kemp, 2001; Mays & Hill, 2004; Johnstone *et al.*, 2009). Note that the latter hypothesis suggests cooperation, while the former involves sexual conflict with older males “urging” females to mate with them while it might be more beneficial to mate with younger males, which may provide more sperm or material benefits (Vahed, 1998; Wedell & Karlsson, 2003; Ferkau & Fischer, 2006).

## **Female mate choice and the role of male sex pheromones**

Females of several species were found to mate preferably with males displaying specific cues such as ornaments, bright colours or sex pheromones (Mays & Hill, 2004; Wong & Candolin, 2005; Velando *et al.*, 2006; Costanzo & Monteiro, 2007; Nieberding *et al.*, 2008, 2012; Rutowski & Rajyaguru, 2012). A preference for those traits should be linked with indirect or direct fitness benefits. Against the background of old male mating advantage the question arose whether females prefer older males on the basis of their sex pheromones, suggesting that pheromones function as an honest signal. Butterflies became important model organisms for sexual selection, as they comprise a broad variety of sexually selected traits like colour patterns or pheromones (Morehouse & Rutowski, 2010; Cook *et al.*, 2012; Rutowski & Rajyaguru, 2012; Karl *et al.*, 2013). Both, chemical and optical cues, are of crucial importance for mating success, underlying a female choice (Costanzo & Monteiro, 2007) and it

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is therefore important to analyse, whether females gain any benefits for their offspring when mating with such preferred males. Note, that female choice combined with males' competitive ability is of importance for male mating success (Mays & Hill, 2004) and that both mechanisms are closely intertwined.

Intraspecific communication is mediated, among other things, by sex pheromones, which are species-specific compounds (Wyatt, 2003). Especially in moths, the role of pheromones as an attractant for mating partners is well understood and most strikingly, some species only need a few molecules to detect their partners even over long distances up to eight kilometres (Wyatt, 2003; Majerus, 2002). Over the intervening years a variety of chemical compounds have been identified and underlying genetic pathways are well known (Ando *et al.*, 2004; Mori, 2004; Löfstedt, 1993; Rafaeli, 2005; Lienard *et al.*, 2014). In *B. anynana* three major sex pheromones have been identified in the males, forming the presumptive MSP, namely (Z)-9-tetradecenol (Z9-14:OH), hexadecanal (16:Ald) and 6,10,14-trimethylpentadecan-2-ol (6,10,14-trime-15-2-ol) (Nieberding *et al.*, 2008). These are produced by the male secondary sexual structures, the androconia (Nieberding *et al.*, 2008).

In *B. anynana*, two pairs of androconia can be found on the dorsal wing surfaces that are each made up of modified epidermal scale cells that form patches of shiny scales, the androconial spots and hairs (Birch *et al.*, 1990). Each group of hairs clearly overlies its respective androconial spot when the wings are in a resting position (Nieberding *et al.*, 2008). In male-male competition experiments, males which have been operated due to blocked androconia had a significantly reduced mating success, compared to the control treatment (Costanzo & Monteiro, 2007). These findings suggest that the compounds, being emitted from the androconia, play an important role in female mate choice (Costanzo & Monteiro, 2007; Nieberding *et al.*, 2008).

Assuming that MSPs comprise honest signals, its production may induce non-trivial costs for males and traits being associated with pheromone titres should be investigated (Johansson *et al.*, 2005; Strauss *et al.*, 2008). Especially in insects, females benefit from larger spermatophores, which contain higher amounts of accessory gland products and sperm (South & Lewis, 2011; Wedell,

1994; Vahed, 1998). Therefore, a positive correlation between pheromone titres and spermatophore mass and/or quality can be predicted in case pheromone titres serve as honest signals. A possible indirect fitness benefit for females might be a survival advantage for the offspring, as they received the “good genes” from the father (Mays & Hill, 2004; Johnstone *et al.*, 2009). Another would be based on the Fisherian run-away process (Trivers & Willard, 1973; Chandler *et al.*, 2013), which predicts the production of so called “sexy sons”, inheriting the attractive signals from the father, which in turn results in an increase in the reproductive success of the offspring.

## Costs of reproduction

The evolution of life-histories involve trade-offs regarding fitness-related traits (Stearns, 1992; Roff, 2002). The investigation of reproductive resource allocation then has become of crucial importance for understanding life-history evolution. It predicts that in an organism current and future reproduction are traded-off against each other (Stearns, 1992; Reznick, 1985). In the past, it has been generally believed that females pay higher costs regarding reproduction, than males (Bateman, 1948; Fowler & Partridge, 1989; Chapman *et al.*, 1998; Harshman & Zera, 2007). Typically, females invest the majority to offspring production and parental care, while males only donate cheap sperm, which for a long time has been assumed to be produced in unlimited amounts (Heinze & Hölldobler, 1993). However, this antiquated point of view has been challenged recently. After mating, males need recovery time before they can produce another ejaculate, respectively, ejaculate production itself needs time (Birkhead, 1991; Wedell, 1994; Bissoondath & Wiklund, 1996), resulting in smaller subsequent ejaculates with decreased quantities of sperm (Salamon, 1962; Oberhauser, 1988; Zvěřina & Pondělíková, 1988; Birkhead, 1991). As in already-mated males spermatophore production seems to be more time-consuming than in virgin males (Wedell & Cook, 1999), mating duration is prolonged in subsequent compared with the first matings (Kaitala & Wiklund, 1995), which in turn is detrimental for both partners as a prolonged copulation

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increases the chance of being injured or killed.

Against this background, the existence of non-trivial costs of reproduction is obvious and includes even survival costs of mating (Kotiaho *et al.*, 1998; Kotiaho & Simmons, 2003; Paukku & Kotiaho, 2005; Janowitz & Fischer, 2010). Consequently, in polyandrous mating systems, males should carefully invest their resources considering current as well as future reproduction (Takeuchi, 2012; Caballero-Mendieta & Cordero, 2013). Another important factor, which has large effects on male reproduction, is age. As in many taxa senescence starts early (Ford, 2000; Boggs & Freeman, 2005; Møller *et al.*, 2009), condition should consequently diminish with age (Karlsson, 1994; Fischer *et al.*, 2008). There is accumulating evidence that mating with older males is highly detrimental to females. The effects range from a reduction of female lifespan (Paukku & Kotiaho, 2005), fecundity (Ofuya, 1995), and egg-hatching success (Jones *et al.*, 2000; Karl & Fischer, 2013), to a higher remating propensity combined with a tendency towards shorter refractory periods (Karl & Fischer, 2013).

Quite recently, the role of physiological factors underlying ageing and reproductive performance have become more and more important. Oxidative stress is under debate to play an important role in effecting reproduction. Reactive Oxygen Species (ROS) (as a by-product of the respiratory chain and cytochrome P<sub>450</sub> oxidoreductase) and antioxidant defences stay in a fragile equilibrium. Any imbalances in favour of ROS may result in a higher oxidative damage. Besides several environmental factors, like ultraviolet radiation or heat, there are several studies indicating that reproduction may lead to lower antioxidant defences and higher oxidative damage (Bize *et al.*, 2008; Helfenstein *et al.*, 2010; Stier *et al.*, 2012). As oxidative damage is getting worse and antioxidant defences are declining with age, older organisms may have reduced possibilities to defend effectively against the negative effects of reproduction on their oxidative status (Williams *et al.*, 2008; Monaghan *et al.*, 2009).

## Study organism

*Bicyclus anynana* or Squinting Bush Brown (Butler 1879, Nymphalidae, Satyriinae) is a tropical, fruit-feeding butterfly. The distribution stretches from southern Africa to Ethiopia, southern Somalia, Kenya, Uganda and eastern Zaïre; it can also be found on the Comoros and on Socotra. *B. anynana* is a savannah-adapted butterfly species which flies in open habitats but inclines to rest in the shade of trees and bushes. Roosts in the open are preferred to a lesser extent only (Larsen, 1991). It exhibits striking phenotypic plasticity with two seasonal morphs, as an adaptation to alternate wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield, 1997; Lyytinen *et al.*, 2004). During the dry-season, *B. anynana* shows an asymmetrically suppressed main eye-spot on the underside of the forewing. Its colloquial name (“squinting”) is derived from that aspect (Larsen, 1991). During the wet-season, larval food plants are abundantly available and the reproduction takes place. Larvae feed on a wide spectrum of grasses belonging to the *Poaceae* family (Brakefield, 1997).

A laboratory stock population was established at the Greifswald University, Germany, in 2007 counting several hundred individuals, derived from the stock population of the Bayreuth University, Germany. This population is a descendent of the stock population of the Leiden University, The Netherlands, which was founded in 1988 from 80 gravid females, caught at Nkhata Bay in Malawi. In each generation several hundred individuals are reared, maintaining high levels of heterozygosity at neutral loci (Van’t Hof *et al.*, 2005). For this study, butterflies of the Greifswald stock population were used.



**Fig. 1.1:** Mating couple of *B. anynana*;  
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## 2 Synopsis

### 2.1 Pheromone blend does not explain old male mating advantage in a butterfly

Succeeding in competition is of crucial importance for the individual fitness of males, as the availability of females is often limited (Chen *et al.*, 2012; Kant *et al.*, 2012). Male mating success depends either on female choice or the competitive superiority of specific males, although a combination of both is also possible (Westerman *et al.*, 2012; Van Bergen *et al.*, 2013). Typically, in holometabolous insects, condition, and also sperm quality, diminishes with age (Karlsson, 1994; Fischer *et al.*, 2008; Cook & Wedell, 1996; Marcotte *et al.*, 2005; Sasson *et al.*, 2012), such that young virgin males should gain the highest mating success (Vahed, 1998; Ferkau & Fischer, 2006). Interestingly, old males across a variety of insect species have been shown to outcompete their younger counterparts (Conner, 1989; Cameron *et al.*, 2005; Fischer *et al.*, 2008; Simmons & Zuk, 1992). Two hypotheses try to explain these interesting patterns. The first suggests a decrease of the resource holding potential, which in turn leads to an enhanced aggressiveness and willingness to persist during courtship (Fischer, 2006; Geister & Fischer, 2007; Fischer *et al.*, 2008). As females are typically the choosy sex, the second hypothesis assumes a female preference for older males, which have proven their survival abilities (Trivers, 1971; Brooks & Kemp, 2001; Johnstone *et al.*, 2009; Kehl *et al.*, 2014).

In the tropical butterfly *B. anynana* old male mating advantage prevails, despite of a poorer condition compared to younger males (Fischer *et al.*, 2008; Karl *et al.*, 2013) but the underlying proximate and ultimate reasons are still under debate. As being predicted by the residual reproductive value hypothesis, older males court more often and for longer time bouts (Karl & Fischer, 2013; Karl *et al.*, 2013). Recent results might challenge the female choice hypothesis, as females having mated with an older male, show a reduced egg-hatching success (Karl & Fischer, 2013). However, male sex pheromones seem

to play an important role in male mating success (Costanzo & Monteiro, 2007; Nieberding *et al.*, 2008, 2012) and there is firm evidence that females use age-specific pheromone profiles to choose preferred males (Nieberding *et al.*, 2012).

In a rigorous approach, this study tested the effects of male sex pheromones on old male mating advantage in *B. anynana*. By manipulating the females' ability to smell (Costanzo & Monteiro, 2007) and by operating older male's androconia, it was investigated whether old male mating advantage prevails in females, even when they are unable to distinguish between older and younger males. This procedure was found to block the production of male sex pheromone 2 efficiently, while male sex pheromones 1 and 3 were still produced at least to some extent (Nieberding *et al.*, 2008; Karl *et al.*, 2013). In order to backup the results, female rejection rates, male courtship frequency, courtship duration, time to copulation and mating duration were also examined. If old male mating advantage is a result of a decline in the residual reproductive value in older males, they should gain a higher mating success in scent-blocked and control females. Furthermore, older males should outcompete their younger counterparts despite the operations of males' androconia.

Strikingly, older males of both, control and operated ones were found to have a significantly higher mating success compared to younger males, when competing for a control as well as a scent-blocked female, throughout. Furthermore, older males of both groups courted more often and for longer time bouts. As the mating success of older males increased, despite a manipulation of the females' olfactory ability or male pheromone blend, this study provided solid evidence for the residual reproductive value hypothesis. Regarding old male mating advantage, it seems that mating decisions in *B. anynana* are primarily driven by male behaviour. All in all, old male mating advantage seems to arise from the older males' higher intrinsic motivation and eagerness to mate, as evidenced by longer and more frequent courtship attempts (Fischer *et al.*, 2008; Karl & Fischer, 2013; Kehl *et al.*, 2014). The study indicates that male behaviour may be important for mating decisions even in systems in which the operational sex ratio is biased towards males, where female choice is expected, as females rarely mate more than once while males

can mate many times (Brakefield *et al.*, 2001; Janowitz & Fischer, 2010, 2012). Of course, male aggression and activity itself might be an important cue for female choice, which makes it evidently difficult to distinguish between the effects of male and female behaviour, as both are expressed simultaneously (Kehl *et al.*, 2014).

## 2.2 Old male paternity advantage in *Bicyclus anynana*

Successful reproduction is defined by the number and proportion of gene copies being passed on to the next generation and as a result, sexual selection should favour traits, increasing reproductive output and consequently individual fitness (Arnqvist & Rowe, 2005). Besides pre-copulatory sexual selection, which favours traits increasing the number of matings, post-copulatory sexual selection enhances the sperm competitive ability (Trivers, 1972; Arnqvist & Rowe, 2005; Wong & Candolin, 2005). Typically, in most animals, the resource holding potential is a key determinant of male mating success (Marden & Waage, 1990; Kemp & Wiklund, 2001; Lappin *et al.*, 2006), with few exceptions (Kemp & Wiklund, 2001; Fischer, 2007; Janowitz & Fischer, 2010). Post-copulatory sexual selection is determined by a competition of sperm for access to oocytes and the fertilization success is affected by a variety of factors like mating order, sperm precedence (Parker & Pizzari, 2010; Arnqvist & Rowe, 2005) or the number of sperm transferred upon mating (Bissoondath & Wiklund, 1997; Wedell & Cook, 1998; Solensky & Oberhauser, 2009).

Last-male precedence, *i.e.* the last male mating partner sires the most offspring, is the dominant fertilization pattern in insects (Walker, 1980; Watt *et al.*, 1985; Platt & Allen, 2001). As evidenced by several studies, spermatophore size, sperm numbers and quality decrease with age (Kaitala & Wiklund, 1995; Cook & Wedell, 1996; Wedell & Ritchie, 2004; Marcotte *et al.*, 2005; Damiens & Boivin, 2006). On the other hand, extended periods before or between mating can lead to sperm accumulation (Oberhauser, 1988; Lehmann & Lehmann, 2009; Vande Velde *et al.*, 2011, 2012). As a conse-

quence of diverging reproductive interests in males and females, called sexual conflict (Arnqvist & Rowe, 2005), females should maximize their individual fitness *via* mating with males that promise fitness benefits, leading to female mate choice (Wong & Candolin, 2005; South & Lewis, 2012) and further to cryptic mate choice, excluding unsuitable males from paternity after mating (Parker & Pizzari, 2010; Watanabe & Sasaki, 2010; Kehl *et al.*, 2013).

In this study, post-copulatory sexual selection has been investigated in *B. anynana*, a species which exhibits the fascinating pattern of old male mating advantage. As nothing is known about post-copulatory sexual selection in this species, females were have been mated twice with older and younger males, allowing for sperm competition and cryptic mate choice. Females should prefer younger males if mating with older males is detrimental and in case of female choice for older males, they should use sperm from those. As spermatophore size and the number of eupyrene has been found to affect paternity success in other studies, those traits were also considered (Rutowski *et al.*, 1987; Bissoondath & Wiklund, 1996; Wedell & Cook, 1998). Females have been double-mated with a young (2-3 days) and afterwards an older male (12-13 days) and *vice versa*. Resulting offspring, as well as the parents have been genotyped using eight microsatellite markers. Spermatophore mass and eupyrene sperm numbers of old vs. young males have been investigated by mating 2-day old virgin males with either an old or a young male and subsequently dissecting the females in order to get hold of the spermatophores.

The results revealed that older males had a higher paternity success than younger ones. This was only the case when old males were the second, *i.e.* last mating partner. Interestingly, the paternity success was balanced across the age classes when older males were the first mating partner. Additionally, older males transferred larger spermatophores with much higher numbers of eupyrene (fertile) sperm than younger males. These findings suggest that *B. anynana* does not exhibit cryptic mate choice and that last-male precedence is the most probable fertilization pattern, which is known to be the most common fertilization pattern across insects (Walker, 1980; de Jong *et al.*, 1998; Wedell & Cook, 1998; Platt & Allen, 2001) and even in *B. anynana*, where

it co-occurs with sperm mixing and first-male precedence (Brakefield *et al.*, 2001). Instead, the higher proportion of offspring sired by older males is a result of significantly increased numbers of fertile sperm compared to younger males. Evidenced by heavier spermatophores and higher amounts of eupyrene sperm and the equal fertilization success in younger males when being the last male to mate, the investigated sperm traits counteract the benefits of last-male precedence. Note that in butterflies paternity success is typically positively related to body mass, spermatophore mass and with sperm numbers (Rutowski *et al.*, 1987; Oberhauser, 1988; Gage & Morrow, 2003; Solensky & Oberhauser, 2009), as spermatophore size, sperm number and quality are predicted to decrease with age (Kaitala & Wiklund, 1995; Wedell & Ritchie, 2004; Marcotte *et al.*, 2005; Damiens & Boivin, 2006) As a consequence, younger males are clearly disadvantaged when competing against older males as the latter sire more offspring anyway. In this context, several questions regarding the dynamics of sperm production and sperm quality remain an interesting field, worthy of consideration in future investigations.

## 2.3 Determinants of mating success

As competition for mates often involves direct physical interactions and risk of injury, strength, and heavy weaponry are often found in males, factors being associated with an individual's resource holding potential (Andersson & Iwasa, 1996; Mesterton-Gibbons *et al.*, 1996). In groups lacking physical means like butterflies, odonates etc., flight endurance, vigour, and agility may be more important for mating success (Waage, 1988; Kemp, 2002). In those groups, energy reserves and energetic efficiency may play a key role in male-male competition (Marden & Waage, 1990; Mesterton-Gibbons *et al.*, 1996). However, female choice may also be involved, as females prefer males displaying specific ornaments, bright colours or even sex pheromones (Mays & Hill, 2004; Wong & Candolin, 2005; Velando *et al.*, 2006; Costanzo & Monteiro, 2007; Rutowski & Rajyaguru, 2012; Breuker & Brakefield, 2002). Note, that both mechanisms are not mutually exclusive, as females may have a preference for males

reflecting a high resource holding potential. Due to a lack of weaponry and any obvious means to inflict harm upon combatants, the factors determining male mating success in butterflies, regardless of whether it is based on male behaviour or female choice, are much less obvious as compared to other animal groups (Kemp & Wiklund, 2001). As male-male interactions occur frequently and may reflect energetic “wars of attrition”, the amount of storage reserves and overall condition may be important (Marden & Waage, 1990; Mesterton-Gibbons *et al.*, 1996). During the last few years, several lines of evidence have proven an important role of male motivation, reflected by aggressiveness and persistence during courtship on male mating success (Fischer *et al.*, 2008; Karl & Fischer, 2008; Karl *et al.*, 2013; Kehl *et al.*, 2014).

Against this background, this study explored determinants of mating success in *B. anynana*, considering morphological and physiological traits by comparing successful versus unsuccessful males in mating trials. Further, it was tested, whether specific males were able to repeatedly win mating trials against a specific counterpart. A variety of traits was investigated, including correlations of body size (body mass, wing length), flight ability (thorax mass, wing length), condition (fat and protein content, immune status), and potentially sexually selected signals (eyespot and eyespot pupil size). It was hypothesized that successful males have a larger body size, a better flight ability, more storage reserves and/or a better immune status, and larger eyespots.

The results indicated striking differences regarding both, the measurement of physiological and morphological traits. Successful males were found to have longer wings, a heavier thorax, a lighter abdomen, a higher fat content, and higher phenoloxidase expression levels. Regarding males competing repeatedly against the same counterpart, in 28 out of 125 mating trials one male won all contests against his counterpart, and in another 45 mating trials the same male won at least the first two trials. The former were characterized by larger forewings, a higher body mass, and a higher fat content. Regarding eye-spot size, no differences were found between the respective males. As mating success is directly linked with proxies of flight performance, *i.e.* a higher body mass, larger forewings (Van Dyck *et al.*, 1997; Martinez-Lendeck *et al.*, 2007), energy

reserves (Zera *et al.*, 1998; Karl & Fischer, 2008), a larger thorax, and a lighter abdomen (Srygley & Dudley, 1993; Peixoto & Benson, 2008), it was concluded that successful males showed a better flight performance, in turn increasing mating success. For butterflies, a high flight performance is substantial due to mate location, conquering and defending territories, aerial fights and courtship (Kemp & Wiklund, 2001; Tiple *et al.*, 2010).

As phenoloxidase is an excellent indicator of condition in insects (González-Santoyo & Córdoba-Aguilar, 2011), expression patterns have been discussed. Although a strong trade-off between immunity and reproduction predicts a decrease in phenoloxidase levels after mating (Rolf & Siva-Jothy, 2002; Schwarzenbach *et al.*, 2005), successful males have shown an increased phenoloxidase expression compared to unsuccessful ones. Hence, the results regarding phenoloxidase levels suggest a better body condition in successful males, which may be favourable in terms of a better flight performance, mentioned above. At this point the discussion is limited as mating involves close contact among individuals, which may result in an upregulation of the immune system (Valtonen *et al.*, 2010; Gupta *et al.*, 2013).

Although male mating success was related to several indicators of condition, the amount of storage reserves may be the most important factor affecting male mating success. Besides that, the differences being observed between males in this study were found to play a leading role in male flight performance. Hence, it was concluded that male agility, manoeuvrability, and persistence in mate location and courtship were the underlying determinants of male mating success in *B. anynana* and potentially also in other insect species.

## **2.4 Old male sex: large ejaculate, many sperm, but few offspring**

The traditional view that costs of reproduction mainly concern females (Bateman, 1948; Chapman *et al.*, 1998; Harshman & Zera, 2007), has changed as males have been found to experience non-trivial costs of mating, regarding ejaculate production and even survival (Birkhead, 1991; Wedell, 1994; Bissoondath

& Wiklund, 1996; Salamon, 1962; Oberhauser, 1988; Birkhead, 1991; Kotiaho *et al.*, 1998; Kotiaho & Simmons, 2003; Paukku & Kotiaho, 2005). As in many taxa, condition diminishes with age (Karlsson, 1994; Fischer *et al.*, 2008) and senescence starts early, the age of an individual may be of crucial importance for its reproduction (Ford, 2000; Boggs & Freeman, 2005; Møller *et al.*, 2009). Young, virgin males may provide better nuptial gifts or have more resources available for signalling or sperm production (Vahed, 1998; Ferkau & Fischer, 2006; Fricke & Maklakov, 2007), so that in consequence, females should prefer those males. Indeed, mating with older males has been found to be detrimental to females, *i.e.* evidenced by a reduction of female lifespan (Paukku & Kotiaho, 2005), and a decrease in fecundity and egg-hatching success (Ofuya, 1995; Jones *et al.*, 2000; Karl & Fischer, 2013). On the physiological level of ageing, oxidative stress and antioxidant defences may play an important role (Williams *et al.*, 2008; Monaghan *et al.*, 2009), as reproduction was found to result in lower antioxidant defences and higher oxidative damage, which may in turn limit the capacity for future reproduction (Bize *et al.*, 2008; Helfenstein *et al.*, 2010; Stier *et al.*, 2012).

In the tropical butterfly *B. anynana*, in which older males have a striking mating advantage compared to younger ones (Karl & Fischer, 2013; Karl *et al.*, 2013; Kehl *et al.*, 2014), sexual conflict seems to be underlying old male mating advantage, as older males show higher flight activity and aggressive courtship behaviour than younger counterparts. Further, females suffered from a reduced egg-hatching success, a shorter refractory period, and a higher remating propensity after mating with older males (Fischer *et al.*, 2008; Karl & Fischer, 2013; Karl *et al.*, 2013; Kehl *et al.*, 2014), suggesting that older males transfer lower quality spermatophores (Kaitala & Wiklund, 1995; Wedell & Ritchie, 2004). Sperm limitation seems to be unlikely, as older males have a higher paternity success, likely due to an accumulation of sperm over time (Kehl *et al.*, 2013).

This study tested the effects of male age and mating number on spermatophore mass, sperm number, male oxidative status, and reproductive success in *B. anynana*. By manipulating male age, mating number, and the time

since the production of the last spermatophore, the effects of male age and mating frequency on spermatophore production, males' oxidative status, and fitness consequences for females were investigated.

The results revealed a significant increase of spermatophore mass and sperm numbers with age, while antioxidant defences and oxidative damage declined. In contrast, repeatedly-mated males produced spermatophores and sperm numbers comparable to young, virgin males, showing only little variation. Mating repeatedly caused an increase of antioxidant defences and a decline in oxidative damage. Although young, virgin males provided small spermatophores with relatively low sperm numbers, female fecundity and even egg-hatching success was highest when being mated with those males. Regarding increasing weights of spermatophores and sperm numbers, a constant sperm production and accumulation in virgin males was discussed (Proshold, 1991; Konagaya & Watanabe, 2013), being further supported by similar sperm numbers in previously mated males. Interestingly, spermatophore mass showed a similar pattern like sperm numbers, indicating that accessory gland products were produced at a constant rate. Contrary to the results achieved here, spermatophore size generally decreases with mating number (Lederhouse *et al.*, 1990; Cook & Wedell, 1996; Watanabe *et al.*, 1998; Wedell & Cook, 1999). In line with earlier studies, females gained highest fitness returns when they mated with young, virgin males, evidenced by the highest fecundity and egg-hatching success (Wedell & Karlsson, 2003; Srivastava & Omkar, 2004; Kovac *et al.*, 2013). As, in the study, antioxidant defences decreased with age, a possible reason for the negatively affected reproductive success in females being mated with older males was found. Additionally, evidenced by a decrease of oxidative damage with age, a reduced production of reactive oxygen species by reducing the metabolic rate (Niitepõld & Hanski, 2013; Niitepõld *et al.*, 2014), may lead to an overall decrease of investment into reproduction, in turn affecting old males' poor reproductive performance.

It was concluded that, contrary to results being observed in other insect species (Lederhouse *et al.*, 1990; Cook & Wedell, 1996; Watanabe *et al.*, 1998), spermatophore mass and eupyrene sperm numbers did not decline with

male mating number. This was evidenced by four-times mated males which reached nearly the same spermatophore mass and eupyrene sperm numbers than 3-day old, single mated males. It was assumed that this results from high sperm production rates in combination with producing comparably small spermatophores (Rutowski *et al.*, 1987; Lauwers & Van Dyck, 2006; Vande Velde *et al.*, 2011). Further, ejaculate quality clearly diminished with age and mating number in *B. anynana*, despite significantly larger spermatophores and higher sperm numbers being found in older males. Consequently, spermatophore size and sperm numbers can not be considered as reliable proxies of male condition. The results hold great potential to underline the notion that old male mating advantage in *B. anynana* arises from sexual conflict (Karl & Fischer, 2013), due to the higher mating success of older males being observed unambiguously in earlier studies (Fischer *et al.*, 2008; Karl *et al.*, 2013; Kehl *et al.*, 2014).

### 2.5 Male sex pheromones as honest signals?

In order to maximize the genetic reward of an individual, traits, increasing the number of mating and sperm competitive ability, should be favoured by selection, despite possible negative fitness consequences for the female partner (Chapman *et al.*, 1995; Kemp & Rutowski, 2004; Kuijper *et al.*, 2006). Beside the mating success, being mediated by traits reflected by an individuals resource holding potential (Marden & Waage, 1990; Kemp & Wiklund, 2001), visual, acoustic, or chemical cues play a decisive role in male mating success in some groups (Velando *et al.*, 2006; Judge, 2011; Verburgt *et al.*, 2011; Nieberding *et al.*, 2008, 2012), in which female choice is clearly important for male mating success (Mays & Hill, 2004). Especially in butterflies, providing sexually selected traits like colour and odour, female choice is important for mating success (Breuker & Brakefield, 2002; Costanzo & Monteiro, 2007). In *B. anynana* sex pheromone patterns show a large variation across age, and have been found to have an important impact on male mating success (Nieberding *et al.*, 2008, 2012; Costanzo & Monteiro, 2007).

Against this background, several questions arise, regarding the function

of male sex pheromones and the underlying fitness benefits to females. Do sex pheromones comprise honest signals, implying that they are costly to produce (Harari *et al.*, 2011; Foster & Johnson, 2011), and which traits might be associated with increased pheromone titres (Johansson *et al.*, 2005; Strauss *et al.*, 2008)? The study investigated whether pheromone titres differ among successful and unsuccessful males in control and scent-blocked females (Kehl *et al.*, 2014) and looked at possible correlations between pheromone titres and sperm production and offspring traits like longevity and reproduction, thus comprising direct and indirect fitness benefits. It was hypothesized that males showing higher pheromone titres, have an overall higher mating success, produce larger spermatophores and higher amounts of eupyrene sperm, affect female fecundity and longevity positively, due to higher amounts of accessory gland products and sperm numbers.

Contrary to the expectations from above, pheromone levels did not differ among successful and unsuccessful males, being mated to control and scent-blocked females. As opposed to all previous studies having found positive effects of pheromone titres in *B. anynana*, naturally occurring variation in pheromone titres was used exclusively in this study. Interestingly, successful males had significantly higher numbers of eupyrene sperm in the female control and the olfaction-blocked group. Eupyrene sperm number turned out to be the only factor affecting male mating success in both treatment groups. Except for MSP 2, spermatophore mass was positively related to pheromone titres, in both treatments, whereas only in olfaction-blocked females, sperm numbers were positively correlated to pheromone titres. As these correlations did not lead to significant variations in pheromone blends, and no correlation between male pheromones and any investigated trait was found, it was suggested that pheromone titres do not provide reliable information on male quality. As honest signals, indicating high fitness returns for the mating partner, were discussed among several species (Velando *et al.*, 2006; Strauss *et al.*, 2008; Helfenstein *et al.*, 2010; Langkilde & Boronow, 2012; Tibbetts, 2014), in *B. anynana* nothing indicates that male sex pheromones fulfil such functions.

Further, no correlations between male sex pheromones, female fitness and

the investigated offspring traits were found. Interestingly, several correlations across life-history traits were observed and discussed due to biological significance. Lifetime fecundity was correlated with early fecundity, indicating that the majority of eggs is laid early within the oviposition period (Brakefield *et al.*, 2001). Lifetime fecundity was positively related to longevity, which indicates that long-living females are able to produce more eggs (Bauerfeind & Fischer, 2007). Early and lifetime fecundity tended to negatively affect pupal development time and survival as well as offspring body size. This trade-off between offspring quality and quantity is under debate since several decades (Smith & Fretwell, 1974). The positive correlations between pupal (and larval) survival and measures of body size indicate fitness benefits of being large (Blanckenhorn, 2000).

To cut a long story short, it was concluded that nothing indicates that male sex pheromones in *B. anynana* play a decisive role in female mate choice. Furthermore, these data cast doubt on the notion that female choice is of any particular importance in our system. The most likely explanation seems to be that successful males generally have a better body condition (e.g. having a higher fat content), being evidenced by a previous study (chapter 2.4). Males in a better condition may show a more vigorous courtship behaviour and produce more sperm due to more resources being available. Hence, a variation in body condition rather than pheromone titres is more likely to determine male mating success.

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# **PUBLICATION LIST**



## 4 Publication list

**Kehl, T.**, Burmeister, M.F.W.T., Donke, E., Köhn, N.A.K., Metschke, K., Pfender, D., Karl, I. & Fischer, K. (2014) Pheromone blend does not explain old male mating advantage in a butterfly. *Ethology* 120:1137-1145.

### **Contribution**

1. Butterfly rearing
2. Supervising experiments and data acquisition
3. Statistical analyses and analysing data
4. Writing and editing the Manuscript

**Kehl, T.**, Karl, I. & Fischer, K. (2013) Old-male paternity advantage is a function of accumulating sperm and last-male precedence in a butterfly. *Molecular Ecology* 22:4289-4297.

### **Contribution**

1. Butterfly rearing
2. Conducting experiments and data acquisition
3. Statistical analyses and analysing data
4. Writing and editing the manuscript

**Kehl, T.**, Bensch, J., Böhm, F., Kniepkamp, B.O., Leonhardt, V., Schwieger, S. & Fischer, K. Fat and sassy: factors underlying male mating success in a butterfly. *Entomologia Experimentalis et Applicata* (in press).

### **Contribution**

1. Butterfly rearing (experiment 2)
2. Supervising experiment 2 and data acquisition
3. Laboratory analyses (experiment 2)
4. Statistical analyses and analysing data

5. Writing and editing the manuscript

**Kehl, T.**, Beaulieu, M., Kehl, A. & Fischer, K. Old male sex: large ejaculate, many sperm, but few offspring. (submitted).

**Contribution**

1. Butterfly rearing
2. Conducting experiments and data acquisition
4. Statistical analyses and analysing data
5. Writing and editing the manuscript

**Kehl, T.**, Dublon, I. & Fischer, K. Male sex pheromones as honest signals? Sperm number and not pheromone titre is key in young male butterfly mating success. (submitted).

1. Butterfly rearing
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4. Statistical analyses and analysing data
5. Writing and editing the manuscript

Prof. Dr. Klaus Fischer

Tobias Kehl

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## RESEARCH PAPER

# Pheromone Blend Does not Explain Old Male Mating Advantage in a Butterfly

Tobias Kehl, Martin F. W. T. Burmeister, Elisabeth Donke, Nora A. K. Köhn, Katja Metschke, David Pfender, Isabell Karl & Klaus Fischer

Zoological Institute & Museum, Greifswald University, Greifswald, Germany

**Correspondence**

Tobias Kehl, Zoological Institute & Museum, Greifswald University, Johann-Sebastian-Bach Str. 11/12, D - 17489 Greifswald, Germany.  
E-mail: tobias.kehl@uni-greifswald.de

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**Abstract**

In several insect species, male mating success is higher in older than in younger males, although condition diminishes dramatically with age. Two hypotheses are under debate to explain the counterintuitive pattern of old male mating advantage: first, an increased eagerness of older males to mate, driven by their low residual reproductive value, and second female preference for older males based on chemical cues such as sex pheromones (female choice hypothesis). In a series of experiments, we manipulated female olfaction, male pheromone blend and female age to test whether old male mating advantage prevails when the influence of male sex pheromones is controlled for, using the tropical butterfly *Bicyclus anynana* as model. We found that older males had a higher mating success than younger ones irrespective of female scent-sensitivity and irrespective of male pheromone blend. Interestingly, older males were found to court more often and for longer time bouts than younger males. These results were independent of female age, although younger males courted younger females more often and for longer bouts than older females. Taken together, our results indicate that male courtship activity (1) is higher in older compared to younger males and (2) increases the mating success of older males. Olfaction and sensing pheromones, in contrast, were not a necessary prerequisite for old male mating advantage to occur and may use other cues than pheromones to assess male quality.

**Introduction**

Male reproductive success is often limited by the availability of receptive females, such that gaining a high mating success is of pivotal importance for male fitness (Chen et al. 2012; Kant et al. 2012). Succeeding in competition for mates may be facilitated by either (1) a competitive superiority of specific males compared to others (intrasexual selection), (2) being preferred by female partners as females are typically the choosy sex (intersexual selection), or (3) both (Wong & Candolin 2005; Westerman et al. 2012; Rutowski & Rajyaguru 2013; Van Bergen et al. 2013). Regarding intrasexual selection, a male's resource holding potential, reflected by proxies of condition such as body mass or fat content, is considered to be of crucial importance for male mating success

(Marden & Waage 1990; Kemp & Wiklund 2001; Lapin et al. 2006). Note that the above traits may well play a role in both intra- and intersexual selection. Other important factors which may affect male mating success include visual, acoustic, and chemical cues, at least some of which may be used by females for selecting preferred males (e.g. Costanzo & Montei 2007; Nieberding et al. 2008, 2012; Verburgt et al. 2011; Win et al. 2013).

As in holometabolous insects condition diminishes with age (Karlsson 1994; Fischer et al. 2008), young virgin males have been predicted to gain the highest mating success (Vahed 1998; Wedell & Karlsson 2003; Ferkau & Fischer 2006). Also, sperm quality and quantity typically decrease with age (Kaitala & Wiklund 1995; Cook & Wedell 1996; Wedell & Ritchie 2004; Marcotte et al. 2005; Damiens & Boivin 2006;

Sasson et al. 2012). However, paradoxically, older males have been shown to have a high mating success in a variety of insect taxa, such as beetles, crickets, fruit flies and butterflies (e.g. Conner 1989; Cameron et al. 2005; Fischer et al. 2008; Karl et al. 2013). Two hypotheses have been put forward to explain this counterintuitive pattern. The first hypothesis suggests an enhanced aggressiveness and willingness to persist during courtship in older males, ultimately being caused by a decreasing residual reproductive value with increasing age (Fischer 2006; Geister & Fischer 2007; Fischer et al. 2008; Karl & Fischer 2013; Karl et al. 2013). Indeed, older males, with fewer future mating opportunities, have 'less to lose' compared to their younger counterparts, which pay a greater cost if being killed or injured during courtship or in male–male interactions (Parker 1974; Enquist & Leimar 1990; Kemp 2002, 2006). Therefore, older males may be more motivated to invest into mate location and courtship than younger males (residual reproductive value hypothesis). The second hypothesis suggests that older males may be preferred by females based on their proven survival abilities, thus reflecting a 'good genes' hypothesis (Trivers 1971; Brooks & Kemp 2001; Mays & Hill 2004; Johnstone et al. 2009).

Old male mating advantage has been convincingly shown in the tropical butterfly *Bicyclus anynana*, where mating success is up to four times higher in older males, despite of a clearly poorer condition compared to younger males (Fischer et al. 2008; Karl et al. 2013). However, the underlying proximate and ultimate reasons are under debate. On the one hand, older males were indeed found to court more often and for longer time bouts, suggesting a higher intrinsic motivation as predicted by the residual reproductive hypothesis (Karl & Fischer 2013; Karl et al. 2013). Additionally, females having mated with an older male show reduced egg hatching success and a higher remating propensity, suggesting that mating with an older male is disadvantageous to females, thus challenging the female choice hypothesis (Karl & Fischer 2013). On the other hand, there is firm evidence that male pheromone profiles play an important role in male mating success (Costanzo & Monteiro 2007; Nieberding et al. 2008; Karl et al. 2013). As the composition of male sex pheromones shows age-specific variation in *B. anynana*, females can use this information to choose specific males (Nieberding et al. 2012). In particular, the male sex pheromone hexadecanal shows large variation across male ages and inbreeding levels and plays a crucial role in female

choice (Nieberding et al. 2012; Van Bergen et al. 2013).

Against this background we here investigate the effects of male sex pheromones on old male mating advantage in *B. anynana*. By blocking the females' olfactory ability (Costanzo & Monteiro 2007), we investigate whether old male mating advantage persists in females, even when they are unable to perceive male sex pheromones. The residual reproductive value hypothesis predicts that old male mating advantage prevails regardless of whether females are olfaction-blocked or not, as old male mating advantage is thought to be proximately caused by male behaviour. If, however, females prefer older males based on pheromone blend, this hypothesis predicts that old male mating advantage only persists in control females, while olfaction-blocked females should show no preference for older or younger males, as they are unable to sense their pheromone blend. In addition to manipulating the females' olfactory ability, we manipulate male pheromone production and also investigate female rejection rates and male courtship behaviour in terms of courtship frequency, courtship duration, time to copulation, and mating duration to back up our results. Finally, we test whether results depend on female age, which may well affect male strategic decisions. Older females should be more eager to mate than younger ones because they have less time left available for oviposition and at the same time, a much higher number of mature oocytes in their abdomen (as oogenesis is a gradual process starting upon adult eclosion in *B. anynana*; Fischer et al. 2004; Steigenga & Fischer 2007).

## Methods

### Study Organism and Rearing Conditions

The Squinting Bush Brown *B. anynana* (Butler, 1879) is a nymphalid, savannah-adapted, fruit-feeding butterfly, which is distributed from southern Africa to Ethiopia (Larsen 1991). For the following experiments, two cohorts of approximately 1000 eggs each were collected from the Greifswald stock population. To set up two age classes of younger (2 d) and older (10–12 d) butterflies, eggs for the second cohort were collected 10 d later than for the first one. Note that a difference in lifespan of 10 d is considered to be ecologically relevant in this species, although lifespans may be substantially longer under laboratory conditions (Brakefield & Reitsma 1991; Fischer & Fiedler 2001; Pijpe et al. 2006; Fischer et al. 2008). In order to minimize confounding effects due to female age on

offspring quality, only young female butterflies were used for the production of both cohorts. Larvae were reared in population cages on potted maize plants under constant conditions with a 12/12-h light–dark cycle, 27°C and 70% relative humidity. The conditions used comply with those at which *B. anynana* develops and reproduces during the favourable wet season in the field (Brakefield & Reitsma 1991; Brakefield 1997). Resulting pupae were collected daily and transferred to cylindrical hanging cages. To avoid any matings prior to experiments, males and females were separated on their eclosion day, when males do not yet mate (Janowitz & Fischer 2010). Butterflies were kept separated by eclosion day and were fed with moist banana and water *ad libitum* throughout all experiments.

### Experimental Design

To investigate the effects of female olfaction, male pheromone blend and male age on male mating success, three experiments were carried out as detailed below.

#### Experiment 1

We performed 142 mating trials to investigate the mating success of older (10–12 d) vs. younger males (2–3 d), competing for a control (sham-treated) or an olfaction-blocked female. Thus, each mating trial involved a younger male, an older male and a control or olfaction-blocked female. All females were young (2 d), virgin individuals from the second cohort. One day prior to the respective mating trial, olfaction-blocked females were treated with a transparent, quickly drying nail polish (Essence; Colour & Go, Cosnova GmbH, Sulzbach, Germany) on the top of their antennae (Costanzo & Monteiro 2007). To control for confounding solution effects on olfaction, the control group received a sham-treatment by applying nail polish on the right anterior forewing. In turns, either the younger or the older male was marked 1 d before the mating trial with a small white dot of correcting fluid (Tipp-Ex GmbH & Co. KG, Frankfurt/Main, Germany) on the ventral surface of the right hind wing. All mating trials were carried out under the same conditions as those used for rearing butterflies, that is, in cylindrical hanging cages (30 cm diameter × 15 cm height). Cages were continuously monitored for a maximum of 4 h (trials were terminated once a mating had occurred), during which mating success ('winning male'), the number of courtship attempts rejected by females, time to copulation and copulation

duration were recorded. We expect that older males have a higher mating success than younger males if male courtship activity is higher in older than in younger males. We also expect that olfaction-blocked females mate as often with old and young males if sex pheromone composition is the only male trait of importance by females to assess male quality.

#### Experiment 2

In the second experiment, both male pheromone blend and female olfaction were manipulated. As above, each of the 152 mating trials involved a younger male (2–3 d), an older male (10–12 d) and either a control or olfaction-blocked female. Again all females were young (2 d), virgin individuals from the second cohort. Males were also manipulated, with all older males being 'operated' while all younger males were 'sham-operated'. In operated older males, the androconia (wing scales releasing pheromones) were surgically removed with fine scissors and a precise punching tool under carbon dioxide anaesthesia on the day of adult eclosion. This procedure basically blocks the production of male sex pheromone 2, while male sex pheromones 1 and 3 may still be produced at least to some extent (Nieberding et al. 2008; Karl et al. 2013). Sham-operated younger males were treated in the same way, except that a part of the wing nearby the androconia was removed, thus allowing for a normal production of male sex pheromones. Importantly, earlier results showed that the pheromone blends of older operated males and younger sham-operated males are very similar, although male sex pheromone 1 and 3 amounts remained significantly higher in older than in younger males (Karl et al. 2013). Thus, in this experiment, females should be unable to distinguish between older and younger males based on their pheromone blend regarding male sex pheromone 2, even if olfaction blocking is not fully efficient. We here recorded male mating success, the number of courtship attempts, courtship duration, time to copulation and copulation duration.

#### Experiment 3

In the third experiment, we investigated to what extent males are choosy if offered females of different age. Each mating trial ( $n = 159$ ) involved one male and two females, using two treatment groups: (1) older males (10–12 d) confronted with a younger (2 d) and an older (10–12 d) female; (2) younger males (2 d) confronted with a younger (2 d) and an older (10–12 d) female. In general, males should

preferentially mate with younger females promising higher fitness returns (Chambers & Klownen 2001; Srivastava & Omkar 2004; Milonas & Andow 2010). The residual reproductive value hypothesis, however, predicts that older males should be less choosy than younger ones. Consequently, we predict that younger males should prefer younger females, while older males should not or only weakly discriminate between female age classes. The general set up was as described above, and we here recorded male mating success, age of mating partner, the number of courtship attempts and courtship duration.

### Statistical Analyses

Frequencies of successful matings for older vs. younger males and older vs. younger females were tested against even distributions using  $\chi^2$ -tests. As data did not meet ANOVA requirements, Mann–Whitney *U*-tests were used to analyse differences in courtship attempts, courtship duration and the number of courtship attempts rejected by females in relation to age. To analyse variation in time to copulation and copulation duration, two-factorial analyses of variance (ANOVAs) with male age and female treatment as fixed factors (control vs. olfaction-blocked) were performed. Throughout only significant interaction terms are reported. All statistical tests were performed using Statistica 8.0 (StatSoft Inc.).

## Results

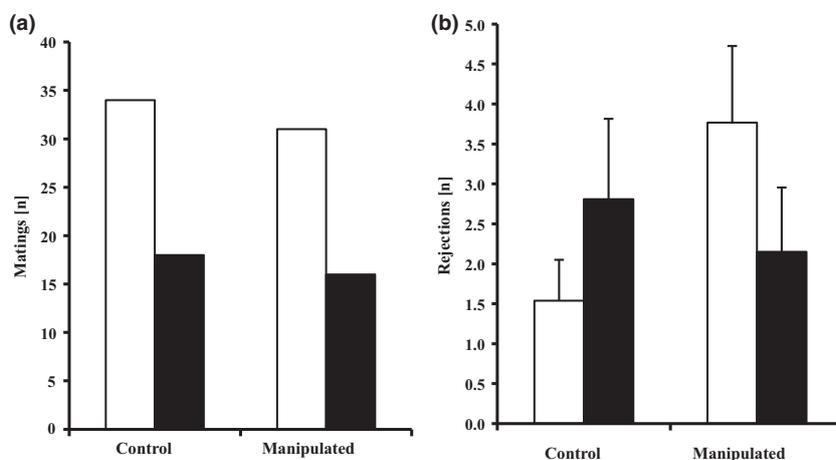
### Experiment 1

When using control females, 52 of 54 mating trials were successful in yielding a mating (96.3%), while the success rate was only 53.4% (47 out of 88 trials)

when using olfaction-blocked females ( $\chi^2_1 = 39.51$ ,  $p \leq 0.0001$ ). Older males had a significantly higher mating success than younger males in both the treatment involving control females ( $\chi^2_1 = 4.92$ ,  $p = 0.0265$ ) and in the treatment involving olfaction-blocked females ( $\chi^2_1 = 7.79$ ,  $p = 0.0287$ ; Fig. 1a). Considering the successful mating trials, there were no significant differences in the number of courtship attempts rejected by females performed by older or younger males in the treatment with control females ( $Z = 0.45$ ,  $p = 0.6550$ ) or in the treatment with olfaction-blocked females ( $Z = 1.09$ ,  $p = 0.2753$ ; Fig. 1b). Taking additionally unsuccessful mating trials ( $n = 47 + 41$ ) into account, blocked females rejected old males significantly more often than younger males ( $Z = 2.53$ ,  $p = 0.0114$ ). Pooled across male age classes, blocked females did not reject males more often than control females ( $Z = 0.83$ ,  $p = 0.4060$ ). Male age and female treatment did not affect time to copulation ( $F_{1,95} = 1.84$ ,  $p = 0.1785$ ;  $F_{1,95} = 0.94$ ,  $p = 0.3355$ ) or mating duration ( $F_{1,95} = 2.80$ ,  $p = 0.0975$ ;  $F_{1,95} = 0.10$ ,  $p = 0.7556$ ).

### Experiment 2

As above, more successful matings were recorded when using control (89.5%; 68 of 76 trials) compared with olfaction-blocked females (71.1%; 54 of 76). Compared to younger sham-operated males, older operated males had a significantly higher mating success in both control ( $\chi^2_1 = 23.53$ ,  $p < 0.0001$ ) and olfaction-blocked females ( $\chi^2_1 = 21.41$ ,  $p < 0.0001$ ; Fig. 2a). *U*-tests revealed significantly more courtship attempts in older operated vs. younger sham-operated males in both control ( $Z = 5.05$ ,  $p < 0.0001$ ) and olfaction-blocked treatment ( $Z = 4.95$ ,  $p < 0.0001$ ; Fig. 2b). Furthermore, older males courted for



**Fig. 1:** Male mating success (a) and the number of male courtship attempts rejected by females (b;  $\bar{x} + 1$  SE) of older vs. younger *Bicyclus anynana* males competing for control or manipulated (= olfaction-blocked) females. Open bars: old males; filled bars: young males.

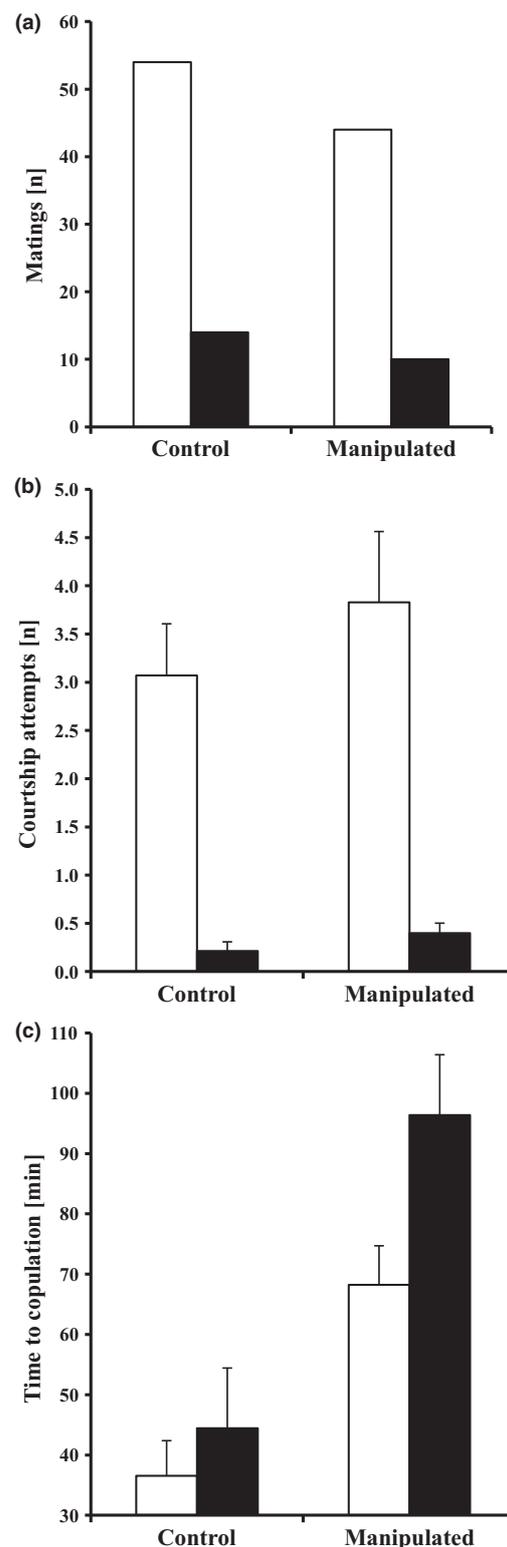
significantly longer time bouts than their younger counterparts (control:  $23.18 \pm 2.78$  vs.  $1.64 \pm 2.78$  s;  $Z = 4.97$ ,  $p < 0.0001$ ; olfaction-blocked:  $40.97 \pm 6.02$  vs.  $3.72 \pm 6.02$  s;  $Z = 5.22$ ,  $p < 0.0001$ ). Time to copulation differed significantly between treatment groups, as both younger and older males mated significantly sooner with control compared to olfaction-blocked females (young:  $44.43 \pm 11.48$  vs.  $96.40 \pm 13.58$  min; old:  $36.54 \pm 5.84$  vs.  $68.23 \pm 6.48$  min;  $F_{1,118} = 17.85$ ,  $p < 0.0001$ ). Older males tended to mate earlier than younger ones irrespective of female treatment. ( $F_{1,118} = 3.32$ ,  $p = 0.0711$ ; Fig. 2c). Copulation duration differed significantly among female treatment groups (control:  $26.16 \pm 1.95$  vs. olfaction-blocked:  $33.40 \pm 2.29$  min;  $F_{1,114} = 5.78$ ,  $p = 0.0179$ ), and between older operated vs. younger sham-operated males (old:  $24.09 \pm 1.35$  vs. young:  $35.46 \pm 2.69$  min;  $F_{1,114} = 14.26$ ,  $p = 0.003$ ). A significant female treatment by male age interaction indicated that younger sham-operated males mated longer with olfaction-blocked females than with control females ( $44.50 \pm 4.11$  vs.  $26.42 \pm 3.47$  min), which was not the case for older operated males ( $25.89 \pm 1.79$  vs.  $22.29 \pm 2.03$  min;  $F_{1,114} = 12.94$ ,  $p < 0.001$ ).

### Experiment 3

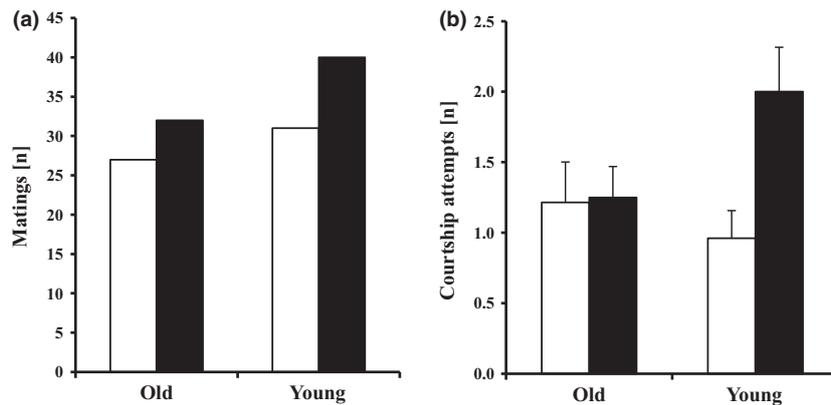
When using older males, 59 of 79 mating trials were successful (74.7%), and when using younger males, 71 of 80 trials (88.8%). Neither in older ( $\chi^2_1 = 0.42$ ,  $p = 0.5151$ ) nor in younger males ( $\chi^2_1 = 1.14$ ,  $p = 0.2855$ ) did mating success differ among female age classes (Fig. 3a). Courtship attempts ( $Z = -0.82$ ,  $p = 0.4126$ ) and courtship duration ( $Z = -1.24$ ,  $p = 0.216$ ) did not differ significantly across female age classes in older males, while younger males courted younger females significantly more often ( $Z = -2.37$ ,  $p = 0.0179$ ; Fig. 3b) and for longer time bouts ( $35.4 \pm 6.28$  vs.  $14.68 \pm 6.28$  s;  $Z = -2.51$ ,  $p = 0.0119$ ) than older females.

### Discussion

Our results yielded support for the residual reproductive value hypothesis, as (1) older males outperformed younger ones regardless of whether females were olfaction-blocked or not and (2) regardless of whether males exhibited differences in pheromone blend or not (experiments 1 and 2), and (3) as older males courted females more often and for longer time bouts than their younger counterparts (in experiment 2) when competing for a female. Thus, information



**Fig. 2:** Male mating success (a), courtship attempts (b), and time to copulation (c;  $\bar{x} + 1$  SE) of older, operated vs. younger, sham-operated *Bicyclus anynana* males competing for control or manipulated (= olfaction-blocked) females. Open bars: old males; filled bars: young males.



**Fig. 3:** Mating success (a) and courtship attempts (b;  $\bar{x} + 1$  SE) of older and younger males confronted with a younger and an older female. Open bars: old females; filled bars: young females.

provided by sex pheromones is not necessary for old male mating advantage to occur.

In experiment 2, any effects of pheromone blend were rigorously controlled for by blocking female olfaction and additionally by using males which smell very similar in spite of differences in age (Karl et al. 2013). The fact that this experiment yielded qualitatively identical results to experiment 1 suggests that male sex pheromone 2 is necessary to increase the mating success of older males in *B. anynana*, although under more natural conditions *B. anynana* females may use sex pheromone composition to assess male quality (Costanzo & Monteiro 2007; Nieberding et al. 2008, 2012; Van Bergen et al. 2013). In fact, the lower mating rate and the longer time to copulation (in experiment 2) in the trials involving olfaction-blocked females rather than control females suggest that female manipulations were highly effective, and that olfaction per se matters in terms of mating success. However, the higher mating success of older males was associated with male courtship activity, although this does not exclude that female choice also contributes to the higher mating success of older males compared to younger ones (Costanzo & Monteiro 2007; Nieberding et al. 2008, 2012; Karl et al. 2013; Van Bergen et al. 2013).

Overall, we found no support for a decisive role of female choice based on pheromone blend in our experiments. If female choice were the most important mechanism underlying old male mating advantage, females should more often reject courting younger as compared with preferred older males, which was not the case. Mating with older males is detrimental to females, challenging the notion that females should prefer older males at all (Karl & Fischer 2013). On principle old females have less time left for oviposition and simultaneously a much higher number of mature oocytes in their abdomen (as oogenesis is a gradual process starting upon adult

eclosion in *B. anynana*; Fischer et al. 2004; Steigenga & Fischer 2007). As a consequence, they should be more eager to mate than younger ones. Although older females consequently need sperm to fertilize their eggs more urgently than younger females, the former did not gain a higher mating success than the latter. We therefore conclude that, in the context of old male mating advantage, mating decisions in *B. anynana* are primarily driven by male behaviour. This does of course not rule out that females exhibit mate choice by preferentially mating with more active and aggressive males, but males initiate mating by courting females (Karl et al. 2013). In line with this notion, old male mating advantage seems to result from the older males' higher intrinsic motivation and eagerness to mate, as evidenced by longer and more frequent courtship attempts (cf. Fischer et al. 2008; Janowitz & Fischer 2010, 2012; Karl et al. 2013).

When given the choice between a younger and an older female in experiment 3, males did not mate more often with the former, which should promise higher fitness returns (Chambers & Klowden 2001; Srivastava & Omkar 2004; Milonas & Andow 2010). While this was expected for more time-constrained older males, we had predicted that younger males would prefer younger females. Although younger females indeed received a few more matings (Fig. 3), this effect was not significant, probably indicating that it is more beneficial for males to mate with any receptive female rather than being choosy, especially because males are able to mate repeatedly (Steigenga et al. 2005). However, younger males at least courted younger females more often and for longer time bouts, which is line with our *a priori* predictions. Note here that the mating trials involving younger males were overall slightly more successful than those involving older males (89% vs. 75% successful mating trials), confirming that a lower mating success of younger males when in competition with older males

is not caused by a lack of sexual maturity or a concomitant inability to mate at this age (Larsdotter Mellström et al. 2012; Karl & Fischer 2013).

Consistent with earlier studies (Janowitz & Fischer 2010, 2012; Karl et al. 2013), we found that copulation duration did not differ across male age classes in experiment 1, although it was shorter for older males in experiment 2. One possible explanation for the latter is that older virgin males, which typically provide larger spermatophores containing more fertile sperm cells compared to younger males (Kehl et al. 2013), are able to form a spermatophore more quickly. Producing larger spermatophores with higher sperm numbers by older virgin males is irrespective of the fact that mating with older males is detrimental to females, which might be related to sperm quality (Karl & Fischer 2013; Kehl et al. 2013).

In summary, we here show that older male *B. anynana* gain a higher mating success than younger ones, even if differences in pheromone blend are controlled for by blocking female olfaction and manipulating male sex pheromone production. We thus show that old male mating advantage in *B. anynana* is associated with male activity and reject the hypothesis that it is proximately related to female preference based on pheromone blend. As older males were more active and aggressive during courtship than younger ones, our results support the residual reproductive hypothesis and a direct link between mating success and male activity (Fischer et al. 2008; Karl & Fischer 2013; Karl et al. 2013). However, we cannot rule out an involvement of additional factors, such as visual cues (Velando et al. 2006; Costanzo & Monteiro 2007; Verburg et al. 2011). Our study indicates that male behaviour may be important for mating decisions even in systems in which the operational sex ratio is biased towards males, where female choice is expected (as females rarely mate more than once while males can mate many times; Brakefield et al. 2001; Janowitz & Fischer 2010, 2012). As male aggression and activity itself might be an important cue for female choice, it is evidently difficult to distinguish between the effects of male and female behaviour, as both are expressed simultaneously. Being amenable to experimental manipulations, the *B. anynana* system holds great potential to resolve the recurrent issue in behavioural ecology of the relative contribution of males and females in mate choice.

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# Manuscript II

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# Old-male paternity advantage is a function of accumulating sperm and last-male precedence in a butterfly

TOBIAS KEHL, ISABELL KARL and KLAUS FISCHER

Zoological Institute & Museum, Johann-Sebastian-Bach Str. 11/12, 17489 Greifswald, Germany

## Abstract

Old-male mating advantage has been convincingly demonstrated in *Bicyclus anynana* butterflies. This intriguing pattern may be explained by two alternative hypotheses: (i) an increased aggressiveness and persistence of older males during courtship, being caused by the older males' low residual reproductive value; and (ii) an active preference of females towards older males what reflects a good genes hypothesis. Against this background, we here investigate postcopulatory sexual selection by double-mating *Bicyclus anynana* females to older and younger males, thus allowing for sperm competition and cryptic mate choice, and by genotyping the resulting offspring. Virgin females were mated with a younger virgin (2–3 days old) and afterwards an older virgin male (12–13 days old) or vice versa. Older males had a higher paternity success than younger ones, but only when being the second (=last) mating partner, while paternity success was equal among older and younger males when older males were the first mating partner. Older males produced larger spermatophores with much higher numbers of fertile sperm than younger males. Thus, we found no evidence for cryptic female mate choice. Rather, the findings reported here seem to result from a combination of last-male precedence and the number of sperm transferred upon mating, both increasing paternity success.

**Keywords:** eupyrene sperm, old-male mating advantage, postcopulatory sexual selection, residual reproductive value, sexual conflict

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## Introduction

Individual fitness is intimately related to successful reproduction, that is, the number and proportion of gene copies passed on to the next generation (Arnqvist & Rowe 2005). Consequently, sexual selection is expected to favour traits that increase the reproductive output (Arnqvist & Rowe 2005). In males, sexual selection may favour (i) traits that increase the number of matings (pre-copulatory sexual selection) and/or (ii) sperm competitive ability (postcopulatory sexual selection; Trivers 1972; Arnqvist & Rowe 2005; Wong & Candolin 2005). Male mating success is throughout the animal kingdom often related to resource holding potential, proxies of

which include body mass, fat content and in flying insects wing wear (Marden & Waage 1990; Kemp & Wiklund 2001; Lappin *et al.* 2006), although that does not always seem to be the case (Kemp & Wiklund 2001; Fischer 2007; Janowitz & Fischer 2010).

Regarding postcopulatory sexual selection, sperm may compete for access to oocytes just as males compete for mates (Arnqvist & Rowe 2005; Parker & Pizzari 2010), with fertilization success being affected by a large array of factors. First, mating order may play a significant role, with possible outcomes including first-male precedence, sperm mixing and last-male precedence. In insects, last-male precedence appears to be the dominant pattern, that is, the last male to mate typically sires most offspring (Walker 1980; Watt *et al.* 1985; de Jong *et al.* 1998; Wedell & Cook 1998; Platt & Allen 2001). Second, paternity success typically increases with the

Correspondence: Tobias Kehl,  
E-mail: tobias.kehl@uni-greifswald.de

number of sperm transferred upon mating, in turn selecting for larger body and spermatophore mass (for insects e.g. Bissoondath & Wiklund 1997; Wedell & Cook 1998; Gage & Morrow 2003; Solensky & Oberhauser 2009). In general, spermatophore size, sperm number and quality are predicted to decrease with the growing age in insects, mainly due to prior matings (Kaitala & Wiklund 1995; Cook & Wedell 1996; Wedell & Ritchie 2004; Marcotte *et al.* 2005; Damiens & Boivin 2006), while extended periods between or prior to mating cause larger ejaculates due to sperm accumulation (Oberhauser 1988; Lehmann & Lehmann 2009; Vande Velde *et al.* 2011, 2012). Such examples suggest that reproductive interests of males and females are often diverging, causing sexual conflict (Arnqvist & Rowe 2005). Concomitantly, females will also try to maximize their individual fitness by mating with males that promise direct or indirect fitness benefits, resulting in female mate choice (e.g. Wong & Candolin 2005; South & Lewis 2012). Thus, male fertilization success may finally depend on female cryptic mate choice, biasing paternity towards preferred males (Edvardsson & Arnqvist 2000; Parker & Pizzari 2010; Watanabe & Sasaki 2010).

Against the above-mentioned background, we here investigate postcopulatory sexual selection in the tropical butterfly *Bicyclus anynana*. This species shows the intriguing pattern of old-male mating advantage, resulting in an up to four times higher mating success in older compared with younger males (Fischer *et al.* 2008). Old-male mating advantage prevails in several other insects such as Fungus beetles (*Bolithotherus cornutus*, Conner 1989), crickets (Zuk 1988; Simmons & Zuk 1992), the potato tuber moth (*Phthorimaea operculella*, Cameron *et al.* 2005) and the Mexican fruit fly (*Anastrepha ludens*, Perez-Staplez *et al.* 2010). These findings are especially interesting for holometabolic insects with finite growth, in which senescence basically starts at adult eclosion and which heavily depends on the resources gathered during the larval phase (Boggs & Freeman 2005). Consequently, older males are expected to show diminished rather than increased performance.

In *B. anynana*, older males gain a higher mating success despite clearly poorer condition, as evidenced by a decrease in body mass, fat content and wing wear with increasing age (Fischer *et al.* 2008). Two hypotheses have been put forward to explain the counter-intuitive pattern. One suggests that male mating success depends on aggressiveness and willingness to persist during courtship (Fischer 2006; Geister & Fischer 2007; Fischer *et al.* 2008; Karl & Fischer 2013; Karl *et al.* 2013), being ultimately driven by the older males' low residual reproductive value. Note that younger males pay generally greater costs than older ones when being killed or injured during mating or courtship (Parker 1974;

Enquist & Leimar 1990; Kemp 2002, 2006). The alternative hypothesis suggests that females actively prefer older males, which have proven a high fitness in terms of survival, potentially reflecting 'good genes' (e.g. Mays & Hill 2004; Johnstone *et al.* 2009). Proximally, old-male mating advantage may, under this scenario, be based on age-specific changes in sex pheromone profiles (Nieberding *et al.* 2008, 2012). Note that the latter hypothesis suggests cooperation, while the former may result in sexual conflict, for which there is indeed some evidence as females having mated with older males exhibit reduced egg hatching success (Karl & Fischer 2013).

While the phenomenon of old-male mating advantage has been extensively analysed in *B. anynana*, nothing is known about the according paternity success, that is, postcopulatory sexual selection. We here address this issue by double-mating *B. anynana* females to older and younger males, thus allowing both sperm competition and cryptic mate choice, and by genotyping the resulting offspring. We specifically aim to test for any biases in paternity success across male age classes. If old-male mating advantage indeed results from sexual conflict and is therefore detrimental to females, females may preferentially use the sperm of younger males. If, on the other hand, females actively prefer older males for mating, they may also preferentially use the sperm of these males. In addition to such biases in sperm use, paternity success may be affected by mating order and sperm number/ejaculate size. These issues are also addressed here by scoring, along with sire identity, spermatophore size and the number of eupyrene (fertile) sperm in older vs. younger males, as these traits may causally underlie differences in paternity success (e.g. Rutowski *et al.* 1987; Bissoondath & Wiklund 1996; Wedell & Cook 1998). For reasons given above, we predict that those males that produce larger ejaculates and are the last to mate have a paternity advantage. Irrespective of that we expect that females bias paternity towards preferred, that is, younger males, which would be indicative of sexual conflict as older males generally have a higher mating success.

## Material and methods

### Study organism

The Squinting Bush Brown *Bicyclus anynana* (Butler, 1879) is a medium-sized butterfly belonging to the family Nymphalidae. Its distribution stretches from southern Africa to Ethiopia (Larsen 1991). *Bicyclus anynana* is a savannah-adapted butterfly, which mainly occurs in open habitats but prefers to rest in the shade of trees

and bushes (Larsen 1991). Larvae feed on a wide spectrum of Poaceae grasses, while adults feed on a variety of fallen and decaying fruit including that from *Ficus* trees (Larsen 1991; Brakefield 1997). This butterfly shows striking phenotypic plasticity (two seasonal morphs) as an adaptation to alternate wet- and dry-seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen *et al.* 2004). A laboratory stock population was established at Greifswald University, Germany, in 2007 based on several hundred individuals derived from the stock population of Bayreuth University, Germany (Kehl & Fischer 2012). The latter population was in turn derived from a well-established stock population at Leiden University, the Netherlands, which was founded in 1988 from 80 gravid females caught at a single locality in Malawi. In each generation, >1000 individuals are reared, all of which are used as parents. This procedure maintains high levels of heterozygosity at neutral loci (Van't Hof *et al.* 2005). For this study, butterflies from the Greifswald stock population were used.

#### *Butterfly rearing and experimental design*

Throughout, all larvae were fed on potted maize plants, being replaced as necessary, while butterflies were provided with moist banana and water *ad libitum*. Unless otherwise stated, rearing and maintenance took place in a climate cell set at 27 °C, 70% relative humidity and a light/dark cycle of 12 h:12 h. To initiate this experiment, two cohorts of approximately eight hundred eggs each were collected from the stock population. To produce two age classes of younger (2–3 days) and older (12–13 days) males, the second cohort was collected 10 days later than the first one. A difference in lifespan of 10 days is considered to be ecologically relevant in this species (Brakefield & Reitsma 1991; Fischer & Fiedler 2001; Fischer *et al.* 2008). To control confounding effects of female age, all females used for egg-laying were 2–4 days old. Thus, both cohorts were produced by different females, all originating from the same stock population. To ensure having sufficient young females available 10 days apart, eggs were collected over an extended period of time in the preceding generation, and larvae were reared at 20 °C resulting in a prolonged period of adult eclosion.

For oviposition, ca. 100 fecund females were placed within each hanging cage being provided with a potted maize plant as oviposition substrate, which was transferred to a population cage for rearing after 3 days of oviposition. Larvae hatched after 4–5 days at 27 °C. Larvae were reared throughout in population cages (50 × 50 × 50 cm), using three cages per cohort. Result-

ing pupae were collected daily from the cages and transferred to cylindrical hanging cages (30 × 39 cm). Males and females were separated on their eclosion day, on which male *B. anynana* do not yet mate. Once a sufficient number of butterflies from the first cohort were available, virgin males and females were set up for mating in a hanging cage. The hanging cage was monitored continuously, and mating couples were immediately removed and individually transferred to 1-L translucent plastic containers covered by gauze. After mating, males were removed from the containers, and 60 mated females were kept individually. They were each provided with a fresh maize leave as oviposition substrate and moist banana for adult feeding to produce full-sib families. The same procedure was repeated 10 days later with the second cohort. Eggs from both cohorts were kept separated by female and transferred to elongated, sleeve-like gauze cages. Each such cage consequently hosted one full-sib family. Resulting butterflies were marked individually and transferred, separated by sex, to hanging cages.

Using the above-mentioned butterflies, two female mating groups were set up to investigate the paternity success of older vs. younger males. Young virgin females from the second cohort were either mated with an older male first (12–13 days, from the first cohort) and on the following day with a younger male (2–3 days, from the second cohort) or vice versa. Throughout the experiment, we decided to use virgin males in order to be able to link the results of the present study to earlier ones on old-male mating success in *B. anynana* (Fischer *et al.* 2008; Karl & Fischer 2013; Karl *et al.* 2013). We thus employed the same design as in earlier studies. Matings took place on days 2 and 3 of the females' adult life in cylindrical hanging cages. All females were mated to unrelated males, which was ensured by taking the female and both males from different full-sib families. Per treatment group, only one female and one male per family were used. Cages were continuously monitored, and mating couples were immediately removed and individually transferred to 1-L translucent plastic containers covered by gauze. After mating, males were removed from the containers and frozen at –80 °C for later paternity analysis. Twenty successfully double-mated females per treatment group were kept and were, after the second mating, set up individually for oviposition as detailed above. From each female, early (oviposition days 1–4) and late eggs (oviposition days 8–12) were collected, after which the females were also stored at –80 °C. Early and late eggs of individual females were kept separated and reared in cages to adulthood. Resulting butterflies (ca. 30 offspring per female in total) were frozen at –80 °C for later paternity analysis on their eclosion day.

**Table 1** Overview of the eight microsatellite loci used for paternity assignment including marker name, literature source, primer sequences, allele size range (in base pairs) and allele count

Marker; Source	Primer (5'–3')	Size range	Allele count
BACA12*; Van't Hof <i>et al.</i> (2005)	F-AGCGGTAACATTTTTTCCTCAGTT R-GCCGACGTGTTCCACACA	255–332	8
BACA8*; Van't Hof <i>et al.</i> (2005)	F-TCCATGGTTACAACGGCTTACC R-ACAATAATTCTGACATGCAAAAATATGAA	227–247	4
BAGA2*; Van't Hof <i>et al.</i> (2005)	F-CAATGACCCGCTCCCCTC R-GTAGGTTTTGTAAAAGTCATTGTTGTAGC	188–210	4
BAATG208*; Van't Hof <i>et al.</i> (2005)	F-GAGGAGACTCGAGCTCAACAGTG R-TCGACTACGAACGATTTTTGGAT	108–249	9
BACA2; Van't Hof <i>et al.</i> (2005)	F-AAATCAACAGCGTTACCAAG R-GCGACTAGCGGAAACTA	222–309	10
BACA7; Van't Hof <i>et al.</i> (2005)	F-TCCGCGTCTGTACCCGTAGA R-TCAGTAGCCGACGCGAAAAAG	135–286	9
BEL1373; Beldade <i>et al.</i> (2008)	F-CACAGGCCAGTATTATGTAAGAGA R-TTCATCATAGTGGGTACAACAAAAA	152–160	6
BEL2339; Beldade <i>et al.</i> (2008)	F-TGTTGAAAACAAAATTAACAAAAACA R-TCTGGATCCTGTAACAAACGTG	125–135	5

\*Redesigned.

### Paternity analysis

For paternity assignment, eight microsatellite markers were used (Table 1). We analysed 7–10 early and late offspring each per female as well as the two possible fathers and the mother. Three hundred and fifty-eight of 380 offspring could be unambiguously assigned to a specific father. The DNA that was used from the individuals for paternity analyses was extracted using the Macherey Nagel Food Kit on silica membrane columns, as specified by the manufacturer. Resulting products were diluted with distilled water (1:10) prior to polymerase chain reactions (PCRs). The PCR conditions for this analysis were as follows: 12 min at 96 °C, 35 cycles of 30 s each at 96 °C, 30 s at 60 °C, 30 s at 72 °C, 10-min extension at 72 °C in 22.05 µL solution containing 19.2 µL water, 0.3 µL *Taq* polymerase (AmpliQ Gold; ABI/Lifetech; 5 U/µL), 2.5 µL PCR buffer (Gold-STR 10X; Promega) and 0.05 µL of each primer (100 pmol). PCRs were set up with three primers each, two marker-specific primers and one 5'-FAM-marked M13 primer, using five different primer mixes: Mix 1 – BEL1373 & BACA8; Mix 2 – BEL2339, BAGA2, BACA12; Mix 3 – BACA7; Mix 4 – BACA2; and Mix 5 – BAATG208 (cf. Table 1). PCR products were separated using capillary electrophoresis (ABI XL 3130 Genetic Analyser; Applied Biosystems) on 36 cm capillaries, POP4 polymer and the size standard ILS 600 (Promega). Data were initially analysed with Gene Scan/Genotyper software (ABI). PCR analyses and redesigning primers (Table 1) were performed by Eurofins Medigenomix GmbH, Ebersberg, Germany. The automatically analysed

data were independently verified and, if necessary, corrected by T.K. and I.K.

### Spermatophore mass and fertile sperm numbers

To analyse spermatophore mass and sperm numbers of old vs. young males, an additional set of 2-day-old virgin females was mated to either 2- or 10- to 12-day-old males. Females were frozen in liquid nitrogen immediately after the mating had been ceased, preventing sperm from dispersing to the spermatheca. To remove the bursa copulatrix, which contains the male spermatophore, females were thawed and dissected in a Ringer's solution for insects (9 g NaCl, 0.25 g MgCl<sub>2</sub>, 0.2 g KCl, 1 g glucose, 1 L aqua dest., pH 6.8). Afterwards, surplus Ringer's solution was removed from the bursa using filter paper. The bursa was then weighed on an electrobalance (Sartorius LE225D) to the nearest 0.01 mg. Thus, the mass of the bursa copulatrix containing the spermatophore (hereafter referred to as spermatophore) was used as a proxy of spermatophore mass, as the mass of the bursa is negligible and as both masses are tightly correlated (Cook & Wedell 1996). After weighing, the spermatophore was transferred to a cavity slide with a droplet of Ringer's solution, opened with forceps and stirred gently to disperse the sperm. Eupyrone (fertile) sperm bundles were counted within the cavity slide using a microscope (at ×40). To calculate the number of eupyrone sperm, the number of bundles was multiplied by 256, owing to the fact that in Lepidoptera, all the sperm in a bundle originate from a single spermatogonium, which undergoes a fixed

number of eight divisions (Virkki 1969; Cook & Wedell 1996, 1999; Fischer 2007).

*Statistical analyses*

Paternity success of older vs. younger males was analysed separately for each treatment group (i.e. OY early offspring, OY late offspring, YO early offspring, YO late offspring) by comparing the distribution of offspring sired by old vs. young males against even distributions with chi-square tests. Additionally, paternity success across treatment groups (females mated to an old male first and then a young one (OY) or vice versa (YO)) was investigated for early and late offspring using a nominal logistic regression on offspring sired by old vs. young males. Differences in spermatophore mass and fertile sperm numbers across male age classes were tested using one-factorial ANOVAS with male age as fixed factor. All tests were performed using JMP 7.0.1 (SAS Institute) or Statistica 8.0 (StatSoft).

**Results**

*Paternity analysis*

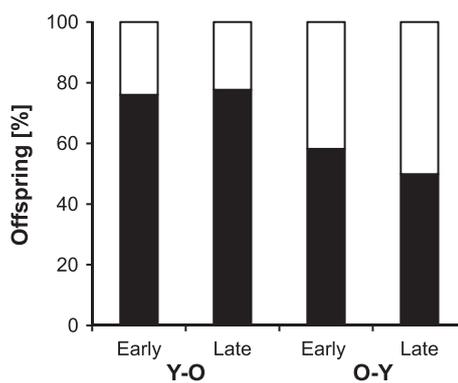
For paternity analysis, 358 offspring from 19 females were used in total (Table 2). Regarding the mode of sperm use, all possible outcomes were found. The numerically dominating pattern was sperm mixing, which was found in 11 of 19 or in 17 of 38 (if considering early and late offspring separately) cases. However, note that in most cases of sperm mixing, last males sired more offspring than first males (in 9 of 11 or in 12 of 17 cases; Table 2). Complete last-male precedence was found in 4 (or 13 of 38) cases, complete first-male precedence in 4 (or 8 of 38) cases. Effects of male age were evident in the group of females having mated with a younger male first. Here, older males had a significantly higher paternity success than younger males (early offspring: 82 vs. 24;  $\chi^2_1 = 17.2, P < 0.0001$ ; late offspring: 80 vs. 23;  $\chi^2_1 = 17.2, P < 0.0001$ ). In contrast, paternity success was equal between older and younger males for females having mated with an older male first (early offspring: 44 vs. 31;  $\chi^2_1 = 1.1, P = 0.2851$ ; late offspring: 38 vs. 36;  $\chi^2_1 = 0.03, P = 0.8694$ ; Fig. 1). Consequently, older males only had a higher paternity success in females having mated with a younger male first, but not in females having mated with an older male first (Fig. 1). Accordingly, the nominal logistic regression indicated that paternity success of older vs. younger males differed significantly among female treatment groups ( $\chi^2_1 = 20.1, P < 0.0001$ ), but with no difference between early and late offspring ( $\chi^2_1 = 0.4, P = 0.5520$ ; interaction:  $\chi^2_1 = 0.5, P = 0.5020$ ).

**Table 2** Number of early and late offspring sired by younger (Young) vs. older (Old) males for females having mated with a younger male first and then with an older male (Y-O) or vice versa (O-Y).% old: percentage of all (i.e. early and late) offspring sired by the respective older male

Female	Early offspring		Late offspring		% old
	Young	Old	Young	Old	
Y-O 1	0	10	0	10	100.0
Y-O 2	6	4	4	5	47.4
Y-O 3	0	10	1	9	95.0
Y-O 4	8	0	10	0	0.0
Y-O 5	1	9	2	8	85.0
Y-O 6	3	6	2	5	68.8
Y-O 7	0	10	1	9	95.0
Y-O 8	5	5	1	7	66.7
Y-O 9	0	10	0	10	100.0
Y-O 10	1	8	2	8	84.2
Y-O 11	0	10	0	9	100.0
Sum	24	82	23	80	

Female	Early offspring		Late offspring		% old
	Old	Young	Old	Young	
O-Y 1	10	0	9	0	100.0
O-Y 2	7	3	0	8	38.9
O-Y 3	6	1	8	1	87.5
O-Y 4	0	10	1	9	5.0
O-Y 5	10	0	10	0	100.0
O-Y 6	10	0	10	0	100.0
O-Y 7	1	9	0	10	5.0
O-Y 8	0	8	0	8	0.0
Sum	44	31	38	36	



**Fig. 1** Paternity success of older vs. younger males across female treatment groups (Y-O: female mated first to a young male, then to an old male; O-Y: vice versa) for early and late offspring. Black bars: offspring sired by old males; open bars: offspring sired by young males. Sample size was 11 (Y-O) and 8 (O-Y) females. Per female, 7–10 early and late offspring each were used ( $n_{total} = 358$  offspring).

### Spermatophore mass and fertile sperm numbers

Older males produced significantly heavier spermatophores compared with their younger competitors ( $F_{1,80} = 7.1$ ,  $P = 0.0091$ ), containing significantly more fertile sperm ( $F_{1,80} = 103.4$ ,  $P < 0.0001$ ; Fig. 2).

### Discussion

In our study, neither older nor younger males had a generally higher paternity success under the mentioned conditions that involved sperm competition. Offspring genotyping revealed that older males were more successful in siring offspring, but only if they were the second mating partner of double-mated females. If, however, younger males were the second mate, paternity success was equal among male age classes. These findings suggest that female *B. anynana* do not exhibit cryptic mate choice by generally preferring males of a given age class, thus providing no conclusive information on whether old-male mating advantage arises from sperm competition or cryptic female choice in our

system. We thus suggest that the paternity advantage of older males, occurring only when older males were the second mates, does not indicate cryptic mate choice. We therefore reject the hypothesis relating to female mate choice. Instead, our findings indicate that a combination of last-male precedence and the number of sperm transferred upon mating is responsible for the obtained pattern.

We thus argue that the higher proportion of offspring sired by older males, when being the last male to mate, is basically a consequence of last-male precedence, what is generally the most common fertilization pattern in butterflies and other insects (Walker 1980; Watt *et al.* 1985; de Jong *et al.* 1998; Wedell & Cook 1998; Platt & Allen 2001). Accordingly, last-male precedence was found to be the most common outcome of sperm competition in *B. anynana*, although first-male precedence and sperm mixing do also occur (Brakefield *et al.* 2001). These earlier findings are in full agreement with our results as we could also observe all three patterns, suggesting that male individual quality has a substantial impact on paternity success.

As, however, younger males did not benefit to the same extent, when being the last male to mate and achieved no advantage in comparison with older males, last-male precedence cannot be the only factor that affects paternity success. We suggest that the larger spermatophores produced by older males, containing much higher numbers of fertile sperm, counteracted the benefits of last-male precedence, thus resulting in an equal fertilization success. This notion is strongly supported by empirical evidence, as in butterflies, paternity success typically increases with body mass (at least if being related to spermatophore mass), with spermatophore mass and with sperm numbers (Rutowski *et al.* 1987; Oberhauser 1988; Bissoondath & Wiklund 1996, 1997; Wedell 1997; Wedell & Cook 1998; Gage & Morrow 2003; Solensky & Oberhauser 2009). While in general spermatophore size, sperm number and quality are predicted to decrease with age (Kaitala & Wiklund 1995; Cook & Wedell 1996; Wedell & Ritchie 2004; Marcotte *et al.* 2005; Damiens & Boivin 2006), we have reported the opposite pattern that is obviously related to the fact that we exclusively used virgin males. Thus, while age per se may cause an increase in spermatophore size and sperm numbers due to accumulation (Lehmann & Lehmann 2009), prior matings reduce both, what also is the case in *B. anynana* (Ferkau & Fischer 2006). Concomitantly, increasing spermatophore size and sperm numbers with age has been reported for several insects (e.g. *Danaus plexippus*, Oberhauser 1988; *Choristoneura rosaceana*, Delisle & Bouchard 1995; *Pararge aegeria*, Vande Velde *et al.* 2011, 2012). Larger ejaculates are not only beneficial to males in terms of sperm

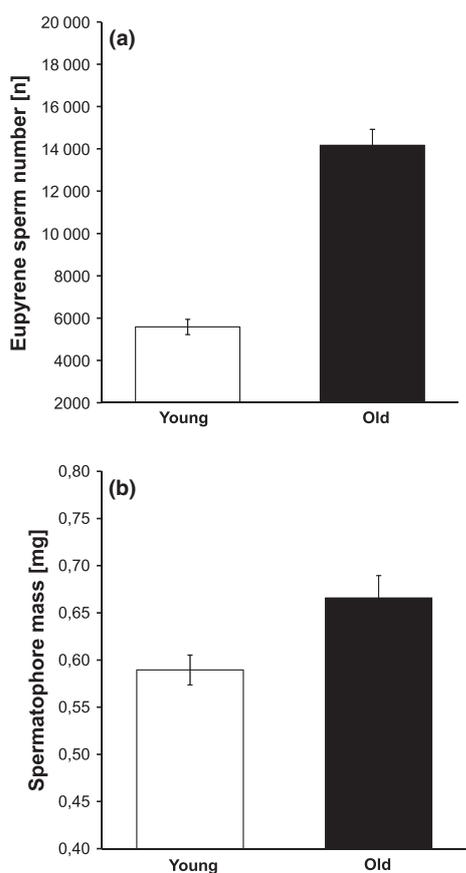


Fig. 2 Spermatophore mass (a) and number of eupyrene (fertile) sperm (b) in younger (open bars,  $n = 41$ ) vs. older (black bars,  $n = 41$ ) *Bicyclus anynana* males.

competition, but also to females, as accessory gland products may increase female reproductive output (e.g. Kaitala & Wiklund 1995; Oberhauser 1997; Wiklund *et al.* 1998; Wedell & Karlsson 2003). However, under field conditions, older males will not be able to generally provide larger ejaculates, as they would have mated previously, at least in some cases. Thus, different patterns may have emerged if old males had had the chance to mate prior to our experiments or if the time since last mating would have been kept constant. Consequently, paternity success may well be equal across age classes if such variation is controlled for.

In summary, several lines of evidence suggest that old-male mating advantage is an example of sexual conflict in *B. anynana* butterflies. Most importantly, females having mated with older compared with younger males show a higher remating propensity and reduced egg hatching success (Karl & Fischer 2013; see also Janowitz & Fischer 2012). Nevertheless, females do not exhibit cryptic mate choice by preferentially using the sperm of younger males, as was shown here. However, we here provide solid evidence that paternity success is a function of last-male precedence and the number of fertile sperm transferred. When females mated with an older male first and then with a younger male, the latter male had the fertilization advantage of last-male precedence, while the older male had the advantage of transferring more sperm per ejaculate. Both effects obviously cancelled each other out, resulting in an equal fertilization success across age classes. When females mated with a younger male first and then with an older male, older males had the advantages of both last-male sperm precedence and sperm accumulation, resulting in a higher fertilization success of older males. Still, the dynamics of sperm production, numbers and quality in relation to age and mating status remain an interesting task for future research, but are beyond the scope of the current contribution.

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K.F. designed the research, T.K. performed the research, T.K., I.K. and K.F. analysed data, and T.K. and K.F. wrote the article.

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#### Data accessibility

Microsatellite data, sperm numbers and spermatophore mass data are available on Dryad doi:10.5061/dryad.3mt25.



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# Manuscript III

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1 **Fat and sassy: factors underlying male mating success in a**  
2 **butterfly**

3

4 **Tobias Kehl\*, Julia Bensch, Friederike Böhm, Björn Ole Kniepkamp, Vivien**  
5 **Leonhardt, Sarah Schwieger & Klaus Fischer**

6

7 Zoological Institute & Museum, Greifswald University, Johann-Sebastian-Bach Str. 11/12,  
8 17489 Greifswald, Germany

9

10 **\*Correspondence:** E-mail: tobias.kehl@uni-greifswald.de

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12 **Running title:** *Factors underlying male mating success*

13

14 *Key words:* *Bicyclus anynana*, flight performance, intrasexual selection, male-male  
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16 condition

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19

1 **Abstract**

2 Theory predicts that male mating success depends on resource holding potential (RHP),  
3 which is reflected by proxies of condition, such as body mass, fat content, strength, or  
4 weaponry. In species lacking any physical means to inflict injuries upon combatants, such as  
5 butterflies, the factors determining mating success are less clear. Against this background we  
6 explored the determinants of male mating success in the tropical butterfly *Bicyclus anynana*  
7 Butler (Lepidoptera: Nymphalidae: Satyrinae), by comparing physiological, immunological,  
8 and morphological traits between winning and losing males. Our results showed that  
9 successful males are characterized by a better flight performance, evidenced by having longer  
10 wings, a heavier thorax, a lighter abdomen, a higher fat content, and higher phenoloxidase  
11 expression levels than their unsuccessful counterparts, when being compared after their first  
12 mating. Males that won three consecutive trials against the same combatant were also  
13 characterized by a better flight performance, having larger forewings, a higher body mass,  
14 and a higher fat content. Thus, successful males were larger and in better condition than  
15 unsuccessful ones. Strikingly, many differences found indicated an enhanced flight  
16 performance for the former, which we suggest ultimately plays the key role for male mating  
17 success in *B. anynana*. As fat is the main energy source for flying insects, being crucial to  
18 flight endurance and in turn presumably to male mating success, it may represent a key  
19 determinant at the proximate level.

20

## 1 **Introduction**

2 Across the animal kingdom, an array of factors is linked to male mating success including,  
3 for instance, the intensity of courtship behaviour, eye-catching ornaments, impressive song,  
4 or condition-dependent traits, such as body size, fat content, or immune status (Candolin,  
5 2005). Various factors associated with an individual's resource holding potential (RHP), such  
6 as strength, weaponry, or agility, increase success in male-male competition and are therefore  
7 often beneficial to male mating success (Andersson & Iwasa, 1996; Mesterton-Gibbons et al.,  
8 1996). The factors underlying RHP vary widely across animal species, depending on the  
9 nature of male-male interactions (Kemp, 2002). If competition for mates involves direct  
10 physical interactions and risk of injury, strength and heavy weaponry are required, whereas in  
11 groups without such direct interactions and/or weaponry (e.g., butterflies and odonates) flight  
12 endurance, vigour, and agility may be more important (Waage, 1988; Kemp, 2002). In the  
13 latter group, energy reserves and energetic efficiency may play a crucial role (Marden &  
14 Waage, 1990; Mesterton-Gibbons et al., 1996).

15 Besides male competitive ability reflected in RHP, female choice is also clearly  
16 important for male mating success (Mays & Hill, 2004). Females frequently mate preferably  
17 with males displaying specific cues such as ornaments, bright colours, or sex pheromones  
18 (Mays & Hill, 2004; Wong & Candolin, 2005; Velando et al., 2006; Costanzo & Monteiro,  
19 2007; Nieberding et al., 2008, 2012; Rutowski and Rayjaguru, 2012). Both mechanisms, male  
20 competitive ability vs. female choice, are not mutually exclusive, as females may mate  
21 preferably with males having a high RHP. Sexually selected cues may covary with traits  
22 reflecting RHP, thus providing honest signals (Velando et al., 2006). For instance, if males  
23 with more vigorous courtship behaviour are more successful than others, this may be due to  
24 female choice directly selecting for increased vigour, or male behaviour per se with females  
25 giving up and mating for 'convenience' in order to avoid further harassment (Parker, 1984).

26 Butterflies have become important models for sexual selection, showing a rich  
27 diversity of presumably sexually selected traits such as colour patterns or odour (Morehouse  
28 and Rutowski, 2010; Cook et al., 2012; Rutowski and Rayjaguru, 2012; Karl et al., 2013).  
29 Costanzo & Monteiro (2007), for instance, showed that both optical and chemical cues were  
30 important for female mate choice. But why should females mate with males showing specific  
31 colour patterns or odours: are there any direct fitness benefits for her or her offspring? Owing  
32 to a lack of weaponry and any obvious means to inflict harm upon combatants, the factors  
33 determining male mating success in butterflies, regardless of whether it is based on male

1 behaviour/capability or female choice, are much less obvious than in other animal groups  
2 (Kemp & Wiklund, 2001). Male-male interactions, however, occur frequently and may  
3 reflect energetic ‘wars of attrition’, in which the amount of storage reserves and overall  
4 condition may be important (Marden & Waage, 1990; Mesterton-Gibbons et al., 1996).  
5 Several studies furthermore suggested an important impact of male motivation, reflected by  
6 aggressiveness and persistence during courtship, on male mating success (Fischer et al.,  
7 2008; Karl & Fischer, 2008; Karl et al., 2013; Kehl et al., 2014).

8         Against this background, we here investigated morphological and physiological  
9 differences that may underlie male mating vigour and/or indicate a higher male fitness to  
10 females, between successful vs. unsuccessful males in mating trials in the tropical butterfly  
11 *Bicyclus anynana* Butler (Lepidoptera: Nymphalidae), the squinting bush brown. Traits  
12 investigated included correlates of body size (body mass and wing length), flight ability  
13 (thorax mass and wing length), condition (fat and protein content and immune status), and  
14 potentially sexually selected signals (eyespot and eyespot pupil size). We predicted more  
15 successful males (1) to be larger, as body size is often related to fitness (Martinez-Lendeck et  
16 al., 2007), (2) to have a better flight ability, providing advantages during flight manoeuvres  
17 (Srygley & Dudley, 1993; Vande Velde et al., 2012), (3) to have a better overall condition,  
18 reflected, e.g., by more storage reserves and/or a higher immune status (Peixoto & Benson,  
19 2008), and (4) to have larger eyespots (Breuker & Brakefield, 2002). We furthermore tested  
20 to what extent male mating success is consistent over time, i.e., whether specific males  
21 repeatedly win mating trials against a specific counterpart.

22         Regarding proxies of condition, we measured fat and protein content, alcohol  
23 dehydrogenase (ADH) activity, and immune function [phenoloxidase (PO) and lysozyme  
24 activity]. ADH is a metabolic enzyme that catalyses the reaction of alcohols to the  
25 corresponding aldehydes and back, and has been shown to affect, e.g., egg hatchability,  
26 survival rate, and development time (Cosmides et al., 1997; Ogueta et al., 2010). This may be  
27 of special importance to *B. anynana* as this butterfly feeds on fermenting fruit. Phenoloxidase  
28 catalyses melanin synthesis, which is needed for tissue repair and defence against pathogens  
29 (Cerenius et al., 2010; Zhao et al., 2011). It is an essential part of extracellular clotting and  
30 cuticle hardening, decreasing infection risk following injuries (Gillespie & Kanost, 1997;  
31 Eleftherianos and Revenis, 2010). Recent studies provide clear evidence that PO is a good  
32 indicator of insect condition, as its synthesis is costly (González-Santoyo & Córdoba-  
33 Aguilar, 2011). Lysozymes, finally, are a class of enzymes which lyse the cell walls of  
34 certain Gram-positive bacteria and inactivate certain viruses (Ibrahim et al., 2001).

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## **Materials and methods**

### **Study organism**

*Bicyclus anynana* is a fruit-feeding butterfly, ranging from southern Africa to Ethiopia (Larsen, 1991). It exhibits striking phenotypic plasticity with two seasonal morphs, which functions as an adaptation to alternate wet-dry seasonal environments and the associated changes in resting background and predation (Lyytinen et al., 2004). Reproduction in this butterfly is essentially confined to the warmer wet season when oviposition plants are abundantly available, and when 2-3 generations occur. During the colder dry season, reproduction ceases and butterflies do not mate before the first rains at the beginning of the next wet season (Brakefield, 1997). A laboratory stock population was established at Greifswald University, Germany, in 2008 from several hundred eggs derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from 80 gravid females caught at a single locality in Malawi. In each generation, several hundred individuals are reared, maintaining high levels of heterozygosity at neutral loci (Van't Hof et al., 2005). For this study, butterflies from the Greifswald stock population were used.

### **Experimental design**

To investigate the factors affecting male mating success, we carried out two experiments for which we collected approximately 1 000 eggs each. Larvae were reared in population cages on potted maize plants [*Zea mais* L. (Poaceae)] at 27 °C, 70% r.h., and L12:D12 photoperiod. The conditions used are similar to those at which *B. anynana* develops and reproduces during the favourable wet season in the field (Brakefield & Reitsma, 1991; Brakefield, 1997). Resulting pupae were collected daily and transferred to cylindrical hanging cages. Individuals were separated by sex on their eclosion day in order to avoid mating prior to experiments. All males and females were kept in groups of about 20 individuals separated by eclosion day, and were supplied with moist banana and water for feeding ad libitum throughout.

*Experiment 1.* On day 3 after adult eclosion, two random males each were set up for mating with one random, 3-day-old virgin female in a cylindrical hanging cage (30 cm diameter, 15 cm high). Afterwards, all cages were monitored for 6 h or until a mating occurred. Following mating, males being successful in gaining a mating were labelled as ‘winners’ and

1 unsuccessful ones as ‘losers’. Immediately after termination of the mating, all males were  
2 placed in a -80 °C freezer for later analyses. In total 50 successful trials were used for  
3 subsequent analyses. The following traits were measured in all males: adult mass, thorax  
4 mass, abdomen mass, protein content, PO activity, ADH activity, lysozyme-like activity, fat  
5 content, forewing length, diameter of the largest eyespot on the forewing, diameter of the  
6 largest eyespot on the hindwing, and diameter of the white pupil in both largest eyespots.

7  
8 *Experiment 2.* In the second experiment, we investigated whether particular males  
9 consistently outperform a given counterpart, or whether mating success changes over three  
10 consecutive matings. Therefore, we performed mating trials as outlined above, though both  
11 males used within a trial were repeatedly set up for mating with a single virgin female on  
12 days 3, 5, and 7 of adult life. Following the first mating trial, each male was individually  
13 numbered on the ventral surface of the hindwing with a permanent marker pen. After the last  
14 trial, all males were transferred to a -80 °C freezer for later analyses. For each trial, the male  
15 gaining the mating was noted. Unsuccessful mating trials were terminated after 6 h. Between  
16 the sequential contests, males were kept in groups of ca. 20 individuals and were fed with  
17 moist banana and water ad libitum. For all males, thorax mass, abdomen mass, protein  
18 content, PO activity, ADH activity, fat content, and forewing length were measured  
19 subsequently. Other traits scored in experiment 1 were not measured here as they had no  
20 measurable impact on mating success (except from protein content needed to assess PO  
21 activity).

## 22 23 **Data collection and biochemical analyses**

24 Frozen butterflies from experiment 1 were first weighed to the nearest 0.01 mg (Sartorius  
25 LE225D; Sartorius, Göttingen, Germany). For butterflies from both experiments, head, legs,  
26 and wings were removed, and thorax and abdomen separated and weighed. Body mass was  
27 calculated as the sum of thorax and abdomen mass for butterflies from experiment 2.

28 Thoraxes, which were used for measuring protein content, PO activity, ADH activity, and  
29 lysozyme-like activity, were transferred to 1.5-ml tubes with 250 µl phosphate buffered  
30 saline (PBS; 11.9 mM Na<sub>2</sub>HPO<sub>4</sub>\*2H<sub>2</sub>O, 137 mM NaCl, 2.7 mM KCl; pH = 7.4). They were  
31 homogenized and centrifuged afterwards at 18 659 g and 4 °C for 20 min.

32 The total protein content was quantified using the Bio Rad protein assay (Bio-Rad  
33 Laboratories, Inc., Hercules, CA, USA), which is based on the Bradford method (Bradford,  
34 1976). Therefore, 1 µl of the hemolymph extract was pipetted into 160 µl distilled water, to

1 which 40 µl Bio Rad solution was added. Following 10 min of incubation at 30 °C, the  
2 absorbance was read at 595 nm with an ELx 808 Absorbance Microplate Reader (BioTek  
3 Instruments, Bad Friedrichshall, Germany). Four (experiment 1) or two (experiment 2)  
4 replicates were measured per individual. Using a concentration series (0-2 mg ml<sup>-1</sup>), a  
5 standard curve was constructed with Albumine Bovine Serum in PBS buffer. The protein  
6 content was quantified using the resulting standard equation and the mean value of  
7 measurements per individual.

8 For PO measurements, 2× 60 µl of the hemolymph extract was pipetted into the wells  
9 of a 96-well plate per individual. Subsequently, 140 µl L-DOPA (1 mM 3,4  
10 dihydroxyphenyl-L-alanine; Fluka Analytica, Sigma-Aldrich, St. Louis, MO, USA) were  
11 added. The optical density (OD) was read at 490 nm and 30 °C for 45 min (90 reads; ELx  
12 808 AMR). The blank samples contained PBS buffer, distilled water, and L-DOPA to control  
13 for a possible increase of the particles in the solutions (Bailey et al., 2010). The respective  
14 values were then subtracted from each individual PO value. For each sample, enzyme activity  
15 was calculated by using the linear slope of the reaction between 15 and 30 min. The mean  
16 value of both measures per individual was used for further analyses.

17 For the measurement of ADH activity, 96-well plates were loaded with 10 µl  
18 supernatant and 190 µl pre-heated reaction solution [30 mM isopropyl (23 µl in 10 ml TRIS  
19 buffer), 3 mM NAD<sup>+</sup> (0.0199 g in 10 ml TRIS buffer), TRIS buffer: 0.1817 g TRIS in 10 ml  
20 distilled water; pH 8.5, 30 °C]. For blank samples, 10 µl PBS was used instead of the  
21 supernatant. Two replicates were measured per individual. Optical density was measured at  
22 340 nm and 30 °C every 15 s for 10 min.

23 To determine lysozyme-like activity, 96-well plates were loaded with 20 µl  
24 supernatant and 80 µl *Micrococcus luteus* (Schröter) Cohn solution (3 mg ml<sup>-1</sup> in PBS) per  
25 individual. Blank samples consisted of 20 µl PBS and 80 µl *M. luteus* solution. Optical  
26 density was measured at 490 nm and 30 °C for 5 h (ELx 808 AMR). By subtracting the final  
27 from the start value, the change of OD over time was determined. Afterwards, the mean of  
28 the blank values was subtracted from the resulting ODs on one plate.

29 For measuring fat content, we followed Fischer et al. (2003) with some minor  
30 changes. We dried the abdomens for 48 h at 60 °C in open 1.5-ml tubes and weighed dry  
31 masses afterwards. Abdomens were then transferred to 4-ml glass tubes, which were  
32 subsequently filled with 2 ml acetone and then closed. After 2 days of fat extraction on a  
33 laboratory shaker at 150 rpm, the solutions were substituted with fresh acetone and the  
34 samples were extracted again for 48 h. Following removal of the acetone solution, abdomens

1 were dried for 48 h at 60 °C and weighed immediately afterwards. By subtracting the fat free  
2 dry weight from the pre-extraction dry weight, the fat content was calculated as absolute  
3 value in mg.

4 To obtain wing measurements, wings were photographed with a digital camera  
5 connected to a stereo microscope (MZ75; Leica, Wetzlar, Germany). Based on the resulting  
6 pictures, forewing length, the diameter of the largest forewing and hindwing eyespot, and the  
7 diameters of the white pupil for both eyespots were measured using the software ImageJ  
8 (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

9

## 10 **Statistical analysis**

11 For thorax mass, abdomen mass, and fat content we used residuals of the regressions against  
12 total body mass for further analyses to produce variables that were independent of body size.  
13 In experiment 2, only males that won all three consecutive matings were considered  
14 ‘winners’, and the respective counterparts as ‘losers’. For both experiments, we initially  
15 examined differences between ‘winners’ and ‘losers’ in the traits measured using paired t-  
16 tests. As this approach incurs problems of multiple testing, we also used principal component  
17 analyses (PCAs) to reduce the number of potentially interrelated variables, followed by  
18 generalized linear models (GLZs). To account for the statistical dependency of the data  
19 derived from individual mating trials, we calculated the difference between winner and loser  
20 male for each trait, thereby generating a single value per male pair. Thus, one male of each  
21 trial was randomly defined as ‘focal’ individual, from which the values of the opponent were  
22 subtracted. This procedure yielded difference values for each mating trial and trait. In  
23 experiment 1, we used the first three principal components (PCs) for further analyses, having  
24 eigenvalues between 1.4 and 4.2 (all other eigenvalues <1.1). PCs 1-3 jointly explained  
25 55.1% of the total variation. In experiment 2, the first two PCs were used for further analyses,  
26 having eigenvalues of 2.1 and 2.4 (all other eigenvalues <0.8). PCs 1-2 jointly explained  
27 75.7% of the total variation. ADH was excluded from both PCAs and PO was also excluded  
28 from the PCA for experiment 2 due to low sample sizes. For both experiments, factor  
29 coordinates were calculated for the selected PCs for all individuals, which were subsequently  
30 used in GLZs with binomial error distributions and logit-link function, by encoding focal  
31 males who lost with ‘0’ and focal males who won with ‘1’. All statistical analyses were  
32 performed using STATISTICA 8.0 (StatSoft, Inc., Tulsa, OK, USA).

33

## 1 **Results**

2 *Experiment 1.* In univariate comparisons, successful compared with unsuccessful males had  
3 on average a significantly higher fat content, and tended to have lower abdomen but higher  
4 thorax mass, higher PO activity, and longer forewings (Table 1). Moreover, they tended to  
5 have a higher ADH activity. The PCA revealed that PC1 essentially represents a wing and  
6 eyespot size variable, that PC2 was positively related to PO expression, forewing length,  
7 residual thorax mass, and residual fat content, but negatively to residual abdomen mass,  
8 whereas PC3 mainly reflected body mass (Table 2). The subsequent GLZ showed that only  
9 PC2 ( $\chi^2 = 20.3$ , d.f. = 1,  $P < 0.0001$ ) was significantly related to mating success, whereas PC1  
10 ( $\chi^2 = 0.5$ , d.f. = 1,  $P = 0.47$ ) and PC3 ( $\chi^2 = 0.2$ , d.f. = 1,  $P = 0.68$ ) were not. PC2 was related  
11 to PO expression, forewing length, residual thorax and abdomen mass, and residual fat  
12 content, indicating that successful males have a better flight performance than unsuccessful  
13 males (see also Table 1).

14  
15 *Experiment 2.* In 28 out of 125 mating trials, one male won all contests against his  
16 counterpart, and in another 45 mating trials the same male won at least the first two trials. In  
17 42 trials, a male died before the last mating (Table 3). In univariate comparisons, successful  
18 males had a significantly higher residual fat and protein content than unsuccessful males  
19 (Table 4). The PCA revealed that PC1 mainly reflects thorax to abdomen, and PC2 body  
20 mass, protein content, residual fat content, and forewing length (Table 2). The subsequent  
21 GLZ showed that only PC2 ( $\chi^2 = 3.4$ , d.f. = 1,  $P = 0.067$ ) tended to be related to mating  
22 success, whereas PC1 ( $\chi^2 = 1.6$ , d.f. = 1,  $P = 0.21$ ) did not. PCA 2 was related to body mass,  
23 protein content, residual fat content, and forewing length.

## 24 25 **Discussion**

### 26 **Factors affecting male mating success**

27 Our experiments revealed that more-successful males differed in physiological and  
28 morphological traits from less-successful ones. Males that had been successful in obtaining a  
29 mating in experiment 1 were characterized by a higher thorax mass, abdomen fat content and  
30 PO activity, longer forewings, and a lighter abdomen. Thus, males having a mating advantage  
31 were larger (forewing length) and in better condition as predicted, evidenced by a higher  
32 relative amount of fat and higher PO expression levels. Successful males also showed a better  
33 flight performance owing to their longer wings, higher energy reserves, larger thorax

1 containing the flight muscles, and their lighter abdomen (Srygley & Dudley, 1993; Peixoto &  
2 Benson, 2008). Flight performance is likely to be of crucial importance for male butterflies in  
3 terms of mate location, conquering and defending territories, aerial fights, and courtship  
4 (Kemp & Wiklund, 2001; Tiple et al., 2010).

5 Larger body size is often positively related to mating success (Marden & Waage,  
6 1990; Kemp & Wiklund, 2001; Candolin, 2005; Lappin et al., 2006). Larger wing size  
7 reduces wing loading and therefore typically increases flight performance in insects (Van  
8 Dyck et al., 1997; Martínez-Lendech et al., 2007). Likewise, fat content is related to flight  
9 performance, as it is the most important storage reserve and essential to fuel the energy-  
10 demanding flight in butterflies (Zera et al., 1998; Karl & Fischer, 2008). Owing to its  
11 importance, fat content has been frequently related to overall condition in insects (Zwaan et  
12 al., 1991). Maintaining high levels of the multifunctional enzyme PO requires tyrosine and  
13 nitrogen, rendering PO expression costly (Schwarzenbach & Ward, 2006; Cerenius et al.,  
14 2008; Kanost & Gorman, 2008); it is therefore predicted to be related to fitness (González-  
15 Santoyo & Córdoba-Aguilar, 2011). Our interpretation regarding PO expression patterns thus  
16 rests on the finding that PO is an excellent indicator of condition in insects (González-  
17 Santoyo & Córdoba-Aguilar, 2011). Although a strong trade-off between immunity and  
18 reproduction has been suggested, causing decreased PO levels after mating (Rolff & Siva-  
19 Jothy, 2002; Schwarzenbach et al., 2005), we cannot rule out that mating has induced an  
20 upregulation of PO in our experiment, as mating involves close contact and the exchange of  
21 material among individuals, which may cause an upregulation of the immune system  
22 (Valtonen et al., 2010; Gupta et al., 2013). Although successful males were overall larger (see  
23 above, wing length), their abdominal mass was lower. A smaller abdomen may shift the  
24 centre of rotation closer towards the thorax and therefore the wings, which also results in a  
25 better manoeuvrability and higher flight speed (Srygley & Dudley, 1993; Marden, 2000).  
26 Finally, the larger thoraces fit the overall pattern, as flight endurance is correlated with the  
27 relative size of flight muscles in insects (Marden, 2000). Interestingly though, the size of  
28 eyespots or their pupils had no obvious influence on male mating success (but see Breuker &  
29 Brakefield, 2002, where females preferred males with larger eyespots).

30 In experiment 2, larger forewings, a higher body mass, and protein and fat content  
31 were the most important factors affecting mating success in *B. anynana*, most of which had  
32 also played an important role in experiment 1. In contrast, PO expression did not differ  
33 between winners and losers in experiment 2, which may be due to the low sample size.  
34 Furthermore, thorax-abdomen ratio did not seem to play a decisive role in experiment 2. This

1 may indicate physiological changes within the males' lifespan, for instance a degradation of  
2 flight muscles. Note in this context that all males analysed in experiment 1 were young, being  
3 frozen after their first mating trial, whereas the males used in experiment 2 were older and  
4 were set up for mating repeatedly.

5

### 6 **Consistency of male mating success over time**

7 Only in 28 out of 125 mating trials did one male win all contests against his counterpart.  
8 However, in another 45 trials, one male won at least the first two trials. Note that in 23 out of  
9 the latter trials the 'winner' was not able to win a third time, as he (or his counterpart) died  
10 before the last trial. Thus, if the comparison is restricted to the first two mating trials, one  
11 male won twice in 73 out of 125 mating trials. These results do not clearly support that given  
12 males predictably outcompete a given counterpart. We can think of three explanations for this  
13 pattern. First, male mating success may be a matter of chance, if, for example, the first male  
14 that happens to encounter the female will mate with her. Although male mating success in our  
15 system may indeed involve chance effects at least to some extent, mating success is clearly  
16 not random as evidenced by the morphological and physiological differences found.  
17 Therefore, a second explanation seems more likely. We suggest that the design of our  
18 experiment caused different rates of ageing among successful and unsuccessful males, owing  
19 to a higher effort for courtship and mating in the former. Note that spermatophore production  
20 might be costly in butterflies (Takeuchi, 2012; Caballero-Mendieta & Cordero, 2013), and  
21 that studies on other insects indicated that courtship alone may entail measureable costs  
22 (Woods et al., 2007; White et al., 2008; Mowles, 2014). Thus, whereas successful males had  
23 to invest repeatedly, the unsuccessful males could save all their energy. Therefore, the latter  
24 may become 'more similar' to the successful males over time. This would also explain why  
25 fewer variables were found to be associated with male mating success in experiment 2, as  
26 only the males being in constantly good condition are likely to repeatedly outperform their  
27 counterpart. The third explanation would be variation in female preference, as each mating  
28 trial involved the same males but different females.

29

### 30 **Conclusions**

31 In summary, our results do not indicate that male mating success is necessarily consistent  
32 over time, perhaps most likely owing to the specific experimental design used. Nevertheless,  
33 our study shows clear differences between more- and less-successful males. Male mating  
34 success was related to several indicators of condition, namely a higher abdomen fat content

1 and PO activity, longer forewings, a heavier thorax and a lighter abdomen. Overall, the  
2 amount of storage reserves may be most important for male mating success. Strikingly,  
3 basically all differences found play a leading role in male flight performance. We therefore  
4 suggest that male agility, manoeuvrability, and persistence during mate location and  
5 courtship play ultimately the key role for male mating success in *B. anynana* and possibly in  
6 other species. To answer the question whether this gives the males a competitive advantage  
7 compared to other males or whether females preferably mate with such males is beyond the  
8 scope of this contribution but comprises a potentially fruitful venue for subsequent studies.

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- 31

1 **Table 1** Comparison of several traits (mean  $\pm$  SD) between successful ('winner') and  
 2 unsuccessful ('loser') males of *Bicyclus anynana*, having competed for a single virgin female,  
 3 including results of paired t-tests

Trait	Winner	Loser	d.f.	t	P
Total body mass (g)	0.041 $\pm$ 0.006	0.042 $\pm$ 0.007	99	-1.320	0.19
Thorax mass (residuals)	0.159 $\pm$ 1.091	-0.159 $\pm$ 0.871	99	2.546	0.013
Abdomen mass (residuals)	-0.152 $\pm$ 0.801	0.152 $\pm$ 1.145	99	-2.235	0.021
Protein content (mg/ml)	0.340 $\pm$ 0.003	0.340 $\pm$ 0.003	99	0.729	0.47
Phenoloxidase activity (mOD/ml)	0.186 $\pm$ 0.793	0.157 $\pm$ 0.782	96	-2.109	0.038
Alcoholdehydrogenase activity (mOD/ml)	0.053 $\pm$ 0.339	0.048 $\pm$ 0.360	51*	-0.019	0.056
Lysozyme activity (mOD/ml)	0.261 $\pm$ 0.118	0.283 $\pm$ 0.121	99	1.392	0.17
Fat content (residuals)	0.222 $\pm$ 0.959	-0.222 $\pm$ 0.996	97	3.683	0.0004
Forewing length (mm)	18.051 $\pm$ 0.600	17.816 $\pm$ 0.705	98	-2.465	0.016
Eyespot diameter (mm) (forewing)	3.344 $\pm$ 0.308	3.344 $\pm$ 0.330	98	0.002	1.0
White pupil diameter (mm) (forewing)	0.676 $\pm$ 0.103	0.682 $\pm$ 0.091	98	0.404	0.69
Eyespot area (mm <sup>2</sup> ) (forewing)	8.857 $\pm$ 1.612	8.898 $\pm$ 1.705	98	0.046	0.96
Eyespot diameter (mm) (hindwing)	2.149 $\pm$ 0.214	2.153 $\pm$ 0.219	98	0.125	0.90
White pupil diameter (mm) (hindwing)	0.389 $\pm$ 0.697	0.386 $\pm$ 0.076	98	-0.377	0.71
Eyespot area (mm <sup>2</sup> ) (hindwing)	3.664 $\pm$ 0.736	3.677 $\pm$ 0.733	98	0.126	0.90

4 \*Forty-nine samples were corrupted, resulting in a lower sample size.

5 Note that after Bonferroni correction only the difference in fat content remains significant.

6

1 **Table 2** Factor loadings for the selected principal components (PCs) in experiments 1 and 2

		PC 1	PC 2	PC 3
Exp 1	Body mass	0.403563	-0.135492	0.462516
	Thorax mass (residuals)	0.314662	0.609102	-0.047314
	Abdomen mass (residuals)	-0.398663	-0.589630	0.297535
	Protein content	0.028926	0.006447	-0.366285
	Phenoloxidase activity	0.098830	0.603544	0.017820
	Lysozyme activity	-0.007489	-0.293011	0.121730
	Fat content (residuals)	0.107327	0.577499	0.160984
	Forewing length	0.556945	0.528665	0.166189
	Eyespot diameter (forewing)	0.821170	-0.245844	-0.409467
	White pupil diameter (forewing)	0.710793	-0.195670	-0.396586
	Eyespot area (forewing)	0.822770	-0.250215	-0.398240
	Eyespot diameter (hindwing)	0.784464	-0.191328	0.457198
	White pupil diameter (hindwing)	0.784883	-0.194282	0.454768
	Eyespot area (hindwing)	0.633777	0.092958	0.121426
Exp 2	Body mass	-0.119121	0.820883	
	Thorax mass (residuals)	0.979037	-0.026589	
	Abdomen mass (residuals)	-0.979037	0.026589	
	Protein content	-0.316513	0.728683	
	Fat content (residuals)	0.012138	0.749716	
	Forewing length	0.630525	0.589011	

2

3

1 **Table 3** Results of 125 mating trials in each of which two specific males of *Bicyclus anynana*  
2 competed for a single virgin female on days 3, 5, and 7 of male adult life. The male who won  
3 the first trial was labelled with '1' for 'winner'. Thus, '111' indicates that the respective male  
4 was successful 3×, whereas his counterpart was unsuccessful throughout; '110' indicates that  
5 the male who has won the first trial also succeeded in the second, but that his counterpart  
6 gained the mating in the third trial, etc.

Group	n
111	28
110	22
11+	23
100	13
101	20
10+	19

7 '+': the individual died after the second trial.

8

1 **Table 4** Comparison of several traits (mean  $\pm$  SD) between successful ('winner') and  
 2 unsuccessful ('loser') male *Bicyclus anynana*, having won three consecutive mating trials on  
 3 days 3, 5, and 7 of male adult life, including results of paired t-tests

Trait	Winner	Loser	d.f.	t	P
Total body mass (g)	0.030 $\pm$ 0.007	0.029 $\pm$ 0.005	24	0.835	0.41
Thorax mass (residuals)	0.007 $\pm$ 0.969	-0.007 $\pm$ 1.036	24	0.047	0.96
Abdomen mass (residuals)	-0.007 $\pm$ 0.969	0.007 $\pm$ 1.036	24	-0.047	0.96
Protein content (mg ml <sup>-1</sup> )	0.315 $\pm$ 0.0003	0.314 $\pm$ 0.0001	24	15.890	<0.0001
Phenoloxidase activity (mOD ml <sup>-1</sup> )	0.041 $\pm$ 0.005	0.042 $\pm$ 0.004	11	-0.150	0.88
Alcoholdehydrogenase activity (mOD ml <sup>-1</sup> )	0.013 $\pm$ 0.005	0.013 $\pm$ 0.004	16	0.024	0.98
Fat content (residuals)	0.416 $\pm$ 0.991	-0.416 $\pm$ 0.811	24	3.816	0.0008
Forewing length (mm)	18.917 $\pm$ 0.884	18.853 $\pm$ 0.830	19	0.296	0.77

4 All differences remain significant after Bonferroni correction.

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# Manuscript IV

## Submitted as:

Kehl, T., Beaulieu, M., Kehl, A. & Fischer, K. Old male sex: large ejaculate, many sperm, but few offspring.



1 **Old male sex: large ejaculate, many sperm, but few offspring**

2

3

4 **Tobias Kehl, Michaël Beaulieu, Alexander Kehl, Klaus Fischer**

5

6

7 Zoological Institute & Museum, Greifswald University, Johann-Sebastian-Bach Str.

8 11/12, 17489 Greifswald, Germany

9

10

11 **Corresponding author:**

12 Tobias Kehl

13 Zoological Institute & Museum

14 Greifswald University

15 Johann-Sebastian-Bach Str. 11/12

16 D - 17489 Greifswald, Germany

17 Tel.: +49-3834-864268

18 Fax: +49-3834-864252

19 Email: tobias.kehl@googlemail.com

20

21 **Abstract**

22 Reproduction induces non-trivial costs, such that both males and females should  
23 choose their mates carefully and invest their resources prudently. Male performance  
24 and thus their investment into ejaculates are often predicted to decrease with age  
25 and mating frequency, which may in turn negatively affect female fitness and thus  
26 feedback on the attractiveness of old males. Such reproductive senescence may be  
27 mediated by changes in the males' oxidative status. Here we investigated the effects  
28 of male mating frequency and age on male reproduction and oxidative status, and  
29 the respective consequences for female reproduction. We used the tropical butterfly  
30 *Bicyclus anynana*, in which counterintuitively older males have a higher mating  
31 success than younger ones. In once-mated males, spermatophore mass and sperm  
32 numbers strongly increased with age, while antioxidant defences and oxidative  
33 damage declined with age. In repeatedly-mated males, spermatophore mass and  
34 sperm number showed little variation being similar to young once-mated males, while  
35 antioxidant defences increased and oxidative damage decreased with mating  
36 frequency. Female reproductive success was highest when mating with young once-  
37 mated males, although these produced small spermatophores with low sperm  
38 numbers. Our findings suggest that in *B. anynana* (1) ejaculate size and sperm  
39 number are not reliable proxies of male quality, (2) ejaculate quality diminishes with  
40 age and mating number, and that (3) old male mating advantage likely results from  
41 sexual conflict owing negative effects on female fitness.

42

43 **Keywords:** *Bicyclus anynana*, costs of reproduction, male reproduction, oxidative  
44 stress, sexual conflict, spermatophore

45

## 46 **Introduction**

47 Historically, it was believed that costs induced by reproduction concern females  
48 rather than males (Bateman 1948; Fowler and Partridge 1989; Chapman et al. 1998;  
49 Harshman and Zera 2007). This was because females typically invest strongly in  
50 offspring production and parental care, whereas males were assumed to essentially  
51 donate nothing but cheap sperm, thus having near unlimited capacities to copulate  
52 (Heinze and Hölldobler 1993). This traditional notion has, however, more recently  
53 been challenged for various reasons. For instance, males typically need some time  
54 for recovery before being able to produce another ejaculate (Birkhead 1991; Wedell  
55 1994; Bissoondath and Wiklund 1996). In addition, subsequent ejaculates are  
56 typically smaller and often contain a decreased number of sperm (Salamon 1962;  
57 Oberhauser 1988; Zvěřina and Pondělícková 1988; Birkhead 1991). Finally, mating  
58 duration is often prolonged in later as compared with the first mating (Kaitala and  
59 Wiklund 1995) suggesting that already-mated males need more time to produce a  
60 spermatophore than virgin males (cf. Wedell & Cook, 1999). These findings  
61 highlighted the existence of non-trivial costs of reproduction in males (Kotiaho et al.  
62 1998; Kotiaho and Simmons 2003; Paukku and Kotiaho 2005; Janowitz and Fischer  
63 2010). Therefore, iteroparous males should invest their resources prudently,  
64 considering current as well as future reproduction (Takeuchi 2012; Caballero-  
65 Mendieta and Cordero 2013).

66

67 Apart from mating frequency, age is yet another factor which may impact on male  
68 reproduction. In many taxa, senescence starts early (Ford 2000; Boggs and Freeman  
69 2005; Møller et al. 2009), and condition diminishes with age (Karlsson 1994; Fischer  
70 et al. 2008). Consequently, females are expected to prefer younger, virgin males,

71 which may have more resources available for signaling, sperm production, and  
72 nuptial gifts than older males (Vahed 1998; Wedell and Karlsson 2003; Ferkau and  
73 Fischer 2006; Fricke and Maklakov 2007). Nuptial gifts, for instance, may be used by  
74 females to produce additional offspring (Vahed 1998; South and Lewis 2011; South  
75 and Lewis 2012). In line with the above considerations, mating with older males has  
76 repeatedly been found to be detrimental to females, causing reduced lifespan  
77 (Paukku and Kotiaho 2005), fecundity (Ofuya 1995), and egg-hatching success  
78 (Jones et al. 2000; Karl and Fischer 2013), as well as a higher remating propensity  
79 combined with a tendency towards shorter refractory periods (Karl and Fischer 2013).  
80 However, male age is often confounded with mating number, as both may covary. If  
81 males age without mating, they may actually accumulate sperm and other resources  
82 available for ejaculate production (Kehl et al. 2013), and may hence be preferred by  
83 females. It is therefore important to disentangle the effects of age from those of  
84 mating number.

85

86 From the female perspective, mate choice is especially important in monandrous  
87 species, that is if females have only one mating partner within their lives. In such  
88 systems no compensation for having chosen a low-quality male is possible. However,  
89 polyandry, i.e. mating with different males, is common and probably even more  
90 widespread than monandry (Parker 1970). This is interesting as theory predicts that  
91 only one or a few matings are typically sufficient to maximise a female's reproductive  
92 success (Bateman 1948; Arnqvist and Nilsson 2000; Larsdotter Mellström and  
93 Wiklund 2010). Females may gain direct and/or indirect benefits from multiple  
94 matings. Regarding direct benefits, receiving nuptial gifts may be of particular  
95 importance (Wedell 1994; Vahed 1998; Arnqvist and Nilsson 2000; South and Lewis

96 2011; South and Lewis 2012). Additionally, mating with different males reduces the  
97 risk of genetic incompatibilities arising from detrimental genome interactions, thus  
98 reflecting indirect fitness benefits (Tregenza and Wedell 1998; Tregenza and Wedell  
99 2000; Zeh and Zeh 2001; Janowitz and Fischer 2012). Overall, the level of female  
100 polyandry is governed by a balance between the costs and benefits of mating  
101 multiply together with the extent of conflict over optimal mating rates between the  
102 sexes (Larsdotter Mellström and Wiklund 2010; Janowitz and Fischer 2012).

103

104 One physiological factor that may underlie detrimental effects of mating frequency  
105 and age on male reproductive performance is oxidative stress, *i.e.* an imbalance  
106 between the production of Reactive Oxygen Species (ROS) and antioxidant defences  
107 that results in higher oxidative damage. Indeed, reproduction may result in lower  
108 antioxidant defences and higher oxidative damage, which may in turn limit the  
109 capacity to reproduce again (Bize et al. 2008; Helfenstein et al. 2010; Stier et al.  
110 2012). Such effects may be exacerbated by age, as oxidative damage may  
111 accumulate and antioxidant defences may decline with age (Williams et al. 2008;  
112 Monaghan et al. 2009), such that older animals may be less efficient than younger  
113 ones at counteracting the effects of reproduction on their oxidative status. However, if  
114 the first reproductive attempt of an individual induces oxidative stress, it may trigger  
115 hormetic effects such that respective individuals may be better protected against  
116 oxidative stress when reproducing again. Despite the presumably important benefits  
117 of hormesis for iteroparous organisms, such effects on the oxidative balance have, to  
118 our knowledge, not yet been described in the context of reproduction (Costantini et  
119 al. 2012; Costantini et al. 2014b).

120

121 Here, we investigated the effects of male age and mating number on spermatophore  
122 mass, sperm number, male oxidative status, and reproductive success in the tropical  
123 butterfly *Bicyclus anynana*. In this species, most females are monandrous but some  
124 may mate repeatedly, the rate of polyandry being up to 25% (Brakefield et al. 2001).  
125 Males provide relatively small spermatophores, indicating that nuptial gifts may play a  
126 minor role in *B. anynana* (Molleman et al. 2004; Ferkau and Fischer 2006).  
127 Interestingly, multiple mating does not seem to increase female fitness, while there is  
128 evidence that they may actually suffer from reduced longevity (Fischer 2007).

129

130 Older males of this species have been described as having a higher mating success  
131 than younger ones (Karl et al. 2013; Karl and Fischer 2013; Kehl et al. 2014).  
132 However, several lines of evidence suggest that old male mating advantage arises  
133 from sexual conflict, *i.e.* from a higher eagerness and persistence of older males  
134 during courtship rather than female preference for older males. This notion is  
135 supported by the fact that older males show higher flight activity and court more  
136 frequently and for longer time bouts than younger males, while females having mated  
137 with older males show a shorter refractory period, a higher remating propensity, and  
138 suffer reduced egg hatching success than females having mated with younger males  
139 (Fischer et al. 2008; Karl et al. 2013; Karl and Fischer 2013; Kehl et al. 2014). The  
140 latter suggests that older males transfer a spermatophore of lower quality to females,  
141 either in terms of reduced nutrients, sperm viability or sperm numbers, as found in  
142 other insects (Kaitala and Wiklund 1995; Wedell and Ritchie 2004). However, once-  
143 mated older males were found to have higher paternity success than younger males  
144 in double-mated females, presumably because of an accumulation of sperm and  
145 accessory gland products over time (Kehl et al. 2013). Thus, the occurrence of sperm

146 limitation in old males is unlikely to be the principal factor reducing female  
147 reproductive success. Based on these intriguing findings, *B. anynana* appears to be  
148 a particularly rewarding study organism to explore the effects of male age and mating  
149 frequency on spermatophore production and the respective fitness consequences for  
150 females. Towards this end, we here employ an experimental design manipulating  
151 male age, male mating number, and the time since the production of the last  
152 spermatophore. Specifically, we investigate the effects of male age and mating  
153 frequency on (1) spermatophore mass and sperm numbers, (2) on the males'  
154 oxidative status, and (3) the respective consequences for female reproduction.

155

156

## 157 **Methods**

### 158 *Study organism and rearing conditions*

159 The Squinting Bush Brown *B. anynana* (Butler, 1879) is a nymphalid, fruit-feeding  
160 butterfly, with a distribution ranging from southern Africa to Ethiopia (Larsen 1991).  
161 The species exhibits two seasonal morphs as a striking example of phenotypic  
162 plasticity, which is thought to be an adaption to alternating wet-dry seasonal  
163 environments and the associated changes in resting background and predation  
164 (Lyytinen et al. 2004). A laboratory stock population was established at Greifswald  
165 University, Germany, in 2008 from several hundred eggs derived from a well-  
166 established stock population at Leiden University, The Netherlands. The Leiden  
167 population was founded in 1988 from 80 gravid females caught at a single locality in  
168 Malawi. In each generation several hundred individuals are reared maintaining high  
169 levels of heterozygosity at neutral loci (Van't Hof et al. 2005). For this study,  
170 butterflies from the Greifswald stock population were used.

171 *Experimental design*

172 To explore the factors underlying reduced female fitness when mating with older  
173 males, we performed two separate experiments following the same experimental  
174 design (see below). While the mated females from experiment 1 were sacrificed for  
175 measuring spermatophore mass and sperm numbers, mated females from  
176 experiment 2 were used for assessing the fitness consequences of mating with males  
177 differing in age and previous number of matings. Additionally, the males from  
178 experiment 2 were used to measure their oxidative status. For each experiment,  
179 approximately 4000 eggs were collected over a period of 12 days of oviposition.  
180 Larvae were reared in population cages on potted maize plants in a climate chamber  
181 under constant conditions with a 12/12h light-dark cycle, 27°C, and 70 % relative  
182 humidity. These conditions are similar to those at which *B. anynana* develops and  
183 reproduces during the favourable wet season in its native habitat (Brakefield and  
184 Reitsma 1991; Brakefield 1997). Resulting pupae were collected daily and  
185 transferred to cylindrical hanging cages (30 x 39 cm). Pupal cages were checked  
186 each day in the morning for eclosed butterflies. All eclosed butterflies were removed  
187 and transferred to new hanging cages, one for males and one for females per day.  
188 Thus, butterflies were always separated by sex within 12 h after eclosion to avoid  
189 mating prior to experiments. Male *B. anynana* butterflies are not able to mate on their  
190 first day of adult life due to sexual immaturity. Males were randomly allocated to one  
191 out of 9 treatment groups, designed to disentangle the effects of male age and  
192 mating status on male reproduction (Table 1). This design allowed us to investigate  
193 (1) the effects of male age on the quality of the first spermatophore, (2) the effects of  
194 male mating number (and age) on spermatophore quality, and (3) the effects of male  
195 age on the quality of second spermatophores. Butterflies were set up for mating in

196 groups of about 10 males and 10 females in cylindrical hanging cages. The cages  
197 were continuously monitored and mating couples were immediately removed and  
198 kept separated in a one-litre plastic container. After mating, males were either kept  
199 separated from females until the next mating or discarded if not needed for further  
200 matings, depending on treatment group. Throughout the experiments all butterflies  
201 were supplied with moist banana and water for feeding *ad libitum*.

202

### 203 *Spermatophore mass and sperm numbers*

204 For later analysis of spermatophore mass and eupyrene (fertile) sperm number,  
205 mated females were frozen in liquid nitrogen immediately after the termination of the  
206 copulation, thus preventing sperm from dispersing to the spermatheca, and  
207 afterwards stored at -80°C. The methods used to examine spermatophore mass and  
208 sperm number followed Kehl et al. (2013). In short, females were thawed and  
209 dissected in insect Ringer's solution. The bursa copulatrix, which contains the male  
210 spermatophore, was taken out. After having removed surplus Ringer's solution from  
211 the bursa using filter paper, the bursa was weighed on an electrobalance (Sartorius  
212 LE225D) to the nearest 0.01 mg. Thus, the mass of the bursa copulatrix containing  
213 the spermatophore was used as a proxy of spermatophore mass, as the mass of the  
214 bursa relative to the spermatophore is negligible (Cook and Wedell 1996). Then, the  
215 spermatophore was transferred to a cavity slide with a droplet of Ringer's solution,  
216 opened with forceps and stirred gently to disperse the sperm. Lepidoptera produce  
217 two types of sperm, anucleate apyrene (infertile) and eupyrene (fertile) sperm (Cook  
218 and Wedell 1996). The function of apyrene sperm is not entirely clear, but they  
219 probably serve as filling substance in order avoid female remating, consequently  
220 reducing the potential for sperm competition (Cook and Wedell 1999). As eupyrene

221 sperm bundles contain 256 single sperm cells (Virkki 1969), they appear much bigger  
222 than a single apyrene sperm cell, which makes them easy to distinguish under the  
223 microscope. Eupyrene (fertile) sperm bundles were counted within the cavity slide  
224 using a microscope (at 40x). To calculate the absolute number of eupyrene sperm,  
225 the number of bundles was multiplied by 256, owing to the fact that in Lepidoptera all  
226 the sperm in a bundle originate from a single spermatogonium, which undergoes a  
227 fixed number of 8 divisions (Virkki 1969; Cook and Wedell 1996; Fischer 2007).

228

### 229 *Male oxidative status*

230 Following the males' last mating (Table 1), males were immediately frozen in liquid  
231 nitrogen and afterwards stored at -80°C for later analysis of their oxidative status.  
232 Frozen abdomens were first separated from the rest of the body (thorax, head, wings,  
233 legs) and weighed to the nearest 0.01 mg (Sartorius LE225D; Sartorius AG,  
234 Göttingen, Germany). Then, abdomen were transferred to 1.5 ml tubes with 50 µl  
235 phosphate buffered saline (PBS; 0.15 M NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>\*2H<sub>2</sub>O, pH = 7.4),  
236 homogenized, and subsequently centrifuged at 15000 g and 4°C for 10 minutes. The  
237 supernatants were transferred to new 1.5 ml tubes and once again centrifuged at  
238 15000 g and 4°C for 10 minutes. The resulting supernatant was used to measure  
239 reactive oxygen metabolites (ROM) and the total antioxidant capacity (OXY). The d-  
240 ROM test measures hydroperoxydes, i.e. reactive oxygen metabolites (ROMs)  
241 resulting from the attack of reactive oxygen species (ROS) on organic substrates  
242 (carbohydrates, lipids, amino acids, proteins, nucleotides), thereby reflecting  
243 oxidative damage. OXY was quantified with the OXY-adsorbent test (Diacron  
244 International). For both tests, we followed the procedure described by Criscuolo et al.  
245 (2010) except that we corrected ROM and OXY values for the protein content of the

246 supernatant, as determined by the Bradford protein assay (Bradford 1976).

247

#### 248 *Female fitness*

249 Mated females were set up individually for egg-laying to assess the fitness  
250 consequences of their male partners' age and mating status. Females were placed  
251 individually into one-litre translucent plastic containers containing a maize leaf as  
252 egg-laying substrate and moist banana for feeding. Eggs were removed every third  
253 day and counted for 12 days after mating. To assess egg hatching success, per  
254 female ca. 30 eggs laid early (days 0-4 of the oviposition period) and late (days 10-  
255 12), respectively, were transferred to petri-dishes and provided with a fresh cutting of  
256 maize and moistened filter paper. Theory predicts that egg quality diminishes with  
257 age (Begon and Parker 1986). Therefore, eggs were collected at different time  
258 points, as detrimental effects of mating with low-quality males may only be found later  
259 in the oviposition period (Karl and Fischer 2013). Eggs were checked until no more  
260 hatchlings were found for at least 48 hours, the number of hatched larvae was  
261 counted and hatching rate calculated (number of hatchlings/number of eggs). The  
262 number of offspring produced for each individual female was estimated by multiplying  
263 the mean hatching rate of early and late eggs with fecundity.

264

#### 265 *Statistical analyses*

266 Differences in spermatophore mass, sperm number, hatching success, offspring  
267 number, and oxidative status (ROM and OXY) were analysed using one-factorial  
268 analyses of variance (ANOVAs) with 'treatment group' as fixed factor. Groups 2, 3, 4,  
269 5, 8, 9 were measured on days 6, 9 or 12 after their first or second mating, resulting  
270 in a full-factorial set-up (cf. Table 1). This allowed us to examine the interaction

271 between 'age' and 'mating frequency' using these groups. Towards this end, we  
272 additionally performed two-factorial ANOVAs with 'age', 'mating number' and their  
273 interaction as fixed factors. As eggs were collected at four different time points,  
274 fecundity was analysed using a repeated measures ANOVA. Tukey HSD Post-hoc  
275 tests were used to localise significant differences between groups. All statistical tests  
276 were performed using Statistica 8.0 (StatSoft Inc.).

277

## 278 **Results**

### 279 *Spermatophore mass and sperm number*

280 Spermatophore mass ( $F_{8,353} = 25.1$ ,  $P < 0.0001$ ) and sperm number ( $F_{8,353} = 30.4$ ,  $P$   
281  $< 0.0001$ ) differed significantly across treatment groups. Spermatophore mass (group  
282 4 (12 d, 1x mated) = group 3 (9 d, 1x mated) > group 2 (6 d, 1x mated)  $\geq$  all other  
283 groups; Tukey HSD after ANOVA) and sperm number (group 4 (12 d, 1x mated) > 3  
284 (9 d, 1x mated) = 9 (12 d, 2x mated) > all others) were both highest in group 4 (12 d,  
285 1x mated), which corresponds to males mated for the first time on day 12 of adult life.  
286 Overall, both traits increased with age in single-mated males, while males having  
287 mated twice or more often did not differ amongst each other and young single-mated  
288 males (group 1 (3 d, 1x mated); except for sperm number in group 9 (12 d, 2x  
289 mated), which was mated twice on days 9 and 12; Fig. 1). In agreement with above,  
290 two-factorial ANOVAs based on a subset of groups revealed that spermatophore  
291 mass and sperm number were significantly affected by male age (spermatophore  
292 mass:  $F_{2,230} = 6.2$ ,  $P = 0.002$ ; sperm numbers:  $F_{2,239} = 44.3$ ,  $P < 0.0001$ ) and mating  
293 number (spermatophore mass:  $F_{1,230} = 82.5$ ;  $P < 0.0001$ ; sperm numbers:  $F_{1,230} =$   
294  $49.9$ ;  $P < 0.0001$ ). For both traits, values increased with age and were higher for  
295 single- than for double-mated males, respectively. The interaction between age and

296 mating number showed that spermatophore mass increased with age in single-mated  
297 males only ( $F_{2,230} = 3.7$ ;  $P = 0.025$ ; Fig. 1a). For sperm number, the age by mating  
298 number interaction was nearly significant, indicating that sperm number tended to  
299 increase with age more strongly in single- than in double-mated males ( $F_{2,230} = 3.0$ ;  $P$   
300  $= 0.050$ ; Fig. 1b).

301

### 302 *Oxidative status*

303 Both antioxidant defences ( $F_{8,79} = 4.4$ ,  $P = 0.0002$ ) and oxidative damage ( $F_{8,80} = 3.6$ ,  
304  $P = 0.0012$ ) showed significant differences across treatment groups. Antioxidant  
305 defences were highest in group 5 (6 d, 2x mated) and lowest in group 4 (12 d, 1x  
306 mated) ( $5 \geq$  all others  $\geq 4$ ; Fig. 1c), while oxidative damage was highest in group 1 (3  
307 d, 1x mated) and lowest in group 7 (12 d, 4x mated) ( $1 \geq$  all others  $\geq 7$ ; Fig 1d).  
308 Overall, these results show that both traits decreased with age irrespective of mating  
309 frequency, while mating frequency appears to increase antioxidant defences and  
310 decrease oxidative damage. In agreement, two-factorial ANOVAs on a subset of  
311 groups revealed significant effects of male age and mating number. Antioxidant  
312 defences and oxidative damage decreased with age ( $F_{2,53} = 10.5$ ;  $P = 0.0001$ ;  $F_{2,54} =$   
313  $4.7$ ;  $P = 0.0135$ ; respectively), and were higher or tended to be lower in double- than  
314 in single-mated males ( $F_{1,53} = 6.2$ ;  $P = 0.0157$ ;  $F_{1,54} = 3.4$ ;  $P = 0.0701$ ; respectively,  
315 Fig. 1). Interactions between age and mating were non-significant for antioxidant  
316 defences ( $F_{1,53} = 1.14$ ;  $P = 0.3276$ ) and oxidative damage ( $F_{1,54} = 1.29$ ;  $P = 0.2832$ ).

317

### 318 *Fecundity, egg hatching success and offspring number*

319 ANOVAs revealed significant differences across treatment groups in total fecundity  
320 ( $F_{24,795} = 12.7$ ,  $P < 0.0001$ ), offspring number ( $F_{8,265} = 8.0$ ,  $P < 0.0001$ ), early ( $F_{8,241} =$

321 5.9,  $P < 0.0001$ ) and late hatching success ( $F_{8,152} = 4.3$ ,  $P = 0.0001$ ). Fecundity and  
322 offspring number were highest in group 1 (3 d, 1x mated) and lowest in group 6 (9 d,  
323 3x mated) ( $1 \geq$  all others  $\geq 6$ ; Fig. 1). Early egg-hatching success was highest in  
324 group 1 and lowest in group 6 ( $1 \geq$  all others  $\geq 6$ ; Fig. 1g), while late egg-hatching  
325 success was highest in group 3 (9 d, 1x mated) ( $3 >$  all others; Fig. 1h). Regarding  
326 fecundity and offspring number, two-factorial ANOVAs based on the groups having  
327 mated on days 6, 9 or 12 for the first or second time (cf. Methods) revealed no  
328 significant differences across male age classes and mating frequency for both traits  
329 (results not shown). Similarly, early egg-hatching success was not significantly  
330 affected by male age or mating number, but the significant age by mating number  
331 interaction indicated differential age effects on hatching success in single- versus  
332 double-mated males ( $F_{2,162} = 4.0$ ,  $P = 0.0206$ ; Fig. 1g). Late egg-hatching success  
333 was significantly higher in single- than in double-mated males ( $F_{1,98} = 8.5$ ,  $P =$   
334  $0.0043$ ), while male age had no significant impact. The latter significance is caused  
335 exclusively by the high hatching success found for group 3 (9 d, 1 x mated), as  
336 evidenced by a significant age by mating number interaction ( $F_{2,98} = 4.7$ ,  $P = 0.0108$ ;  
337 Fig. 1h).

338

## 339 **Discussion**

340 Our experiments revealed significant effects of male age and mating number on most  
341 traits investigated, *i.e.* on male ejaculates and oxidative status as well as on female  
342 reproduction. Spermatophore mass and sperm number strongly increased with age in  
343 once-mated males. The near linear relation between sperm numbers and age (Fig.  
344 1b) suggests continuous sperm production at a constant rate, and a concomitant  
345 accumulation of sperm in virgin males (cf. Proshold 1991; Heinze and Hölldobler

346 1993; Konagaya and Watanabe 2013). This notion is further supported by the similar  
347 sperm numbers in previously mated males, all of which had exactly three days for  
348 recovery before being remated. Therefore, sperm numbers are expected to be  
349 comparable among already repeatedly mated males and the three day-old, once-  
350 mated males of group 1, which is indeed the case except for group 9 that was mated  
351 twice on days 9 and 12 of adult life, respectively. The increased sperm number found  
352 here is likely caused by the long period of time prior to the first mating, such that not  
353 all available sperm was transferred to the female at the first mating.

354

355 Spermatophore mass showed an overall similar pattern (Fig. 1a), but (1) its increase  
356 (by 39 %) was far less pronounced as compared with sperm number (281 %), (2)  
357 group 4 (12 d, 1x mated) showed hardly any further increase compared with group 3  
358 (9 d, 1x mated), and (3) group 9 (12 d, 2x mated) did not differ from the other  
359 multiply-mated groups as well as group 1 (3 d, 1x mated). These findings are hardly  
360 surprising, as the sperm ampulla typically comprises a minor fraction of the  
361 spermatophore in butterflies (Cook and Wedell 1996). Therefore, there does not need  
362 to be a tight correlation between spermatophore mass and sperm number. Similar to  
363 sperm numbers, our findings indicate a constant production of accessory gland  
364 products, although perhaps at a lower rate as compared with sperm. If so, the lack of  
365 a further increase in spermatophore mass in 12 days old, once-mated males (group  
366 4) may suggest that an upper limit has been reached, which may be caused by the  
367 limited size of the female bursa copulatrix (Sugawara 1979). Some other studies  
368 indicated that spermatophore size generally decreases with mating number  
369 (Lederhouse et al. 1990; Cook and Wedell 1996; Watanabe et al. 1998; Wedell and  
370 Cook 1999), which was not the case here. However, male *B. anynana* butterflies

371 produce relatively small spermatophores compared with other species (Ferkau and  
372 Fischer 2006), such that mating number is expected to affect resource availability  
373 and thereby allocation to reproduction less strongly.

374

375 Although older once-mated males produced larger spermatophores containing much  
376 more sperm than young once-mated males, females gained, in line with other studies  
377 (Wedell and Karlsson 2003; Srivastava and Omkar 2004; Kovac et al. 2013), highest  
378 fitness returns when mating with young virgin males (Fig. 1f). This pattern mainly  
379 arises from the fact that females having mated with three day-old virgin males  
380 produced by far the highest egg numbers (Fig. 1e), while variation in egg hatching  
381 success was less pronounced (Fig. 1g, f). The particularly poor performance of group  
382 6, (9 d, 3x mated), seems difficult to explain as no comparable effects were visible in  
383 group 7 (12 d, 4x mated), in which males had mated even four times. Therefore, the  
384 results for group 6 (9 d, 3x mated) most likely represent an experimental artefact,  
385 caused by a shortage of females for this particular group such that slightly older  
386 females than in the other groups had to be used. Moreover, we have no explanation  
387 for the extraordinarily high egg hatching success for the late eggs produced by  
388 females having mated with males from group 3 (9 d, 1x mated), though note that this  
389 had very little impact on the overall performance of this group.

390

391 The general patterns arising from our data on female reproductive output suggest  
392 that both older and previously mated males produce ejaculates of poorer quality  
393 compared with young virgin males, which allows for several conclusions. First,  
394 neither spermatophore size nor sperm number indicate ejaculate quality, although  
395 both are widely used as proxies for male and ejaculate quality (Snook 2005; Wedell

396 2005). Second, the fitness advantages found for females having mated with young  
397 virgin males must result from a higher quality of sperm and / or accessory gland  
398 products. Sperm quality often diminishes with age resulting in reduced sperm viability  
399 and / or motility (Wedell and Ritchie 2004; Damiens and Boivin 2006). The high egg-  
400 hatching success, found at least for early eggs when having mated with young virgin  
401 males, may point in this direction. Regarding accessory gland products,  
402 spermatophores produced by young virgin males may contain more resources (e.g.,  
403 nitrogenous compounds) being of critical importance to females (South and Lewis  
404 2011, 2012). These compounds are to a large extent carried over from the larval  
405 stage in holometabolous insects and cannot be replenished in the adult stage (Cook  
406 and Wedell 1996; Boggs 1997; Jervis et al. 2005). Thus, the higher reproductive  
407 output found for females having mated with young virgin males may be caused by  
408 using resources transferred by the males upon mating, which may be less abundant  
409 in older and previously mated males, either because they have been used for  
410 somatic maintenance or were transferred to a previous mating partner. Note though  
411 that spermatophore size in *B. anyana* is relatively low compared with gift-giving  
412 species (Ferkau and Fischer 2006). Third, the above considerations suggest that  
413 older or mated males may cheat females by producing spermatophores of a similar  
414 size compared with young once-mated males, but of a much lower quality. Indeed, it  
415 has been described in *B. anynana* that the spermatophores produced by older males  
416 contain more 'filling substances' that are cheap to produce such as water or apyrene  
417 sperm (Ferkau and Fischer 2006). Such a strategy is straightforward as female  
418 remating propensity is triggered by stretch receptors located in the bursa copulatrix in  
419 Lepidoptera, with larger spermatophores delaying remating for longer periods of time  
420 (Cook and Wedell 1999; Wedell 2005). Indeed, it is believed that the main function of

421 the occurrence of non-fertile apyrene sperm in the Lepidoptera is to delay remating  
422 and thus sperm competition (Cook and Wedell 1999; Solensky and Oberhauser  
423 2009; Wedell et al. 2009).

424

425 In line with the hypothesis that resources decrease with age, we found that  
426 antioxidant defences decreased with age in male *B. anynana*. As described in other  
427 animal species, low antioxidant defences in older males may cause reduced sperm  
428 quality (Aitken et al. 2014), which in turn may have negatively affected the  
429 reproductive success of females. However, we also found that oxidative damage  
430 decreased with age. This suggests that older butterflies have reduced their  
431 production of ROS compared with younger ones, for instance by reducing metabolic  
432 rate as observed in other Lepidoptera (Niitepõld and Hanski 2013; Niitepõld et al.  
433 2014). Antioxidant defences were found to increase while oxidative damage tended  
434 to decrease with mating frequency. These results are in agreement with recent  
435 studies that found positive effects of reproduction on the oxidative status of  
436 reproducing animals (Ołdakowski et al. 2012; Garratt et al. 2013; Costantini et al.  
437 2014) instead of the usually-assumed deleterious effects (Speakman and Garratt  
438 2014). One possible explanation may be that mating triggers a hormetic response by  
439 exposing males to higher ROS production, which subsequently increased antioxidant  
440 defences. This may cause a better protection against oxidative stress when  
441 reproducing again. In iteroparous organisms, this supposed hormetic effect is likely to  
442 play an important role to alleviate the reproductive cost of consecutive reproduction  
443 attempts.

444

445 All in all our study suggests that ejaculate quality clearly diminishes with age and  
446 mating number in *B. anynana*, although older once-mated males produce  
447 substantially larger spermatophores containing much more fertile sperm than  
448 younger ones. Therefore, both spermatophore size and sperm number should not be  
449 considered reliable proxies of male reproductive quality. The lower ejaculate quality  
450 of older males is clearly evidenced by pronounced detrimental effects on female  
451 reproduction. As in *B. anynana* older males were previously found to have a much  
452 higher mating success than younger males (Fischer et al. 2008; Karl et al. 2013; Kehl  
453 et al. 2014), these findings support the notion that old male mating advantage here  
454 arises from sexual conflict rather than female preference for older males (Karl and  
455 Fischer 2013). Given the rather strong fitness reductions in females having mated  
456 with older males reported here, it seems difficult to imagine that potential indirect  
457 genetic benefits would be able to compensate for the negative effects. Unlike in other  
458 insects (Lederhouse et al. 1990; Cook and Wedell 1996; Watanabe et al. 1998),  
459 spermatophore mass and eupyrene sperm numbers did not decline with male mating  
460 number, as even four-times mated males reached nearly the same spermatophore  
461 mass and eupyrene sperm numbers than 3-day old, single mated males. We assume  
462 this to result from high sperm production rates in combination with producing  
463 comparably small spermatophores (Rutowski et al. 1987; Lauwers and Van Dyck  
464 2006; Vande Velde et al. 2011). Finally, we found some evidence that variation in the  
465 oxidative status of male *B. anynana* butterflies may cause variation in reproductive  
466 traits, in turn affecting female fecundity, egg-hatching success, and reproductive  
467 success.

468

469

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473

474

475 **Conflict of interest**

476 The authors of this manuscript declare to have no conflict of interest.

477

478

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- 687

688 **Tables**

689

690 **Table 1** Overview of the experimental design used in experiments 1 and 2.

691

692 In total, 9 groups were used differing in mating number and / or age at mating.  
 693 Groups 1-4 were all mated only once, with mating taking place on day 3 (white), 6  
 694 (light grey), 9 (dark grey) or 12 (black) of adult life, thus investigating effects of male  
 695 age on the quality of first spermatophores. Groups 5-7 were either two-, three- our  
 696 four-times mated, being mated every three days (the single-mated males are  
 697 represented by group 1), thus investigating the effects of mating number (and age)  
 698 on spermatophore quality. Groups 8-9 were both mated two-times, with the first  
 699 mating occurring on day 6 and 9, respectively, thus investigating (along with group 5)  
 700 the effects of male age on the quality of second spermatophores by keeping the  
 701 number of matings and the time since the last mating constant. Numbers within grid  
 702 cells reflect mating numbers (first, second etc.). Note that throughout only the  
 703 spermatophore / data from the last mating of the respective male (given in bold) was  
 704 used to avoid dependent data.

705

Group	Day 3	Day 6	Day 9	Day 12
1	<b>1</b>			
2		1		
3			1	
4				<b>1</b>
5	1	2		
6	1	2	3	
7	1	2	3	<b>4</b>
8		1	2	
9			1	<b>2</b>

706

707

708 **Figure legends**

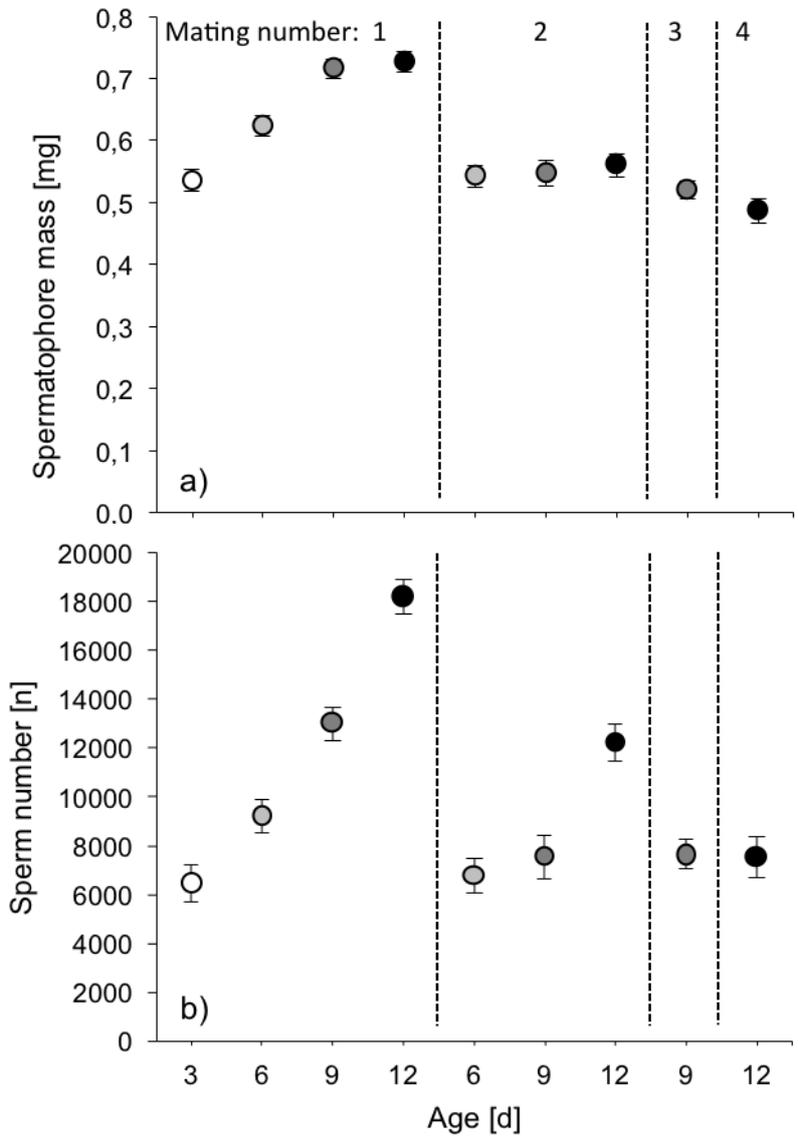
709

710 **Fig. 1** Spermatophore mass (a), eupyrene sperm numbers (b), total antioxidant  
711 capacity (OXY; c), oxidative damage (ROM; d), fecundity (e), estimated offspring  
712 number (f), early (g) and late egg-hatching success (h) as a function of age (3 days =  
713 white; 6 days = light grey; 9 days = dark grey; 12 days = black) and mating number  
714 (1: single-mated; 2: double-mated etc.) in male *Bicyclus anynana* butterflies (means  
715 + SE). OXY =  $\text{mmol}^{-1}\text{HOCl}$  neutralized \*  $\text{mg}^{-1}$ ; ROM =  $\text{mg H}_2\text{O}_2 \text{ dl}^{-1}$ \*  $\text{mg}^{-1}$ .

716

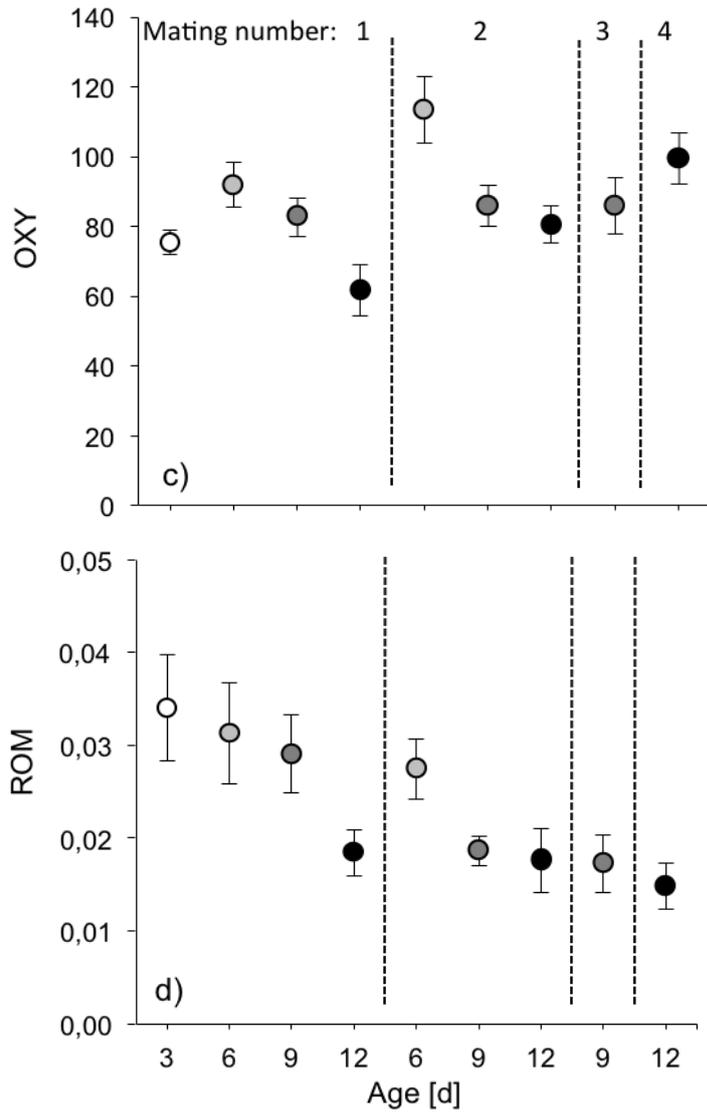
717

718 **Figures**



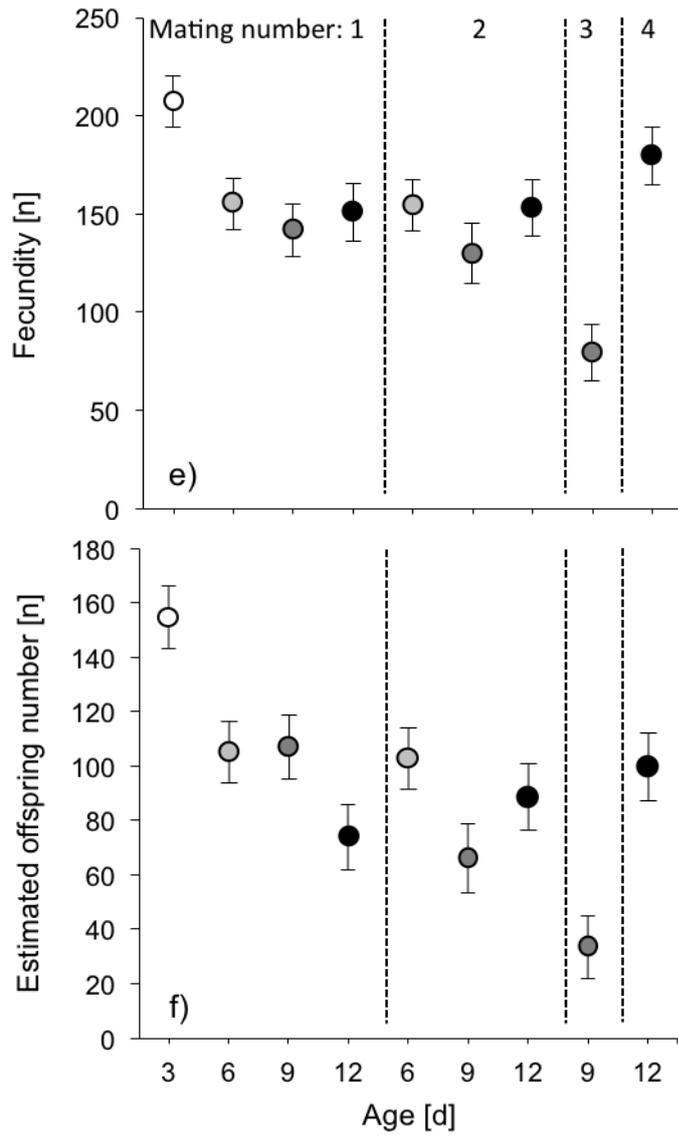
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720 Fig. 1 a & b



721

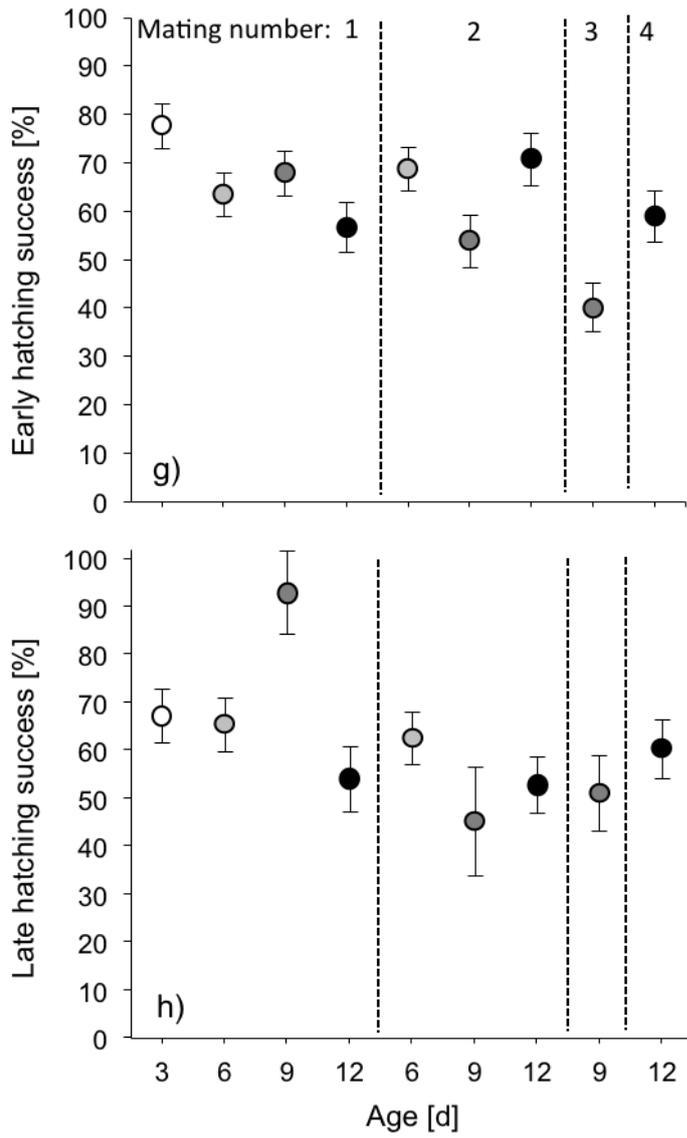
722 Fig. 1 c & d



723

724 Fig. 1 e & f

725



726

727 Fig. 1 g & h



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# Manuscript V

## Submitted as:

Kehl, T., Dublon, I.A.N. & Fischer, K. Male sex pheromones as honest signals?  
Sperm number and not pheromone titre is key in young male butterfly mating  
success.



1                   **Male sex pheromones as honest signals?**  
2                   **Sperm number and not pheromone titre is key**  
3                   **in young male butterfly mating success**

4  
5                   **Tobias Kehl<sup>1</sup>, Ian A.N. Dublon<sup>2</sup> & Klaus Fischer<sup>1</sup>**

6  
7                   <sup>1</sup>Zoological Institute & Museum, Greifswald University,

8                   Johann-Sebastian-Bach Str. 11/12, 17489 Greifswald, Germany

9                   <sup>2</sup>Evolutionary Ecology and Genetics Group, Biodiversity Research Centre,

10                  Earth and Life Institute, Université catholique de Louvain (UCL), Croix du Sud

11                                   4, 1348 Louvain-la-Neuve, Belgium

12  
13                  **Corresponding author:**

14                  Tobias Kehl

15                  Zoological Institute & Museum

16                  Greifswald University

17                  Johann-Sebastian-Bach Str. 11/12

18                  D - 17489 Greifswald, Germany

19                  Tel.: +49-3834-864268

20                  Fax: +49-3834-864252

21                  Email: [tobias.kehl@googlemail.com](mailto:tobias.kehl@googlemail.com)

22  
23                  **Keywords:** honest signals, individual fitness, intrasexual selection, male produced  
24                  sex pheromones, pheromone titre quantification, GC-FID.

25 **Abstract**

26 Intraspecific communication is of crucial importance throughout the animal kingdom  
27 and may involve a combination of visual, gustatory, olfactory and acoustic cues.  
28 Variation in male sex pheromone amount and composition may convey important  
29 information to females. However, whether increased male pheromone titres are  
30 associated with fitness benefits for the female conspecific, thus indicating a role as  
31 an honest signal, is currently largely unknown. Against this background, we tested in  
32 the butterfly *Bicyclus anynana* (1) whether young males being successful or  
33 unsuccessful in gaining a mating differ in sex pheromone titres and (2) for  
34 associations between male pheromone titres and spermatophore mass, fertile sperm  
35 number, and a variety of female and offspring life-history traits. Successful and  
36 unsuccessful males did not differ in pheromone titres, however fertile sperm number  
37 was much higher in successful males. Consequently, sperm count was the only  
38 factor significantly affecting male mating success. Pheromone titres were not  
39 associated with any fitness-related female or offspring trait measured, though  
40 correlation analyses yielded evidence for trade-offs among specific traits. Patterns  
41 did not differ among control and olfaction-blocked females. In young *B. anynana* we  
42 suggest that pheromone titres do not indicate male quality and thus do not amount to  
43 honest signals.

44

## 45 **1. Introduction**

46 Whilst historically reproduction has been regarded as being a largely harmonic event,  
47 a consensus has been reached during the last decades that sexual conflict is all  
48 pervasive [1]. The latter notion rests on the prediction that sexual selection should  
49 maximize an individual's own genetic reward [1,2], even if concomitant traits have  
50 detrimental effects on the respective partner [3–5]. Indeed, empirical studies have  
51 documented a wealth of examples for the occurrence of sexual conflict [5–9].  
52 Although there are exceptions, the operational sex ratio is typically shifted towards  
53 males throughout the animal kingdom, i.e. within a given population there are more  
54 receptive males than females available at any point in time [10–12]. Consequently,  
55 male reproductive success is often limited by access to receptive females, while  
56 females are able to choose among several potential partners [10–12]. Therefore,  
57 sexual selection in males often favours traits increasing the number of matings and /  
58 or sperm competitive ability [4,13]. Correlates of male reproductive success are, for  
59 instance, traits related to the resource holding potential such as body mass, fat  
60 content, weaponry, persistence and aggressiveness [14–16] or sperm number and  
61 motility [17,18].

62

63 In addition to male competitive ability, female choice is clearly important in  
64 determining male reproductive success [19,20]. Females may preferably mate with  
65 males showing a high resource holding potential which may directly benefit their  
66 offspring, or with males displaying specific cues typically being regarded as sexually  
67 selected signals [21,22]. The latter may involve visual (e.g. colours [23,24]), acoustic  
68 [25,26], olfactory [22,27–29] or gustatory cues [29,30]. If female mating decisions are  
69 at least partly based on sexual signals such as ornaments, the principal challenge

70 from the female perspective is to reliably assess male quality based upon these cues.  
71 Which information may specific colour patterns or odours convey to the females, and  
72 are they associated with any direct or indirect fitness benefits? Several studies  
73 provided convincing evidence for a covariation between sexually selected cues and  
74 male quality, thus suggesting honest signalling [23,31,32]. However, given that  
75 sexual conflict is widespread, such signals are prone to cheating as inferior males will  
76 be selected to nonetheless produce sexually attractive signals [33].

77

78 Sex pheromones are commonly used throughout the animal kingdom [34,35] where  
79 they play an important role in conspecific recognition and mating decisions [36–40]. If  
80 they were to function as honest signals indicative of mate quality, the production of  
81 sex pheromones is predicted to be costly for which there is indeed some evidence  
82 [41–43]. In insects, several studies revealed evidence that male sex pheromone  
83 (MSP) profiles may convey detailed information on mating partners [28,44,45],  
84 though evidence for a function as honest signals indicating male quality is scarce (but  
85 see [31,41,46])

86

87 Displaying a wide range of presumably sexually selected traits such as colour  
88 patterns or odour and undergoing rapid reproduction in the laboratory environment,  
89 butterflies have become important models for sexual selection [24,47,48]. Importantly  
90 multiple traits like chemical and optical cues have been suggested to be involved in  
91 mating decisions [22]. In Lepidoptera the role of sex pheromones as species-specific  
92 attractants facilitating long-distance intraspecific communication is very well  
93 documented with over 600 species [49] having their sex pheromones identified [34].  
94 Over shorter distances, communication may be facilitated by male produced

95 pheromones [36] though more work needs to be done in elucidating the role of these  
96 compounds and their effects upon male mating success [50]. Investigating factors  
97 affecting male mating success is especially interesting in groups such as butterflies,  
98 lacking any physical means or weaponry to defeat competitors, therefore largely  
99 excluding simple explanations [51].

100

101 In the butterfly *Bicyclus anynana* it has been recently shown that successful  
102 compared with unsuccessful males were characterised by higher MSP titres  
103 [27,28,48]. Therefore, this species is a suitable model for exploring the ultimate basis  
104 of variation in sexual signals. Are MSP titres associated with male quality or any  
105 fitness benefits for the females? Are they thus an honest signal indicative of male  
106 quality? Which benefits may females possibly derive from mating with males  
107 producing more sex pheromones, i.e. which traits might be associated with increased  
108 pheromone titres [41,52]? Alternatively, increased production of MSPs may be  
109 associated with male condition, in turn increasing competitive ability (e.g. more  
110 vigorous courtship) and thus mating success. This hypothesis rests on the  
111 assumption of a positive covariance between condition and sex pheromone  
112 production: males in a good shape may be able to both court more vigorously and  
113 produce more sex pheromones [53].

114

115 In principle, female preference for males with increased pheromone titres may be  
116 based on direct or indirect fitness benefits. Possible direct benefits in the *Bicyclus*  
117 system include the transfer of larger spermatophores, containing more accessory  
118 gland products and / or sperm, upon mating. Thus, a positive correlation between  
119 pheromone titre and spermatophore mass or sperm numbers is predicted.

120 Additionally, females may receive indirect benefits by ensuring that their offspring  
121 inherits 'good genes' from the preferred males by e.g. providing a survival advantage  
122 (e.g. [19,54]) or by the production of 'sexy sons' inheriting sexually attractive signals  
123 from the father, which will increase their reproductive success (Fisherian run-away  
124 process [33,55]).

125

126 Based on this background we here investigate associations between MSP titres and  
127 potential direct and indirect fitness benefits for females of the butterfly *B. anynana*.  
128 Partly, we compare patterns among control and olfaction-blocked females. If female  
129 choice was decisive in determining male mating success, control females (with intact  
130 olfactory receptors in their antennae) should prefer males with high pheromone titres,  
131 while a lack of difference among groups would favour alternative hypotheses such as  
132 a prominent role of male vigour. As proxies of direct fitness benefits we investigated  
133 spermatophore mass, the number of fertile sperm, and female longevity.  
134 Furthermore, we measured various offspring traits indicative of indirect fitness  
135 benefits.

136

137

## 138 **2. Material and methods**

### 139 **(a) Study organism and rearing conditions**

140 The Squinting Bush Brown *B. anynana* (Butler, 1879) is a nymphalid, fruit-feeding  
141 butterfly, whose distribution ranges from southern Africa to Ethiopia [56]. It exhibits  
142 striking phenotypic plasticity with two seasonal morphs, as an adaptation to alternate  
143 wet-dry seasonal environments and the associated changes in resting background  
144 and predation [57]. Reproduction takes place during the warmer wet season when

145 oviposition plants are abundantly available, and where 2-3 generations occur.  
146 Reproduction ceases during the colder dry season in which butterflies do not mate  
147 before the first rains at the beginning of the next wet season [58]. A laboratory stock  
148 population was established at Greifswald University, Germany, in 2008 from several  
149 hundred eggs derived from a well-established stock population at Leiden University,  
150 The Netherlands. The Leiden population was founded in 1988 from 80 gravid  
151 females caught at a single locality in Malawi. In each generation several hundred  
152 individuals are reared maintaining high levels of heterozygosity at neutral loci [59].  
153 For this study butterflies from the Greifswald stock population were used.

154

#### 155 **(b) Experimental design**

156 We performed two experiments to test for associations between MSP titres and other  
157 male traits and collected approximately 1000 eggs for each. Larvae were reared in  
158 population cages on potted maize plants under constant conditions at a 12/12h light-  
159 dark cycle, 27°C and 70 % relative humidity. The conditions used are similar to those  
160 at which *B. anynana* develops and reproduces during the favourable wet season in  
161 the field [58,60]. Resulting pupae were collected daily and transferred to cylindrical  
162 hanging cages. Following eclosion, individuals were separated by sex and eclosion  
163 day in order to avoid mating prior to experiments. Throughout the experiments all  
164 butterflies were supplied with moist banana and water for feeding *ad libitum*.

165

166 In experiment 1 we examined differences in MSP titre, spermatophore mass, and  
167 eupyrene (fertile) sperm number between successful and unsuccessful males. We  
168 performed 110 mating trials, 91 of which were successful in gaining a mating. In each  
169 trial two 2-day old males competed for either a control or an olfaction-blocked 2-day

170 old female in a cylindrical hanging cage (30 cm diameter, 15 cm height). One day  
171 prior to the respective mating trial, olfaction-blocked females were treated with a  
172 transparent, quickly drying nail polish (Essence; Colour & Go, Cosnova GmbH,  
173 Sulzbach, Germany) on the club surface of their antennae [22]. To control for  
174 confounding solution effects, the control group received a sham-treatment by  
175 applying nail polish on the right anterior forewing as opposed to the antennae. Cages  
176 were monitored for a maximum of 6 hours or until a mating occurred. Unsuccessful  
177 males (n = 91) were afterwards (i.e. on the same day) mated in groups with at least  
178 as many naïve females to obtain their spermatophores. In order to avoid MSP  
179 volatilisation and to prevent sperm cells from dispersing out of the bursa copulatrix  
180 into the spermatheca, all males and females were placed into glassine envelopes  
181 and placed into a container (Air Liquide, Voyageur 12), cooled with liquid nitrogen  
182 immediately after mating and subsequently stored in a freezer at -80°C.

183

184 In experiment 2 we investigated associations between MSPs and female and  
185 offspring traits to test for direct and indirect fitness benefits associated with increased  
186 pheromone titres. We successfully performed 100 mating trials, in each of which one  
187 random 2-day old virgin male and one random 2-day old virgin female were set up for  
188 mating per cage (no-choice assays). As above, males were frozen immediately after  
189 mating had ceased, as described above and their wings were subsequently used to  
190 measure MSPs. Females were, in contrast to above, set up individually for egg-laying  
191 in 1 L translucent plastic pots containing a maize leaf as an egg-laying substrate and  
192 moist banana for feeding [61–63]. The first ca. 30 eggs produced per female were  
193 used to score egg-hatching success. The other eggs were transferred, separated by  
194 female, to elongated sleeve-like gauze cages. Each ‘sleeve’ cage thus contained one

195 full-sib family ( $n = 87$ ). Thirteen females produced no offspring or were lost during  
196 egg-laying. Larval density was standardised to a maximum of 30 larvae per sleeve.  
197 We scored the female partner's longevity, lifetime fecundity, and egg hatching  
198 success as well as the following offspring traits: larval and pupal survival, pupal  
199 development time, pupal mass, adult mass, and wing size. To investigate egg-  
200 hatching success, eggs were transferred to petri dishes containing moist filter paper  
201 in order to prevent desiccation. Eggs were checked daily until no more larvae  
202 hatched for at least 48 hours. Pupal and adult mass were measured on a  
203 microbalance (Kern ABJ 120-4M) to the nearest 0.01 mg. Wings were photographed  
204 with a digital camera (Leica DC300) connected to a stereo microscope (Leica M275)  
205 to subsequently measure forewing length using the software NIS Elements (Nikon  
206 Instruments).

207

### 208 **(c) Quantification of MSPs**

209 Male wings were used for pheromone extractions following established protocols for  
210 this species [27,29,64]. For each male, one fore and one hind wing were carefully  
211 removed from the thorax using dissection scissors. Afterwards, wings were  
212 submerged for 10 minutes in 350  $\mu\text{L}$  hexane (98%, HPLC grade) containing an  
213 internal C15 standard ( $10 \text{ ng } \mu\text{l}^{-1}$  trans-4-tridecenyl acetate (Sigma Aldrich)).

214

215 For pheromone chromatography and quantification we used a gas chromatograph  
216 (Agilent GC7890A) in conjunction with a flame ionisation detector (Agilent  
217 Technologies, Belgium; GC-FID). A  $30 \mu\text{m} \times 320 \mu\text{m} \times 0.25 \mu\text{m}$  DB-5 phase column  
218 (Agilent, 19091J-413) was run in constant flow mode with laboratory generated  $\text{H}_2$   
219 carrier gas. In the 20-minute temperature program, the initial temperature of  $75^\circ\text{C}$

220 was held isothermally for 3 minutes, then ramped at  $20^{\circ}\text{C min}^{-1}$  until  $220^{\circ}\text{C}$ , after  
221 which the ramping rate was increased to  $30^{\circ}\text{C min}^{-1}$  until  $300^{\circ}\text{C}$ . The final  
222 temperature was held constant for 7 min. The FID was heated to  $250^{\circ}\text{C}$  with  $\text{H}_2$  flow  
223 set to  $30\text{ ml min}^{-1}$ , air (Standard air, Praxair, Schoten, Belgium) at  $350\text{ ml min}^{-1}$ , and  
224  $\text{N}_2$  (Praxair) makeup at  $20\text{ ml}^{-1}$ . Hydrogen was generated from high purity distilled  
225 water (Barnstead Easy Pure II, Thermo Fisher Scientific, Erembodegem Belgium)  
226 using a Peak PH300 gas generator (Peak Scientific, Inchinnan, Scotland).

227

228 Hexane samples containing extracted pheromones were injected into the GC-FID  
229 using a 7693 ALS autosampler (Agilent), injecting  $1\ \mu\text{l}$  in the standard injection  
230 mode.. Injections were made in splitless mode and samples were deposited into a 2  
231 mm quartz direct injection liner (Agilent 518-8818) providing  $250\ \mu\text{l}$  volume. Injector  
232 temperature was held at  $250^{\circ}\text{C}$  and 14.23 psi with a septum purge flow of  $3\text{ ml min}^{-1}$ ,  
233 and a purge time of 1.5 min at  $40\text{ ml min}^{-1}$ . Male *B. anynana* butterflies have three  
234 MSPs (Z9-14:OH (MSP1), 16:Ald (MSP2) and 6,10,14-trime-15-2-ol (MSP3) [27].  
235 Under the chosen conditions trans-4-tridecenyl acetate eluted on average at 7.74  
236 min, MSP1 at 7.57 min, MSP2 at 8.39 min, and MSP3 at 8.56 min. MSP retention  
237 times were confirmed through injection of a 1:1:1 pheromone mixture [ $3\text{ ng }\mu\text{l}^{-1}$ ]  
238 prepared from external standards (kindly synthesized at Mittuniversitetet, Sundsvall,  
239 Sweden). All acquisitions and integrations were conducted with GC Chemstation  
240 B.04.03-SP2 [105] (Agilent). No column compensation algorithms were used as  
241 bleed was insignificant during the relevant portion of the temperature cycle.

242

243 **(d) Analyses of spermatophore mass and eupyrene sperm cells**

244 To analyse spermatophore mass and eupyrene sperm numbers, females were  
245 thawed and dissected in Ringer's solution [65]. The bursa copulatrix, which contains  
246 the male spermatophore, was removed. Surplus Ringer's solution was removed from  
247 the bursa using filter paper, and afterwards the bursa was weighed on an  
248 electrobalance (Sartorius LE225D) to the nearest 0.01 mg. Thus, the mass of the  
249 bursa copulatrix containing the spermatophore was used as a proxy of  
250 spermatophore mass, as the mass of the bursa is negligible [66]. After weighing, the  
251 spermatophore was transferred to a cavity slide with a droplet of Ringer's solution,  
252 opened with forceps and stirred gently to disperse the sperm. Eupyrene (fertile)  
253 sperm bundles were counted within the cavity slide using a microscope (Zeiss ICS  
254 KF2, at 40x). To achieve the absolute number of eupyrene sperm the number of  
255 bundles was multiplied by 256, owing to the fact that in Lepidoptera all the sperm in a  
256 bundle originate from a single spermatogonium, which undergoes a fixed number of 8  
257 divisions [66–68].

258

### 259 **(e) Statistical analyses**

260 In experiment 1, differences between successful and unsuccessful males in the traits  
261 measured were first analysed using paired t-tests. Afterwards, generalized linear  
262 models with binomial error distribution and logit-link function were constructed for  
263 both control and olfaction-blocked female groups. As MSP1, MSP2, and MSP3 were  
264 highly correlated to each other (all pairwise  $r$ -values  $> 0.45$ ;  $p < 0.001$ ) we performed  
265 a principal component analysis for each data set to reduce the number of interrelated  
266 variables. For further analyses we used in each case the first principal component  
267 (PC). PC1 had an eigenvalue of 2.19 (all other eigenvalues  $< 0.58$ ) and explained  
268 73.1 % of the total variation in the control group, and an eigenvalue of 2.16 (all other

269 eigenvalues < 0.64) and explained 72.1 % of the total variation in the olfaction-  
270 blocked female group. Furthermore, to account for the statistical dependency of the  
271 data derived from individual mating trials, we calculated the difference between  
272 successful and unsuccessful males for each trait, thereby generating a single value  
273 per male pair. One male of each trial was randomly defined as the 'focal' individual,  
274 i.e. the one from which the values of the opponent were subtracted. This procedure  
275 yielded difference values for each mating trial and trait, which were subsequently  
276 used in the generalised linear models by encoding unsuccessful focal males with '0'  
277 and successful focal males with '1'. Models were constructed based on  
278 spermatophore mass, sperm number, and the PC reflecting spermatophore titres by  
279 stepwise forward inclusion of significant factors. Pearson correlations were used to  
280 investigate correlations between sex pheromones and other traits.

281

282 To analyse the data obtained in experiment 2 we used Pearson correlations testing  
283 for associations between MSPs and female and offspring traits. As above we  
284 performed a principal component analysis based on the three males sex  
285 pheromones. We used the resulting first PC for correlation analyses, having an  
286 eigenvalue of 2.7 (all other eigenvalues < 0.7) and explaining 90.9 % of the total  
287 variation in MSPs. We additionally computed a Pearson correlation matrix involving  
288 all female and offspring traits. All statistical analyses were performed using Statistica  
289 8.0 (StatSoft).

290

291

## 292 **3. Results**

### 293 **(a) Experiment 1**

294 Univariate comparisons revealed that in both treatments successful compared with  
295 unsuccessful males had significantly higher numbers of eupyrene sperm, whereas  
296 differences in spermatophore mass and MSPs were not significant (Table 1).  
297 Accordingly, generalised linear models revealed that eupyrene sperm number was  
298 the sole factor significantly affecting male mating success in both the female control  
299 (Wald- $\chi^2 = 10.24$ ,  $p = 0.0014$ ) and the olfaction-blocked group (Wald- $\chi^2 = 9.09$ ,  $p$   
300  $= 0.0026$ ). Correlation analyses revealed that spermatophore mass was significantly  
301 positively related to pheromone titres (except for MSP2) in both treatment groups  
302 (Table 2). Sperm numbers though were significantly positively related to pheromone  
303 titres (except for MSP2) in olfaction-blocked females only.

304

## 305 **(b) Experiment 2**

306 The titres of the three MSPs were strongly correlated with each other and the  
307 resulting PC (Table 3), but not with any other trait measured except for marginal  
308 positive correlations of MSP2 and the pheromone PC with male pupal development  
309 time (Table 3). The latter correlations though are not significant after Bonferroni  
310 correction. However, several significant correlations were detected between female  
311 and offspring traits (Table 4). As the majority of these would not be significant after  
312 Bonferroni correction, we refrain from mentioning each significant correlation.  
313 Instead, we highlight patterns that may bear biological relevance. Longevity was  
314 positively related to lifetime fecundity. Early fecundity was positively related to lifetime  
315 fecundity, but negatively to pupal survival and several body size measures. Lifetime  
316 fecundity was positively related to male pupal time, but negatively to pupal survival  
317 rate and male wing length. Pupal survival rate was positively related to body size.

318 Different size measures were generally positively correlated, as was the case for  
319 male and female pupal development time.

320

#### 321 **4. Discussion**

322 In our model system the operational sex ratio is clearly shifted towards males, as  
323 female *B. anynana* typically mate only 1-2 times within their lifespan while males are  
324 able to mate multiple times [60,69]. Therefore, sexual selection is predicted to favour  
325 increased male competitive ability while females are predicted to be the choosy sex.  
326 Indeed, evidence suggests that both male aggressiveness and willingness to persist  
327 and female choice contribute to male mating success in *B. anynana* [21,70–72].

328

329 Here, experiment 1 did not reveal evidence for a decisive role of naturally occurring  
330 variation in pheromone blends of young males for female mating decisions, as  
331 successful and unsuccessful males did not differ in pheromone titres. While a lack of  
332 differences was expected for the olfaction-blocked females, the control females did  
333 on principle have the possibility to discriminate between males based on pheromone  
334 titres, which obviously was not the case. This finding contrasts with those of [48]. An  
335 important difference to all previous studies having found positive effects of  
336 pheromone titres in *B. anynana* is that we here exclusively used naturally occurring  
337 variation. In [48] pheromone titres were experimentally manipulated by surgically  
338 removing the androconia and afterwards perfuming males with different synthetic  
339 pheromones blends. Perhaps differences in pheromone titres among random males  
340 are much smaller than those induced by manipulative conditions. Thus, positive  
341 results based on experimental manipulations [48,71] may actually indicate an  
342 important role of sex pheromones in species recognition rather than female choice.

343 This notion is supported by the fact that olfaction-blocked females are generally much  
344 more reluctant to mate when compared with control females, and that control females  
345 strongly discriminate against largely pheromone-deprived males [22,71]. In this work,  
346 we tested young males and while they are sexually mature, they are in accordance  
347 with previous studies [48] very low in MSP2 (hexadecanal).

348

349 Most interestingly preferred males transferred substantially higher sperm numbers to  
350 females compared with non-preferred males, suggesting that mating success is not  
351 random despite a lack of observed differences in pheromone blend. Note that  
352 unsuccessful males were mated in groups with many males and females. Thus, the  
353 perceived risk of sperm competition should be high in unsuccessful males such that a  
354 lack of sperm competition risk cannot explain their reduced sperm numbers. Either  
355 way these data cast doubt on the notion that female choice is of particular importance  
356 in this system. The most straight-forward explanation seems to be that successful  
357 males generally have a better body condition (e.g. having a higher fat content) as has  
358 been found in a previous study [73]. A better condition may allow for both more  
359 vigorous courtship and the production of more sperm. Interestingly sperm numbers  
360 differed among successful and unsuccessful males while spermatophore mass did  
361 not, supporting the notion that the latter may be a poor proxy of male quality [73].  
362 Male *B. anynana* may cheat females by increasing the water content of the  
363 spermatophore, which may delay female remating and thus reduce the males' risk of  
364 sperm competition [74] .

365

366 Although only eupyrene sperm numbers differed among successful and unsuccessful  
367 males, spermatophore mass and partly euyprene sperm numbers were positively

368 related to MSP titres. The fact that such correlations did not result in significant  
369 variation in pheromone blends at least suggests that the latter are of subordinate  
370 importance. This notion is further supported by the data derived from our no-choice  
371 experiment. No significant correlation between male pheromones and any trait  
372 investigated was found, suggesting that pheromone titres in young males do not  
373 provide reliable information on male quality. Thus, if females would prefer males with  
374 higher titres, they would not be able to derive fitness benefits, at least not with regard  
375 to the traits investigated here.

376

377 Taken together our findings suggest that in young males, condition and perhaps  
378 courtship vigour are important for mating success, while we found no evidence for a  
379 significant role of MSPs for female mating decisions. This is in line with earlier  
380 findings suggesting a prominent role of male behaviour rather than female choice  
381 based on MSPs in this species [48,70,71,75]. For instance, old male mating  
382 advantage persisted in spite of a manipulating female olfaction or male pheromone  
383 blend, presumably based on their more aggressive behaviour [48,71]. Moreover, we  
384 found no evidence that pheromones are associated with any fitness-related trait  
385 measured in either females or their offspring. Of course, our findings do not rule out  
386 though that sex pheromones may be associated with other fitness-related parameters  
387 not investigated here.

388

389 Experiment 2 revealed several correlations across life-history traits. Although testing  
390 for such correlations was not the principal aim of our experiment, we would like to  
391 highlight at least some patterns presumably bearing biological significance. As  
392 expected, lifetime fecundity was strongly related to early fecundity, indicating that the

393 majority of eggs are laid early within the oviposition period [69]. Lifetime fecundity  
394 was positively related to longevity, indicating that females living longer produce more  
395 eggs (cf. [73,76]). Both early and lifetime fecundity tended to negatively affect pupal  
396 development time and survival as well as offspring body size. These findings clearly  
397 suggest trade-offs between offspring quality and quantity [77]. The positive  
398 correlations between pupal (and larval) survival and measures of body size support  
399 the wide-held notion of fitness benefits of being large [78].

400

401 We conclude that in young male *B. anynana* male sex pheromones do not seem to  
402 function as an honest signal, indicative of male quality. We suggest that in this case  
403 and for young males first encountering a female the role of male sexual pheromones  
404 in conveying information on male quality may have been overestimated. Most results  
405 testing for an effect of MSPs on mating success cannot rule out that patterns arise  
406 from (1) a vital role of MSPs in species recognition [44,70,71] or (2) a positive  
407 correlation of MSPs with overall condition, such that males in a good shape may be  
408 able to both court more vigorously and produce more sex pheromones [53]. Thus,  
409 variation in condition rather than pheromone titres may truly affect mating success.  
410 Future experiments should aim at disentangling these alternative hypotheses.

411

412

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419

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422 wrote the paper: T.K., I.A.N.D. and K.F.

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427

428 **Competing interests.** We have no competing interests.

429

430

431

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## Tables

**Table 1.** Comparisons of spermatophore mass, eupyrene sperm number, MSPs 1-3, and the principal component extracted from MSPs 1-3 (means  $\pm$  1 SD) between successful and unsuccessful males having competed for a single virgin female, including results of paired t-tests. Data are presented separately for control and olfaction-blocked females. Significant *p*-values are given in bold.

Trait	Successful	Unsuccessful	df	t	p
<b>a) Control</b>					
Spermatophore mass [mg]	0.468 ± 0.091	0.439 ± 0.136	40	1.140	0.2610
Eupyrene sperm [n]	7686.2 ± 3000.5	4583.0 ± 2828.1	40	4.901	<b>&lt; 0.0001</b>
MSP 1 [ng * 350 µl <sup>-1</sup> ]	1680.9 ± 673.2	1487.1 ± 819.5	41	1.444	0.1565
MSP 2 [ng * 350 µl <sup>-1</sup> ]	87.2 ± 42.5	96.5 ± 49.8	33	-0.987	0.3306
MSP 3 [ng * 350 µl <sup>-1</sup> ]	5556.6 ± 2780.2	5159.8 ± 2889.7	41	0.784	0.4378
PC MSP	-0.0007 ± 0.975	-0.0413 ± 1.074	33	0.208	0.8365
<b>b) Blocked</b>					
Spermatophore mass [mg]	0.476 ± 0.090	0.457 ± 0.155	35	0.746	0.4609
Eupyrene sperm [n]	8732.4 ± 3429.6	4394.7 ± 2186.4	35	6.990	<b>&lt; 0.0001</b>
MSP 1 [ng * 350 µl <sup>-1</sup> ]	1726.7 ± 720.1	1731.8 ± 903.7	42	-0.038	0.9698
MSP 2 [ng * 350 µl <sup>-1</sup> ]	83.3 ± 39.3	89.8 ± 41.6	38	-0.741	0.4632
MSP 3 [ng * 350 µl <sup>-1</sup> ]	5595.3 ± 2959.9	5519.9 ± 3138.2	42	0.149	0.8824
PC MSP	0.0067 ± 0.940	-0.0823 ± 1.090	38	0.526	0.6016

**Table 2.** Pearson correlations between MSPs 1-3 as well as the principal component (PC) extracted from MSPs 1-3 and spermatophore mass and eupyrene sperm number. Given are correlation coefficients and *p*-values. Data are presented separately for control and olfaction-blocked females. Significant *p*-values are given in bold.

	MSP 1	MSP 2	MSP 3	PC MSP
<b>a) Control</b>				
Spermatophore mass	0.4896	0.1984	0.5018	0.4692
	<b><i>p</i> &lt; 0.001</b>	<i>p</i> = 0.090	<b><i>p</i> &lt; 0.001</b>	<b><i>p</i> &lt; 0.001</b>
Sperm number	0.2019	0.1680	0.1172	0.1850
	<i>p</i> = 0.084	<i>p</i> = 0.152	<i>p</i> = 0.320	<i>p</i> = 0.115
<b>b) Blocked</b>				
Spermatophore mass	0.3106	0.1349	0.2946	0.3001
	<b><i>p</i> = 0.007</b>	<i>p</i> = 0.252	<b><i>p</i> = 0.011</b>	<b><i>p</i> = 0.009</b>
Sperm number	0.2573	0.1284	0.2962	0.2761
	<b><i>p</i> = 0.027</b>	<i>p</i> = 0.275	<b><i>p</i> = 0.010</b>	<b><i>p</i> = 0.017</b>

**Table 3.** Pearson correlations between MSPs 1-3 as well as the principal component (PC) extracted from MSPs 1-3 and an array of traits. Traits 1-4 were measured in female mating partners, traits 5-14 in the respective females' offspring, and traits 15-17 in male mating partners. Given are correlation coefficients and *p*-values. Significant *p*-values are given in bold.

No.	Trait	MSP 1	MSP 2	MSP 3	MSP PC
1	Female longevity	-0.0780 <i>p</i> = 0.492	-0.0806 <i>p</i> = 0.477	-0.0754 <i>p</i> = 0.506	0.0815 <i>p</i> = 0.472
2	Early fecundity (until day 10)	0.0924 <i>p</i> = 0.415	0.1211 <i>p</i> = 0.285	0.0900 <i>p</i> = 0.427	-0.1055 <i>p</i> = 0.353
3	Lifetime fecundity	0.1094 <i>p</i> = 0.334	0.0951 <i>p</i> = 0.401	0.0878 <i>p</i> = 0.439	-0.1019 <i>p</i> = 0.369
4	Egg-hatching success	-0.0264 <i>p</i> = 0.816	-0.0986 <i>p</i> = 0.384	-0.0968 <i>p</i> = 0.393	0.0769 <i>p</i> = 0.498
5	Larval survival rate	0.0891 <i>p</i> = 0.432	0.1401 <i>p</i> = 0.215	0.0820 <i>p</i> = 0.469	-0.1079 <i>p</i> = 0.341
6	Pupal survival rate	-0.0531 <i>p</i> = 0.640	-0.0375 <i>p</i> = 0.741	-0.0384 <i>p</i> = 0.735	0.0450 <i>p</i> = 0.692
7	Pupal mass (males)	-0.0216 <i>p</i> = 0.849	0.0438 <i>p</i> = 0.699	-0.0225 <i>p</i> = 0.843	0.0007 <i>p</i> = 0.995
8	Adult mass (males)	-0.0377 <i>p</i> = 0.740	-0.0660 <i>p</i> = 0.561	-0.0163 <i>p</i> = 0.886	0.0414 <i>p</i> = 0.715
9	Wing length (males)	-0.0379 <i>p</i> = 0.739	0.0277 <i>p</i> = 0.807	-0.0274 <i>p</i> = 0.809	0.0137 <i>p</i> = 0.904
10	Pupal time (males)	0.2164 <i>p</i> = 0.054	0.2506 <b><i>p</i> = 0.025</b>	0.1841 <i>p</i> = 0.102	-0.2263 <b><i>p</i> = 0.044</b>
11	Pupal mass (females)	-0.0299 <i>p</i> = 0.792	0.0070 <i>p</i> = 0.951	-0.0497 <i>p</i> = 0.661	0.0258 <i>p</i> = 0.820
12	Adult mass (females)	0.0565 <i>p</i> = 0.619	0.0472 <i>p</i> = 0.678	0.0651 <i>p</i> = 0.566	-0.0590 <i>p</i> = 0.603
13	Wing length (females)	-0.0306 <i>p</i> = 0.788	0.0232 <i>p</i> = 0.838	0.0183 <i>p</i> = 0.872	-0.0035 <i>p</i> = 0.975
14	Pupal time (females)	0.1345 <i>p</i> = 0.234	0.1929 <i>p</i> = 0.087	0.1261 <i>p</i> = 0.265	-0.1574 <i>p</i> = 0.163
15	MSP 1		0.8653 <b><i>p</i> &lt; 0.001</b>	0.9281 <b><i>p</i> &lt; 0.001</b>	0.9742 <b><i>p</i> &lt; 0.001</b>
16	MSP 2			<b>0.8227</b> <b><i>p</i> &lt; 0.001</b>	<b>0.9350</b> <b><i>p</i> &lt; 0.001</b>
17	MSP 3				<b>0.9599</b> <b><i>p</i> &lt; 0.001</b>

**Table 4.** Pearson correlation matrix among various traits measured in females (traits 1-4) and their offspring (5-14). Given are correlation coefficients and *p*-values. Significant *p*-values are given in bold.

	2	3	4	5	6	7	8	9	10	11	12	13	14
1 Female longevity	0.2017	0.0018	0.4543	0.0741	-0.2062	0.1700	-0.0215	0.1730	0.1261	0.1524	0.0785	0.0456	0.0395
2 Early fecundity (until day 10)	<i>p</i> = 0.073	0.987	<b><i>p</i> &lt; 0.001</b>	<i>p</i> = 0.513	<i>p</i> = 0.066	<i>p</i> = 0.132	<i>p</i> = 0.850	<i>p</i> = 0.125	<i>p</i> = 0.265	<i>p</i> = 0.177	<i>p</i> = 0.489	<i>p</i> = 0.688	<i>p</i> = 0.728
3 Egg-hatching success		0.0816	0.8082	-0.1074	-0.3382	-0.2714	-0.1278	-0.4017	0.1784	-0.4408	-0.2509	-0.3486	0.0867
4 Lifetime fecundity		<i>p</i> = 0.472	<b><i>p</i> &lt; 0.001</b>	<i>p</i> = 0.343	<b><i>p</i> = 0.002</b>	<b><i>p</i> = 0.015</b>	<i>p</i> = 0.259	<b><i>p</i> &lt; 0.001</b>	<i>p</i> = 0.113	<b><i>p</i> &lt; 0.001</b>	<b><i>p</i> = 0.025</b>	<b><i>p</i> = 0.002</b>	<i>p</i> = 0.444
5 Larval survival rate			0.0629	-0.0265	-0.0220	-0.0837	-0.0647	-0.1766	0.0533	0.0695	0.1178	-0.0200	0.0643
6 Pupal survival rate			<i>p</i> = 0.579	<i>p</i> = 0.815	<i>p</i> = 0.846	<i>p</i> = 0.460	<i>p</i> = 0.568	<i>p</i> = 0.117	<i>p</i> = 0.639	<i>p</i> = 0.540	<i>p</i> = 0.298	<i>p</i> = 0.860	<i>p</i> = 0.571
7 Pupal mass (males)				-0.0526	-0.3188	-0.1262	-0.0895	-0.3135	0.3264	-0.2072	-0.1096	-0.2189	0.1460
8 Adult mass (males)				<i>p</i> = 0.643	<b><i>p</i> = 0.004</b>	<i>p</i> = 0.285	<i>p</i> = 0.430	<b><i>p</i> = 0.005</b>	<b><i>p</i> = 0.003</b>	<i>p</i> = 0.065	<i>p</i> = 0.333	<i>p</i> = 0.051	<i>p</i> = 0.196
9 Wing length (males)				-0.1533	-0.1960	-0.2666	0.0069	0.0837	-0.0637	-0.1112	0.0498	0.1242	
10 Pupal time (males)				<i>p</i> = 0.175	<i>p</i> = 0.081	<b><i>p</i> = 0.017</b>	<i>p</i> = 0.951	<i>p</i> = 0.460	<i>p</i> = 0.575	<i>p</i> = 0.326	<i>p</i> = 0.661	<i>p</i> = 0.272	
11 Pupal mass (females)					0.2968	0.3201	0.2692	-0.0662	0.3484	0.1911	0.2899	-0.1917	
12 Adult mass (females)					<b><i>p</i> = 0.008</b>	<b><i>p</i> = 0.004</b>	<b><i>p</i> = 0.016</b>	<i>p</i> = 0.559	<b><i>p</i> = 0.002</b>	<i>p</i> = 0.090	<b><i>p</i> = 0.009</b>	<i>p</i> = 0.088	
13 Wing length (females)					0.5860	0.7311	0.0890	0.5120	0.2440	0.3823	-0.0952		
14 Pupal time (females)					<b><i>p</i> &lt; 0.001</b>	<b><i>p</i> &lt; 0.001</b>	<i>p</i> = 0.432	<b><i>p</i> &lt; 0.001</b>	<b><i>p</i> = 0.029</b>	<b><i>p</i> &lt; 0.001</b>	<i>p</i> = 0.401		
					0.3006	-0.1226	0.2264	0.1365	0.1468	-0.1861			
					<b><i>p</i> = 0.007</b>	<i>p</i> = 0.279	<b><i>p</i> = 0.043</b>	<i>p</i> = 0.227	<i>p</i> = 0.194	<i>p</i> = 0.098			
					-0.0756	0.4754	0.2755	0.4204	-0.0584				
					<i>p</i> = 0.505	<b><i>p</i> &lt; 0.001</b>	<b><i>p</i> = 0.013</b>	<b><i>p</i> &lt; 0.001</b>	<i>p</i> = 0.607				
					-0.0236	-0.0705	-0.1476	0.4015					
					<i>p</i> = 0.835	<i>p</i> = 0.534	<i>p</i> = 0.191	<b><i>p</i> &lt; 0.000</b>					
					0.6358	0.6490	0.1671						
					<b><i>p</i> &lt; 0.001</b>	<b><i>p</i> &lt; 0.001</b>	<i>p</i> = 0.139						
					0.5876	0.0345							
					<b><i>p</i> &lt; 0.001</b>	<i>p</i> = 0.761							
					-0.0452								
					<i>p</i> = 0.690								



# Publication list

**Kehl, T.**, Dublon, I. & Fischer, K. Male sex pheromones as honest signals? Sperm number and not pheromone titre is key in young male butterfly mating success. (submitted).

**Kehl, T.**, Beaulieu, M., Kehl, A. & Fischer, K. Old male sex: large ejaculate, many sperm, but few offspring. (submitted).

**Kehl, T.**, Bensch, J., Böhm, F., Kniepkamp, B.O., Leonhardt, V., Schwieger, S. & Fischer, K. Fat and sassy: factors underlying male mating success in a butterfly. *Entomologia Experimentalis et Applicata* (in press).

**Kehl, T.**, Burmeister, M.F.W.T., Donke, E., Köhn, N.A.K., Metschke, K., Pfender, D., Karl, I. & Fischer, K. (2014) Pheromone blend does not explain old male mating advantage in a butterfly. *Ethology* 120:1137-1145.

**Kehl, T.**, Karl, I. & Fischer, K. (2013) Old-male paternity advantage is a function of accumulating sperm and last-male precedence in a butterfly. *Molecular Ecology* 22:4289-4297.

**Kehl, T.** & Fischer, K. (2012) Larval starvation reduces responsiveness to feeding stimuli and does not affect feeding preferences in a butterfly. *Journal of Insect Physiology* 58:1028-1035.

