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Mate choice and sexual selection in a model butterfly species, *Bicyclus anynana*: state of the art

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Bicyclus anynana (Lepidoptera: Nymphalidae) is a widely used model species in evolutionary and developmental biology. This species is part of a highly diversified subtribe (Mycalesina) resulting from a rapid species radiation in which sexual selection, in concert with adaptation to new environment may have played an important role. In this paper, we review the current knowledge about sexual selection in *Bicyclus anynana*. We discuss in an integrated view all traits identified so far as being relevant in mate choice: white pupil of fore wing dorsal eyespots, male sex pheromone and to a lesser extent wing size. We discuss also the potential functional roles of these traits for species and sex recognition and as information source on mate quality. We aim at highlighting the consequences of the large diversity of methodological approaches used so far and we briefly underline understudied traits (*i.e.*, behavior) that could potentially be important for mate choice.

Keywords: courtship, sexual role, plasticity, sex pheromone, natural selection, eyespot, wing pattern, Nymphalidae, Satyrinae

A striking sexual dimorphism with males displaying secondary sexual structures, such as the feathery antennae of male moths, was described early in modern Evolutionary Biology (Darwin 1871). It caused astonishment because male exaggerated ornaments were likely to decrease individuals' survival and as such were in disagreement with the increasingly accepted theory of natural selection. Darwin himself proposed that sexual selection, rather than natural selection, could account for the presence and the evolution of these sex-specific traits (Darwin 1871). Sexual selection can be seen as a component of natural selection in which the reproductive success is determined by one sex in the population, not by the rest of the environment. Simply stated, sexual selection is a consequence of the fact that, in most species, females invest more in reproduction than do males. Females are therefore the limited resource in male-female sexual interactions: males usually compete for getting access to females and females

play the role of the choosy sex. Female choice is based on some male traits which, if heritable, will become more common and potentially more remarkable in the population across generations. In species in which males invest a lot in parental care, such as some species of waders, sexual selection is reversed and males play then the role of the choosy sex (Andersson 1994).

Since *The Descent of Man, and Selection in Relation to Sex* (Darwin 1871), the original debate between Darwin and Wallace about the relative importance of sexual vs. natural selection in producing sexual dimorphism has taken the front scene again. The importance of natural selection in driving sexual dimorphism in the Lepidoptera was recently reviewed in details (Allen *et al.* 2011). Mating success depends on (1) the ability to find and (2) be chosen by mates (mate choice). Firstly, the ability to find a mate includes the steps of mate prospection efficiency, and pugnacity or motivation during courtship, which are likely to be partly under natural rather than sexual selection. Second, mate choice is also composed of several steps, some of which may also be under natural rather than sexual selection: intra-sex conflicts (e.g., male-male fights), pre-copulation mate choice (i.e., female choosing between males), and post-copulation mate choice (e.g., cryptic female choice between the sperm of several mates) (Arnqvist & Rowe 2005).

Sexual selection is commonly explained by the following, nonexclusive, mechanisms (reviewed in Andersson & Simmons 2006):

-*Direct phenotypic effect or direct benefit*: male ornaments indicate their ability to provide a material advantage in case of reproduction (nuptial gifts, large territory or parental care). For example, many butterfly males transfer a spermatophore to the female during mating. Spermatophores may contain nutrients enhancing the female reproductive potential.

-*Indicator mechanism or indirect benefit*: male ornaments are linked to a genetic advantage it will transmit to the offspring.

-*Sensory bias*: female preference is here supposed to have initially evolved by natural selection (e. g. preference for a specific colour indicative of food quality) and secondarily exploited in male ornaments.

-*Fisherian runaway, theory of the sexy sons*: Female preference is directed towards male ornaments preferred by the majority of females, so that male offspring will display the preferred ornaments and will thus be preferred too. If both male ornament and female preference are variable and heritable, a self-reinforcing correlation between trait and preference can appear (runaway mechanism). The ornament or signal targeted by the preference can be arbitrary (i.e. not linked to any direct or indirect advantages), yet the runaway mechanism can strengthen other mechanisms of sexual selection.

-*Genetic compatibility*: mate quality may depend on mutual genetic affinities in a one-to-one combination rather than associated to intrinsic absolute differences

between individuals. For example, a selective advantage is provided by the maximisation of the genetic diversity of the major histocompatibility complex (genes coding for immunity), as more genetically divergent individuals produce better offspring. Whereas aforementioned mechanisms allow for the most part females to assess male quality, in this last mechanism females may also assess whether the encountered individual is of the right sex or of the right species (sex and species recognition systems) (Ryan & Rand 1993), in order to minimize genetic incompatibilities.

BICYCLUS AS A MODEL SPECIES

Here we will summarize the accumulating information affecting mating success in the tropical butterfly *Bicyclus anynana* (Butler, 1879) (Nymphalidae: Satyrinae). *Bicyclus anynana* was at first brought to the laboratory in order to study phenotypic plasticity of multicoloured rings present on the wings (eyespot) (Brakefield & Reitsma 1991). The most remarkable feature of this African species is indeed its seasonal polyphenism: a form typical of colder and drier seasonal environment (DSF) and a form representative of a hot and wet season (WSF) (Fig. 1; reviewed in Brakefield 2010). To fit the different ecological constraints of these alternating dry and wet seasons typical of the tropics, the differential expression of several traits is induced by the developmental temperature experienced by the larvae. Differences between the two forms concern morphological differences in wing patterns, physiology and life history traits (Brakefield 2010). Since these early studies, *B. anynana* has become a model invertebrate for more fields of research including evo-devo of butterfly eyespots, and the study of life-history and ageing processes (Brakefield 2010). The relative convenience of its all-year-round rearing, its short generation time, its size allowing marking-recapture protocols and the species richness of the *Bicyclus* genus (over 80 subspecies described; Condamin 1973) as a generous source of comparative data have made this species a model well suited for a broad range of techniques to study ecology, evolution and development (Brakefield *et al.* 2009).

Several studies have investigated sexual selection issues in *B. anynana*, and recently the courtship behaviour of male *B. anynana* was described in details (Nieberding *et al.* 2008). Male courtship is composed of a ritualized succession of four steps (orientation, flickering, thrust and attempting copulation) which allow the male to display its sexual ornaments and the female to accept or reject it. Previous studies showed that the presence of the white pupils in the centre of the dorsal forewing eyespots (Robertson & Monteiro 2005) and the production of male sex pheromone (Nieberding *et al.* 2008) contribute to male mating success. Mating success is also affected by wing size (Frankino *et al.* 2005, 2007) but see the corresponding discussion. Additional traits likely affect mate choice and sexual selection but have not been tested so far: male vigour during ‘fights’ (flight display of several males that seem to discourage each other of approach-

ing females) and courtship, and acoustic communication during courtship. We here discuss in an integrated view all traits identified so far as being relevant in mate choice and suggest the mechanism of sexual (or natural) selection acting on each trait. We also aim at highlighting the consequences of the large diversity of methodological approaches used so far in producing somehow biased or contradictory results across studies.

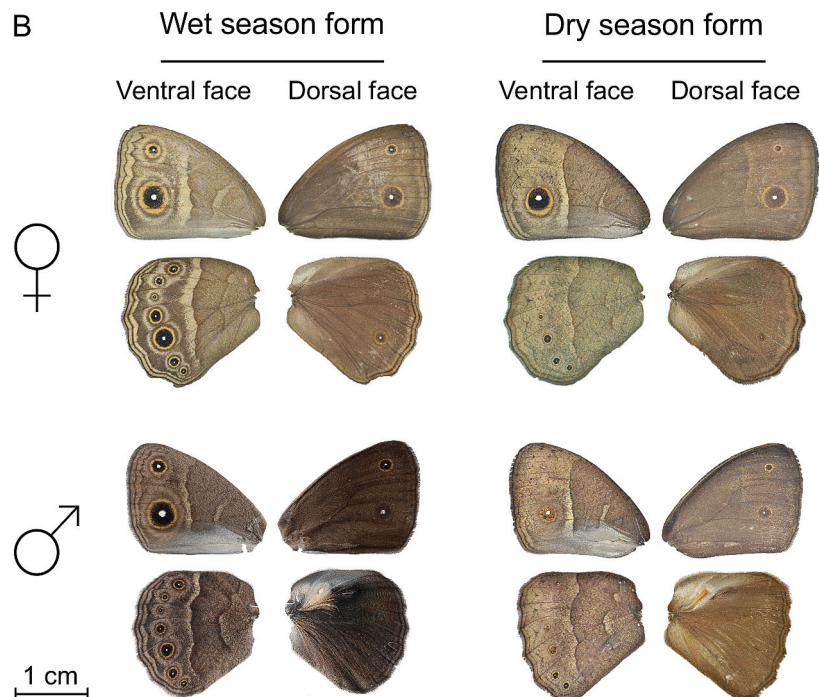
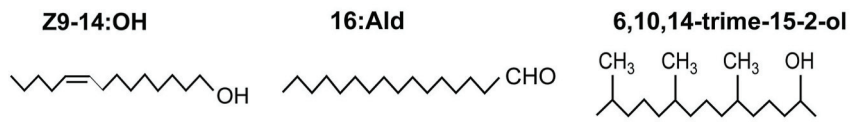
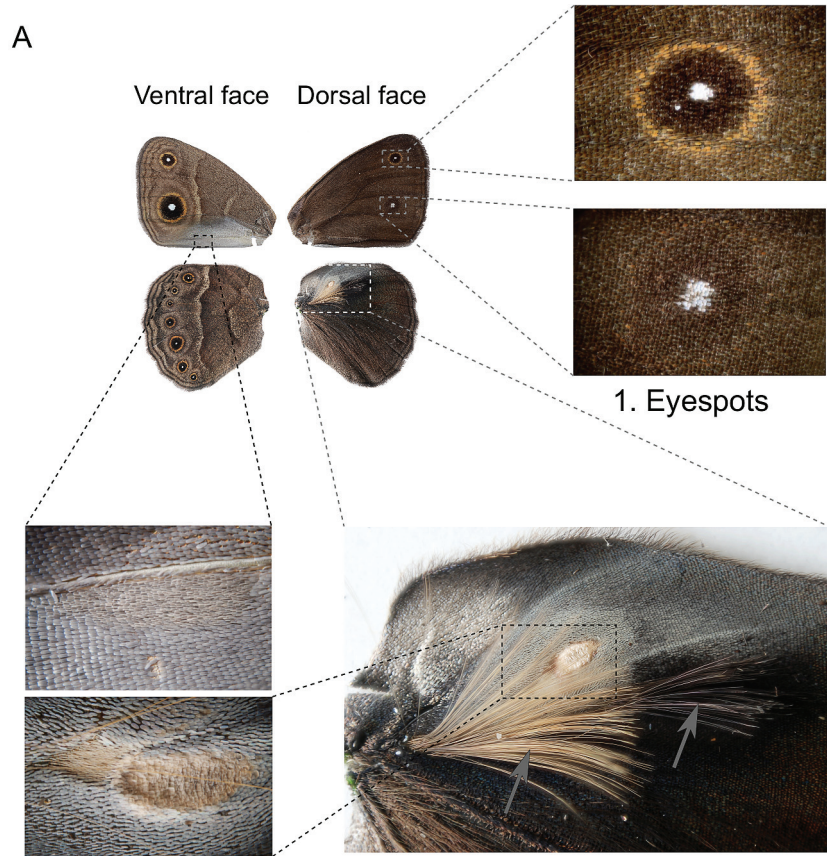
The experimental setup matters

Male mating success is assessed using behavioural experiments in which males are submitted to different treatments or put in different groups based on morphological differences and then competed in a 2:1 to 4:1 male-female sex ratio for mating success to wild-type virgin young females. Two main setups are used. The one most commonly used consists of a small cage (ca. 30 × 40 cm) in which a female is given the choice between 2-4 males, and couples are removed once a mating was initiated. Breuker & Brakefield (2002) add a second female which chooses between the remaining two males after the first couple has been removed. The second setup consists of releasing a large number of males and females in a tropical greenhouse to mimic their natural environment and densities (Frankino *et al.* 2005, 2007, Nieberding *et al.* 2008) [Fischer *et al.* (2008) used a large cage of about ca. 2 m³]. Male genitalia are then marked with coloured fluorescent powders which are transferred to the female genitalia during mating. The necessity for validations of behavioural data in semi-natural conditions was highlighted in *B. anynana* as the conditions of captivity may influence behavioural responses and affect the expression of key courtship traits (Joron & Brakefield 2003). In the large-scale greenhouse setup, both the ability of the male to locate and reach a potential mating partner and the close-range male courtship are assessed, so that the global male mating success is tested. This setup mimics the natural conditions of courtship better than small competition arenas, in which the first step of male mating success is not permitted. Additionally, mate availability is limited in small competition arenas, which is also likely to affect female choosiness.

Eyespots

Wing eyespots in *B. anynana* are composed of a white pupil surrounded by black and gold rings. They are present on the dorsal and ventral sides of the forewing and on the ventral hindwing of both sexes (Fig. 1). So far, most of the work on

Figure 1. (A) Traits known to be under sexual selection in *Bicyclus anynana* illustrated for a male of the wet season form: 1) UV reflectance of the white pupil on the forewing dorsal face eyespots; 2) Male sex pheromone components produced by the androconia situated on the ventral face of the forewing and the dorsal face of the hindwing (arrows point the hairpencils). (B) Ventral and dorsal coloration of the wet and dry season forms in males and females.



mating success in *B. anynana* focused on several features of these eyespots, which were tested for their effect in mate choice and sexual selection. Features include global size of eyespots, relative size of gold and black rings, and size, shape and UV reflectance of white pupils. Eyespot size treatments are based on the choice of extreme morphologies naturally occurring in the lab population (Robertson & Monteiro 2005), or produced by artificial selection (Beldade 2002), or by wing injury performed during wing development. In the latter case, injury of the future position of the eyespot or ectopic injury produces smaller than normal eyespots if the injury is done shortly after pupation, whereas later injury induces bigger than normal eyespots (French & Brakefield 1992; Brakefield & French 1995; Breuker & Brakefield 2002). Males with contrasting sizes of black and gold rings and of white pupils were obtained using artificial selection (Beldade *et al.* 2002; Allen *et al.* 2008). Eyespot pupils can be suppressed by applying black paint or a transparent solution that decreases UV reflectivity (Robertson & Monteiro 2005; Costanzo & Monteiro 2007; Prudic & Monteiro 2011).

Size and brightness of the ultraviolet reflecting pupils present on the dorsal forewing appear to be the only eyespot feature that matters to females. Pupil absence is strongly selected against, as are artificially enlarged pupils, whereas small to intermediate (normal sized) pupils seem to function equally well (Robertson & Monteiro 2005). To our knowledge, the relative importance of upper and lower dorsal forewing pupils has not been tested so far.

The other eyespots features (ventral forewing and hindwing eyespots, black and gold rings, eyespot global size) are not under direct mate choice. However, correlations between these features and white pupils [$R = 0.8-0.86$ for pupil and eyespot diameters (Nieberding *et al.* in prep.)] could lead to apparent sexual preference on the other traits. Moreover, contradictory results found by Breuker & Brakefield (2002), in which females preferred normal-sized black rings of the dorsal forewing eyespots compared to smaller ones, may arise from their methodological protocol. Breuker & Brakefield (2002) produced smaller than normal eyespots by damaging the cells positioned on the future eyespots early during development, and it is likely that such smaller eyespots had no pupil left (French & Brakefield 1995). As such, the absence of white pupils rather than eyespot size was likely counter-selected by females in their experiment.

Interestingly, although it is generally admitted that there is no sexual dimorphism in *B. anynana* eyespots (Brakefield *et al.* 1998; Breuker & Brakefield 2002), it was recently shown that pupils display seasonal, but also sexual, cryptic dimorphism: female pupil size is stable across the wet and dry seasons, but male pupils are approximately 3-6× smaller than female pupils in the wet or dry seasons, respectively (Prudic & Monteiro 2011). UV-reflectance of these white pupils varies significantly between seasons for both sexes (Prudic & Monteiro 2011). Such size differences are biologically meaningful as females display marked mate preferences for males with pupils within this range of size differ-

ences (4× larger pupils for ‘large’ males compared to ‘smaller’ ones; Robertson & Monteiro 2005). The existence of sexual dimorphism in this eyespot feature may matter for sex recognition: pupil size may indicate sex status as females avoid males with larger than normal pupils, a female characteristic (Breuker & Brakefield 2002; Robertson & Monteiro 2005). The presence of a male-specific sex pheromone (see below) is likely an additional signal for sex recognition but we cannot exclude multiple cues for sex recognition.

So far there is little experimental evidence about the information conveyed by eyespot features to females, which prevent us from predicting the long-term evolution of the traits across *Bicyclus* species. Oliver *et al.* (2009) suggest that the function of eyespots depends on the wing surface considered: dorsal forewing is under sexual selection, whereas ventral hindwings are associated to predator escape, a function often invoked for butterfly eyespots. Across *Bicyclus* species, dorsal forewing eyespots evolve more rapidly than eyespots from other wing surfaces in agreement with the higher rate of evolution usually displayed by sexually selected traits (Oliver *et al.* 2009). Moreover, dorsal forewing eyespots, together with three other traits involved in mate choice, evolve at different rates between males and females, further supporting their hypothesis.

Ventral hindwing eyespots would thus only be under natural selection pressures. However, the literature gives little evidence that *B. anynana* eyespots are associated to predator escape (Lyytinen *et al.* 2003, 2004; Vliieger & Brakefield 2007), but the efficiency of eyespots as predator escape features could depend on the context, *e.g.*, the light conditions during the experiment (Olofsson *et al.* 2010). Regarding dorsal forewing eyespots, varying levels of fluctuating asymmetry of eyespot pupils, a feature usually associated to male quality, was not associated to female mate choice (Breuker & Brakefield 2002, 2003). The left-right symmetry of the black rings and not of the white pupil size was here measured but it is likely that the treatment had an impact on both features of the eyespots (French & Brakefield 1995).

Thus, so far there is little information about the role of eyespots in conveying information about male quality to females. In addition to the aforementioned potential role of eyespots in sex recognition, eyespots may also play a role in species recognition, *i.e.*, in reproductive isolation, as a large variation is observed in the size of forewing dorsal eyespot size and of the black and gold rings across *Bicyclus* species (Brakefield & Roskam 2006; Brakefield 2010). Oliver *et al.* (2009) found also that several pairs of *Bicyclus* sister species differ in the composition of dorsal wing ornaments. However, these studies do not focus on the specific features of the eyespots (pupils) that play a role in female mate choice in *B. anynana*. Part of the interspecific variation of eyespots is probably correlated to pupil variation, but the range of phenotypic variation for the pupils (presence or absence of the two pupils, limited range of size) is limited compared to the variability of the other features of eyespots. We propose (but this remains

totally speculative) that the simple presence or absence or size of the pupils is not very species-specific but that the combination of their presence and of the male courtship behaviour (e.g., frequency of wing flickering) acts as a species-specific visual ‘Morse code’ to the female. It would also be timely to test whether females from species other than *B. anynana* use additional eyespot features in mate choice.

Wing size

Fore- and hindwing sizes are strongly correlated in *B. anynana* (Frankino *et al.* 2005). The first behavioural experiments were performed in small cages using the variation in size naturally occurring in the lab-population. These experiments concluded that mate choice is not influenced by differences in wing size (Breuker & Brakefield 2002; Robertson & Monteiro 2005). In contrast, ‘normal’-sized males displayed a higher mating success than large- or small-winged males obtained by artificial selection (fore- and hindwings selected in the same direction) and competed in large-scale, more natural-like, conditions (greenhouse experiments) (Frankino *et al.* 2005). The allometric relationship between fore- and hindwings is also important as the mating success of ‘normal’-sized males is much higher than males with artificially modified allometries (fore- and hindwings selected in the opposite directions, producing ‘large forewing and small hindwing’ males, and vice-versa) (Frankino *et al.* 2007). Alternatively, artificial selection on wing size could have modified correlated traits known to affect mate choice: pupil and androconia size (see below), which was not checked. Lastly, these results may be either due to female mate choice (and corresponding stabilizing sexual selection on wing size or correlated traits) or to the male aptitude to reach the female (natural selection on male flight ability).

Male sex pheromone (MSP)

Sex pheromone production is suppressed by applying a ‘scent-blocking’ nail solution on the androconia of young males (Robertson & Monteiro 2005; Nieberding *et al.* 2008), or by removing the androconia with fine scissors (Nieberding *et al.* 2008), before they start pheromone production. Costanzo & Monteiro (2007) recently showed using a small cage set-up that the androconia are important to *B. anynana* male mating success. Males for which the putative pheromone production of the fore- and hindwing androconial structures was blocked mated less often than control males in male-male competition experiments. They found that the chemicals emitted by both androconia were equally important for female choice. Moreover, they showed that the chemicals are likely to be perceived by the female antenna, because when the latter were blocked, females were no longer able to discriminate between scented and unscented males. Costanzo & Monteiro (2007) suggested that the MSP in *B. anynana* could be involved in sex recognition, because they observed that males would often attempt to court males that had their androconial structures blocked.

Recently, we have shown that the transfer of pheromone from males to females in *B. anynana* is probably associated with the flickering and thrust phases of courtship (Nieberding *et al.* 2008). We identified by gas chromatography coupled to electro-antennograms the male components that induced a female antennal response, characterized their chemical structure, namely (Z)-9-tetradecenol (Z9-14:OH), hexadecanal (16:Ald) and 6,10,14-trimethylpentadecan-2-ol (6,10,14-trime-15-2-ol), hereafter MSP₁, MSP₂ and MSP₃, respectively. We confirmed that the androconia are involved in their production which occurs after adult emergence. We also assessed the behavioural activity of these components in semi-natural conditions (Nieberding *et al.* 2008). Taken together, the absence of supplementary secondary sexual structures on the body of males (Condamin 1973), the absence of novel synthesized components by males reared on the native food diet, and the recovery of male mating success of perfumed operated males, suggest that the three identified components form the full MSP blend of *B. anynana* (Nieberding *et al.* 2008).

These results together highlight that the production of a MSP is important for mate choice. Yet, there is so far no published information about the existence of sexual selection acting on the MSP blend. Accumulating observations suggest that sexual selection on MSP composition is likely: MSP titres and ratios display a high variability in stock males of *B. anynana* providing an opportunity for sexual selection (Nieberding *et al.* 2008). Our analysis of courtship also revealed that virgin females frequently reject one or more males before accepting a mate, and that female rejection usually occurs directly after the flickering and thrust phases when MSP transfer probably occurs suggesting that females could use MSP to assess the quality of individual males. If sexual selection is acting on MSP composition, variation in MSP blend may be associated with variation in male ‘quality’ (*i.e.*, direct or indirect genetic benefits). The toolkit developed for *B. anynana* (*e.g.*, methodology of micro-surgical manipulations, synthetic pheromone components and experimental competition assays) enables the relevant experiments to be performed to investigate the type of information that is conveyed by *B. anynana* MSP. Finally, ongoing work on MSP diversity in the whole *Bicyclus* genus reveals large differences in MSP composition between species. Rapid evolution of the MSP signal suggests Fisherian runaway dynamics, and the differences between species could allow females to use MSP as a species recognition tool.

Sexual (and natural?) selection on the male side

Cost of reproduction in females is well known and studied, and one generally expects to observe trade-offs between life-history traits related to reproduction. For example, larval and adult diets affect egg size, a trait correlated to female fitness in most insect species (Bauerfeind & Fischer 2009)

It is now generally accepted that reproduction is costly for males as well, thus males too may be subjected to trade-offs between investment in reproduction

and other life-history traits such as longevity. We start to understand at which level male cost for reproduction may be positioned. *Bicyclus anynana* males produce a spermatophore that contains both fertile and non-fertile sperm. Both types are transferred to the female during copulation and non-fertile sperm can constitute up to 95% of an ejaculate (Lewis & Wedell 2007). Spermatophores in *B. anynana* are small in comparison to other Lepidoptera and do not contain large amounts of nuptial gift (Ferkau & Fischer 2006). Yet, spermatophores decrease in size between first and subsequent matings, and later spermatophores have a decreased dry mass and a higher water content suggesting that spermatophore production is indeed physiologically costly (Ferkau & Fischer 2006). Similarly, in *Bicyclus* butterflies, which feed on rotten fruits as adults, adult diet did not affect male mating rate or sperm production except that the first transmitted spermatophore was bigger for males fed on fruit than with sugar. Bigger males produce bigger spermatophores with both more non-fertile and fertile sperm (Lewis & Wedell 2007). Lastly, variation in the number of previous successful copulations or the sodium composition of the male diet did not affect female reproduction in terms of number of eggs, sodium contents of eggs, but may slightly affect egg hatchability (Molleman *et al.* 2004).

It remains generally unclear how life-history factors have shaped lifetime investment into male sexually selected traits (Fischer *et al.* 2008). Fischer *et al.* (2008) investigated male reproductive success in relation to age and nutritional status, condition and wing damage. They propose that older males should be more willing to invest in potentially costly behaviour (*e.g.*, courtship) as they age, as older males stand to lose substantially less in trials than younger ones ('residual reproductive value' hypothesis). In agreement with this hypothesis, older males had a considerably higher mating success than younger males in small cage experiments. Yet, the higher mating success of older males may be either due to male motivation for courtship increasing with age (Fischer *et al.* 2008), or to females preferring older males (Nieberding *et al.*, unpubl. data), or most likely to a combination of the two factors. The higher mating success of older males faded away in experiments using larger cages in which male flight to locate and reach females is also tested. This suggests that the potential differences in male motivation may be masked under more natural conditions. This outcome was expected, as mating success does not only depend on male motivation but also on male physiological factors, such as flight endurance, manoeuvrability, energy reserves, responsiveness, (*e.g.*, Joron & Brakefield 2003), all of which are likely to diminish with age (Fischer *et al.* 2008) and may be under natural rather than sexual selection pressure. Noticeably, there is a positive correlation between male age at first mating and both size of the first spermatophore and proportion of non-fertile sperm [Z. Lewis, unpubl. data, in Lewis *et al.* (2010)].

Additional support in favour of the importance of male behaviour in female mate choice was recently provided (Janowitz & Fischer 2010). Ample mating

opportunities during male lifetime is correlated to a reduced mating success (Janowitz & Fischer 2010). Courtship and flight activity tended to directly affect male mating success, with more active males gaining more matings, and courtship activity was reduced in males having had regular access to females during their life. Male access to mates did not affect the longevity, fecundity, egg hatching rate of mated females. There was no evidence either for a cost of mating on male survival or fertility (Janowitz & Fischer 2010).

Other male-specific traits under natural selection likely affect their mating success; one of them being developmental time. Many animal species display sexual dimorphism in developmental time: the mean adult emergence is usually shorter in males compared to females (protandry). Protandry can be advantageous for males because it increases their probability of mating (Zwaan *et al.* 2008). Early emerged males are more likely to encounter virgin females, which is important as only approximately one third of *B. anynana* females remate in the field (Brakefield & Reitsma 1991) [one quarter in the lab; Brakefield *et al.* (2001)]. After 30 generations of artificial selection on protandry, lines selected for lower protandry (males emerging later and females emerging earlier than in the stock population) had lower egg-to-adult survival and lower rates of hatching, which suggests that interactions with fertility might constrain certain directions of change in patterns of protandry (Zwaan *et al.* 2008). The proportion of non-fertile and fertile sperm produced in spermatophores, as well as the time necessary to mate were also affected in artificially selected lines for lower or higher protandry, which suggests that there might be a trade-off between developmental time and sperm production (Lewis *et al.* 2010).

PERSPECTIVES

Recently, it was shown that the alternative male (competition) and female (choosiness) roles typical of sexual selection are not necessarily fixed within a species (Prudic & Monteiro 2011). Plasticity in sexual roles arises within *B. anynana* in relation to temperature experienced during larval development and reversed sexual selection takes place (male choosiness and female competition for accessing males) (Prudic & Monteiro 2011). This switch is correlated with a change in direct benefits to females and mating costs in males. Thus, the discrete seasonal environments known to produce these two developmental forms impose alternating, symmetrical patterns of sexual selection, one season on male ornaments, the following season on female ornaments. Studied ornaments are the dorsal eyespot pupil size and UV reflectance, which are shown here to be dimorphic between seasonal forms (Prudic & Monteiro 2011), but also between sexes (see above). This symmetry through time in male and female sexual roles results in the maintenance of sexual ornaments in both sexes. It leads to the question of which phenotypic traits are used by choosy males to recognize females of their own species. So far, the only traits likely involved in species

recognition are the dorsal forewing pupils which provide limited variability, as discussed above. Courtship behavioural differences across species – currently understudied – may provide an answer to this question. Lastly, this recent study challenges our ideas about the long-term evolution of the previously discussed male traits under the pressures of sexual, or natural, selection in such a dynamic process.

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