ECOLOGY LETTERS

Ecology Letters, (2012) **15**: 415–424 doi: 10.1111/j.1461-0248.2012.01748.x

LETTER

Cracking the olfactory code of a butterfly: the scent of ageing

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Abstract

Although olfaction is a primary mode of communication, its importance in sexual selection remains understudied. Here, using the butterfly *Bicyclus anynana*, we address all the parameters of importance to sexual selection for a male olfactory signal. We show that variation in the male sex pheromone composition indicates male identity and male age. Courting males of different ages display small absolute (*c.* 200 ng) but large relative (100%) change of one specific pheromone component (hexadecanal) which, unlike the other components, showed no heritability. Females prefer to mate with mid-aged over younger males and the pheromone composition is sufficient to determine this preference. Surprisingly refined information is thus present in the male olfactory signal and is used for sexual selection. Our data also reveal that there may be no 'lek paradox' to resolve once the precise signal of importance to females is identified, as hexadecanal is, as expected, depleted in additive genetic variation.

Keywords

(2*R*, 6*R*, 10*R*)-6,10,14-trimethylpentadecan-2-ol, (*Z*)-9-tetradecenol, 16:Ald, 6,10,14-trime-15-2-ol, age, *Bicyclus anynana*, butterfly, hexadecanal, identity, Lepidoptera, male sex pheromone, mate choice, olfactory communication, sexual selection, synthesis, Z9-14:OH.

Ecology Letters (2012) **15**: 415–424

INTRODUCTION

Olfactory communication is predominant in most living organisms but remains understudied compared to other modes of communication such as vision and audition (Wyatt 2003, 2009). Olfactory communication usually relies on combinations of chemical components and this chemical complexity provides the potential to convey sophisticated information about individuals, as documented in some vertebrates (e.g. Osada *et al.* 2003; Greenwood *et al.* 2005; Brennan & Zufall 2006; Boulet *et al.* 2009), and in social invertebrates (e.g. Moore *et al.* 1997; Thom *et al.* 2007; Guerrieri *et al.* 2009). In particular, work on honey bees *Apis mellifera* revealed that waggle-dancing individuals produce a unique scent that recruits additional workers to coordinate their foraging efforts (Thom *et al.* 2007). Similarly, efficient discrimination between 'friends' (nest-mates) and 'foes' (non-nest-mates) in some ant species is based on the presence of specific cuticular hydrocarbons (Guerrieri *et al.* 2009). Refined information can thus be transferred among individuals based on the presence or absence of specific chemical components in the blend, reflecting the complexity of the social interactions observed among individuals.

Olfactory signals should convey a wide diversity of information regarding the identity or quality of potential mating partners (Andersson 1994; Wyatt 2003; Johansson & Jones 2007). Thus olfactory communication is expected to be essential in mate choice and sexual selection issues as well. Communication by female sex pheromones has been well documented, especially in nocturnal moths, where females attract males from long distance and the composition of the pheromone is used for species recognition (Svensson 1996; Carde & Minks 1997).

Much less is known about sexual communication via male sex pheromones (MSP), although the strength and direction of sexual selection may differ markedly from sexual selection occurring on female sex pheromones in moths (Svensson 1996; Carde & Minks 1997). In *Drosophila*, two male-specific chemical components increase male attractiveness towards females in laboratory conditions, (Z)-11-octadecenyl acetate (cis-vaccenyl acetate, cVA) and Z-7-tricosene (7-T) (Grillet *et al.* 2006; Lacaille *et al.* 2007). Yet, the role of cVA and 7-T on male mating success in natural conditions is unclear (Scott *et al.* 2011). Males of some species of moths and of butterflies also produce sex pheromones but their study has not led yet to fully comprehensive chemical and behavioural case studies (e.g. Pliske & Eisner 1969; Baker *et al.* 1981; Sappington & Taylor 1990; Dussourd *et al.* 1991; Nishida *et al.* 1996; Hillier & Vickers 2004; Andersson *et al.* 2007; Lassance & Löfstedt 2009; for a full reference list, see Nieberding *et al.* 2008). These studies indicated that MSP are usually employed at short range during the courtship sequence (Myers 1972; Birch *et al.* 1990; Vane-Wright & Boppre 1993) and they may inform females about social status, genetic relatedness or immunocompetence (Carde & Minks 1997; Cardé & Millar 2004; Johansson & Jones 2007). Yet, the parameters of male chemical signals that are important to sexual selection are only known in rather rudimentary terms: do they vary

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among individuals? Are they heritable? Are they reliable indicators of male phenotype and, if so, what are the components of the signals relevant to female choice? Determining which specific item(s) of information is extracted from sexual signals by females is crucial to predicting their choice, and thus to understanding how sexual selection has influenced the evolution of the signals (Endler 1992; Andersson & Simmons 2006; Steiger *et al.* 2011).

Recently, it was shown that females of the long-lived African butterfly *Bicyclus anynana* (Butler, 1879) rely on the perception of a three-component MSP for mate choice, making this species an interesting model to study the complexity of information conveyed by male olfactory signals under sexual selection (Costanzo & Monteiro 2007; Nieberding *et al.* 2008). Here, we provide demonstrative experimental evidence that a single olfactory signal can provide several pieces of information about males, namely age and individuality. Indeed, the MSP composition enables females to finely assess age differences between courting males. Moreover, individual males have specific blend characteristics that remain stable throughout their life. Thus, variation with age does not blur a pheromonal signature unique to each male, providing a foundation for interindividual recognition and assessment of male identity and, possibly, quality. We also showed that the fine variation in the composition of the male olfactory signal is sufficient for female to select male partners, we identified the specific part of the olfactory signal under sexual selection and assessed its heritability. This understanding enables predictions to be made about the longer term evolution of olfactory communication under sexual selection in this butterfly system.

MATERIAL AND METHODS

Insects

An outbred laboratory stock of the African butterfly, *B. anynana*, was established in 1988 from over 80 gravid females collected in a single source population in Malawi and maintained at a large population size since (Brakefield & Reitsma 1991). The experiments were performed on individuals reared in climate rooms under a standard temperature regime (27 °C), 12 : 12 L : D regime and high relative humidity (75%). All larvae were fed on maize *ad libitum*, adult butterflies were fed *ad libitum* on banana. We showed previously that feeding the larvae on maize or on the natural host plant *Oplismenus africanus* did not affect MSP composition (Nieberding *et al.* 2008).

Chemicals

(*Z*)-9-tetradecenol (Z9-14:OH, 'MSP1') is available from Sigma-Aldrich (Chem. Co., P. O. Box 355, Milw., WI 5329). Hexadecanal (16:Ald, 'MSP2') was synthesised following the procedure established in Nieberding *et al.* (2008). Stereoisomerically pure (2*R*,6*R*,10*R*)-6,10,14-trimethylpentadecan-2-ol (6,10,14-trime-15-2-ol, 'MSP3') was synthesised following the published method for synthesis of stereoisomeric mixtures of 6,10,14-trimethylpentadecan-2-ol (Nieberding *et al.* 2008; Text S1). Please also consult Text S1 for information about the procedure used to quantify MSP composition on butterfly wings.

Heritability of pheromonal and morphological traits

Five male offspring per family for 65 families were reared in controlled conditions and, after emergence, were mated to a female and then killed at 8 days old when all three MSP components are present at significant levels in the pheromone blend (Nieberding *et al.* 2008), and are correlated to MSP composition at older ages (see Results). We measured MSP composition (Text S1), length and area covered by androconial hairpencils and spots, respectively, and wing area (Fig. 1). In addition, repeatability for the morphological traits was estimated using 50 stock males, each measured three times. The intraclass correlation coefficient was calculated using variance components derived from one-way ANOVA (Lessels & Boag 1987; Falconer & Mackay 1996). Repeatability of MSP composition depends only on GC accuracy and is over 99% (Nieberding *et al.* 2008).

Heritabilities were estimated by mixed-effect models using the above full-sib families, with pheromonal and morphological traits as dependent variables and 'family' as a random factor. MSP ratios were log transformed prior to analysis to normalise residual distribution. The full-sib breeding design allows the estimation of the covariance among full sibs (covFS). In terms of genetic variance components, the covFS equals half the additive genetic variance (V_A) together with a combination of non-additive components (dominance and epistasis) (Falconer & Mackay 1996). Heritabilities can thus be estimated as $H^2 = 2 \times$ cov FS. Permutation tests were run 10 000 times to determine the level of significance of the heritabilities. Phenotypic correlations between traits were estimated by Pearson product–moment correlation of individual measurements in the data set for sons, and *P*-values were Bonferoni-corrected for multiple comparisons.

Correlation between MSP composition and morphological and physiological traits

The data set gathered for estimating the heritabilities of MSP composition and of the five morphological traits were used to estimate Pearson product-moment correlations between these traits. In an additional experiment, the resting metabolic rate (RMR), MSP composition, and dry thorax and abdomen masses were measured for 50 males. Individual rate of CO_2 respiration (mL h^{-1}) was measured from 3-day-old males in the morning during the dark phase of the diurnal cycle. The levels of CO_2 produced were measured with a Li-Cor LI-6251 CO_2 analyser in a respirometer setup (Sable Systems; http://www.sablesys.com) with a push-through flow of 100 mL min^{-1}over a period of 20 min. Data on CO_2 from two consecutive replicate measurements were analysed using Datacan 5.4 (Sable Systems) and averaged. The next day, butterflies were frozen at −80 °C. MSP composition and body mass were measured as dry weight to the nearest 0.01 mg after removal of wings, legs, and antennae and drying at 40 °C for 48 h. Multiple regressions examined the relationships between dry weight, RMR and MSP composition.

Mating success of males of different age classes

To test the role of age in male mating success we performed mating competition experiments in semi-natural conditions using males of three different age classes (2 to 4-day, 13- to 15-day and 27- to 29-day old). Most males survive over 35 days in the laboratory (Pijpe *et al.* 2008) and can live up to 6 months in the field (Brakefield & Reitsma 1991); thus our experiments focus on ages within the known lifespan (natural and in the laboratory) for *B. anynana* males. The genitalia of each competing group of males were dusted using a differently coloured fluorescent dust (Nieberding *et al.* 2008). Forty virgin males

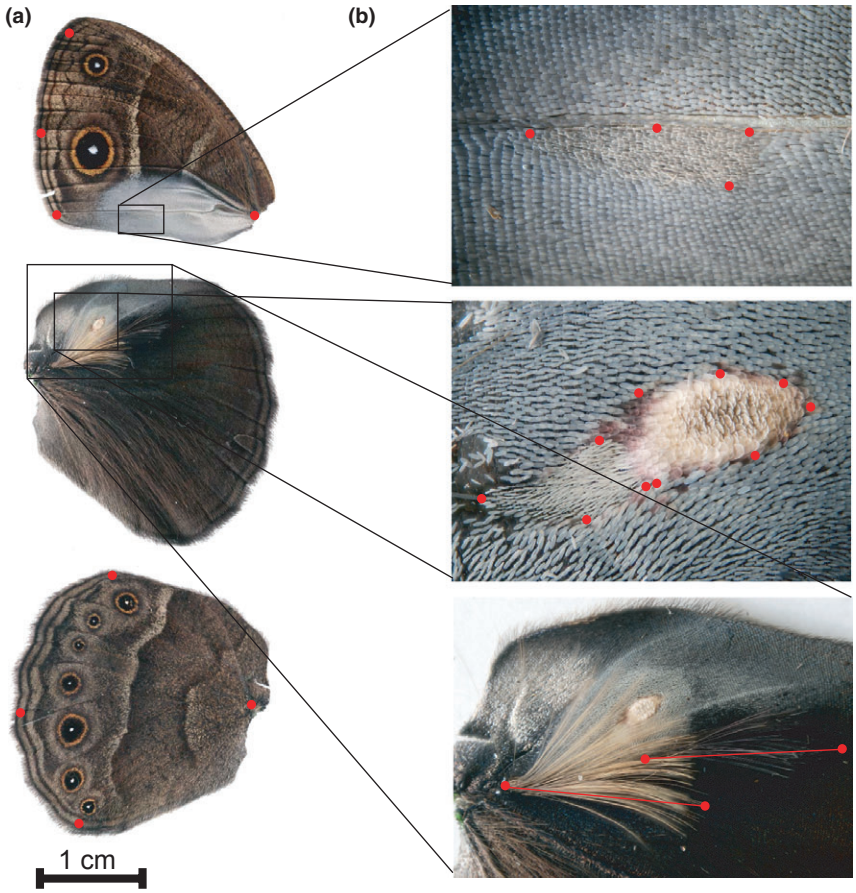

Figure 1 Measurement of the size of wings and of androconial structures. Males display two androconia each formed by a spot of differentiated scales and a hairpencil. Androconia 1 is on the hindwing and produces MSP2; androconia 2 is on both the forewing (spot) and the hindwing (hairpencil) and produces MSP1 and MSP3; each MSP component affects male mating success (Costanzo & Monteiro 2007; Nieberding *et al.* 2008). From top to bottom: (a) the right ventral forewing, dorsal and ventral right hindwings. (b) the spot 2, the spot 1 after removing the hairpencils, and the hairpencils. Morphological traits are represented by the area within a polygon formed by, or the length between, a series of landmarks (red dots).

of each age-class were released in a tropical environment (cf. Joron & Brakefield 2003) such that density was comparable to mating in the field (Brakefield & Reitsma 1991). The next morning, sixty 3- to 5-day-old virgin stock females were released to reach a 2 : 1 male:female ratio. A second group of 20–30 females was released 1 day later. All butterflies were collected 1.5 days after the final group of females was released. We assessed the group-identity of female mating partners by the transfer of fluorescent dust under UV light, and occasional double matings were scored as 1 : 1. Two trials were performed sequentially. Data analysis used G-tests. In each replicate, male survival and mating distributions were compared against an equal distribution of mating success across age classes. We tested for heterogeneity among replicates using a Pearson's Chi-squared test (or a Fisher's Exact test if the expected frequencies were close to 5 or less) before performing the same analyses on the pooled data set.

Mating success of males perfumed with '3-day' or '14-day' MSP synthetic perfumes

Both variance and mean values in MSP composition differ between 3- and 14-day-old males. We produced two synthetic perfumes mimicking the MSP composition of 3- and 14-day-old males ('young' and 'mid-aged' perfumes, respectively). The total MSP titre of both perfumes was standardised at 12 μg per male and the titres of the three MSP components were scaled accordingly to maintain natural ratios between components in the two perfumes (Table S1). The actual MSP composition of the 'young' and the 'mid-aged' perfumes, as estimated by GC, are close to the natural values (Table S1).

The 'young' and 'mid-aged' perfumes were prepared for 120 males each, each male receiving a 10-male equivalent of synthetic MSP on its wings. A 10-male equivalent load was chosen to attempt counteract the elimination of the hairs which normally help to distribute the MSP during courtship, and because it was shown that more than 50% of the extract is lost immediately after application (Nieberding *et al.* 2008). The perfumes were used for testing (i) whether their rates of evaporation from butterfly wings differed (Text S2), and (ii) for the behavioural experiments described here under.

Two-day old stock males were operated (*op*-males) to surgically remove their androconia using fine scissors and a fine punch tool. This treatment avoided damage of any wing veins and male flight ability was maintained (Nieberding *et al.* 2008). Male activity, measured as RMR, was estimated for groups of forty 3- to 4-day-old *op*-males as described above. On day 4 or 5, *op*-males were randomly assigned to one of the two

competing groups and dusted with red or yellow colours as in the previous experiment to track male mating success. Each male group was then perfumed with either the 'young' or 'mid-aged' perfume and the competition experiment was immediately started (i.e. within 15 min after the perfuming step was started) by releasing the males in the cage in which females had been freed an hour before. The compositions of the perfumes were checked before each trial by GC analysis. Competition between the two groups of 20 perfumed males for matings with 20 3- to 5-day-old virgin stock females was performed in a 3 × 3 × 3 m net-covered, wood-framed cage placed in a greenhouse kept at 27–29 °C. The cage was lit by a set of SHP sodium, HTX Metal Halide and high UVA and UVB lamps, and mirrors were placed on three sides of the cage to limit light dispersion outside the cage. This set up allowed us to mimic as closely as possible the range and amount of wavelengths observed in bright daylight. In addition, we could release and recapture all scented butterflies within the time limit imposed by the evaporation rate of the synthetic MSP. Four replicates were performed sequentially. The relative mating success of 'young' vs. 'mid-aged' males was determined as previously mentioned.

Statistical analyses

All statistical analyses were carried out using the R language and environment for statistical computing and graphics (http://www.r-project.org/).

RESULTS

MSP composition is a reliable indicator of male age

The MSP of *B. anynana* comprises three components, namely (*Z*)-9-tetradecenol (hereafter MSP1), hexadecanal (MSP2) and [(2*R*, 6*R*, 10*R*)-6,10,14-trimethylpentadecan-2-ol; MSP3] (Nieberding *et al.* 2008). Variability of MSP composition through male lifetime was assessed by measuring MSP titres and ratios using one wing sample per virgin male (n = 230 3-day to 21-day old males). We observed a large variation between males in MSP titres and ratios: for MSP1 titre, 3.00 ± 1.87 μg (mean ± SD), for MSP2 titre, 0.40 ± 0.26 μg, and for MSP3 titre, 13.54 ± 7.39 μg (Fig. 2A). MSP titres and ratios varied significantly between age classes (Fig. 2A). We tested whether male age could be assessed based on their MSP composition. Factorial discriminant analyses (FDA) on MSP titres and ratios discriminated successfully among the different age classes, as over 97% of the variance in MSP composition across age classes was explained by the first two axes (n = 230, $P < 0.0001$; Fig. 2B). MSP2 titre is likely the most indicative signal for age as it is represented by axis 1 (projection is 0.73; Fig. 2B), which explained 65.3% of the total variance. MSP1 and MSP3 titres may also participate in the signal for age, as they are represented by axis 2 (projection of 0.71 and 0.85, respectively), which explained an additional 31.7% of the variance. While axis 1 separates 3 vs. 28 day-old males, it is axis 2 that differentiates middle-aged males from the former groups. Using axes 1 and 2, assignment for an age class based solely on MSP information was efficient as the mean rate of false reclassification between pairs of age classes was 3.1% using MSP titres and 4.1% using MSP ratios, while the sum of false reclassifications across all pairs of age classes reached 37 and 40%, respectively. Mean false reclassification rate was higher between flanking age classes (3.9%) than between more distant pairs of age classes (2.3%), and the maximum rate of false reclassification was observed between age classes 14 and 21 days old (6.93%). Consequently, all MSP titres and ratios show clear variation with male age, information that can be used to assess male age.

MSP composition is a reliable indicator of male individuality

We tested whether part of the large variation observed for MSP composition could be due to interindividual differences, with some males producing consistently more, and others consistently less, MSP throughout their lifetime. We measured MSP titres and ratios at two successive ages in 230 virgin males, first on one hindwing, and then on the contra-lateral hindwing. For this purpose, we removed male wings either on days 3 and 14, or on days 8 and 21, or 8 and 14, or 14 and 21 (only males that survived to the second time point were analysed). This design allowed us to compare variability of MSP composition within and between individuals through male lifetime. To test whether individual males have a unique MSP profile, Pearson product-moment correlations on MSP titres and ratios were performed between both hindwings from the same individuals and contrasted against correlations between hindwings of two random males of corresponding age classes. In most cases, correlations coefficients between hindwings of individual males across age classes were consistently higher (mean $r = 0.53$, $P < 0.05$) than those between hindwings of random males (mean r = 0.08, NS for all but one comparison) (Fig. 3). A sign test confirmed a significant difference in r-values between the two groups (n = 56 comparisons, $P < 0.001$). The olfactory signal of individuality was strongest over shorter (8- to 14-day and 14- to 21-day comparisons) rather than longer (3- to 14-day and 8- to 21-day comparisons) periods of time and when using MSP titres rather than MSP ratios (Fig. 3). These results together support that male MSP composition is a reliable indicator of male individuality over several weeks.

MSP composition is partly heritable

We estimated the heritability of MSP composition and related morphological structures in order to estimate the potential of evolution of these traits under sexual selection. The repeatability of measurements of pheromonal and morphological traits ranged from 75 to 99% (Table 1), indicating that potential heritability was high (Falconer & Mackay 1996). A full-sib analysis involving 65 families of sons revealed significant heritabilities in all morphological traits including wing size, size of the androconial spots and length of the androconial hairpencils, and in MSP1 and 3 titres, but not in MSP2 titre and MSP ratios despite high repeatabilities (Fig. 1; Table 1).

MSP composition is partly correlated to morphological or physiological traits

We tested whether MSP composition was correlated to the size of the androconial structures, and to morphological and physiological traits often associated to male mating success in butterflies. There were significant phenotypic correlations ($r > 0.4$, $P < 0.003$) between total wing area and the area of the androconium producing MSP1 and MSP3, and MSP1 and MSP3 titres themselves. In contrast, there was no correlation between wing area and the androconium producing the MSP2 titre, nor with MSP2 titre. Similarly, there was a strong relationship between dry weight and both MSP1 and MSP3 titres

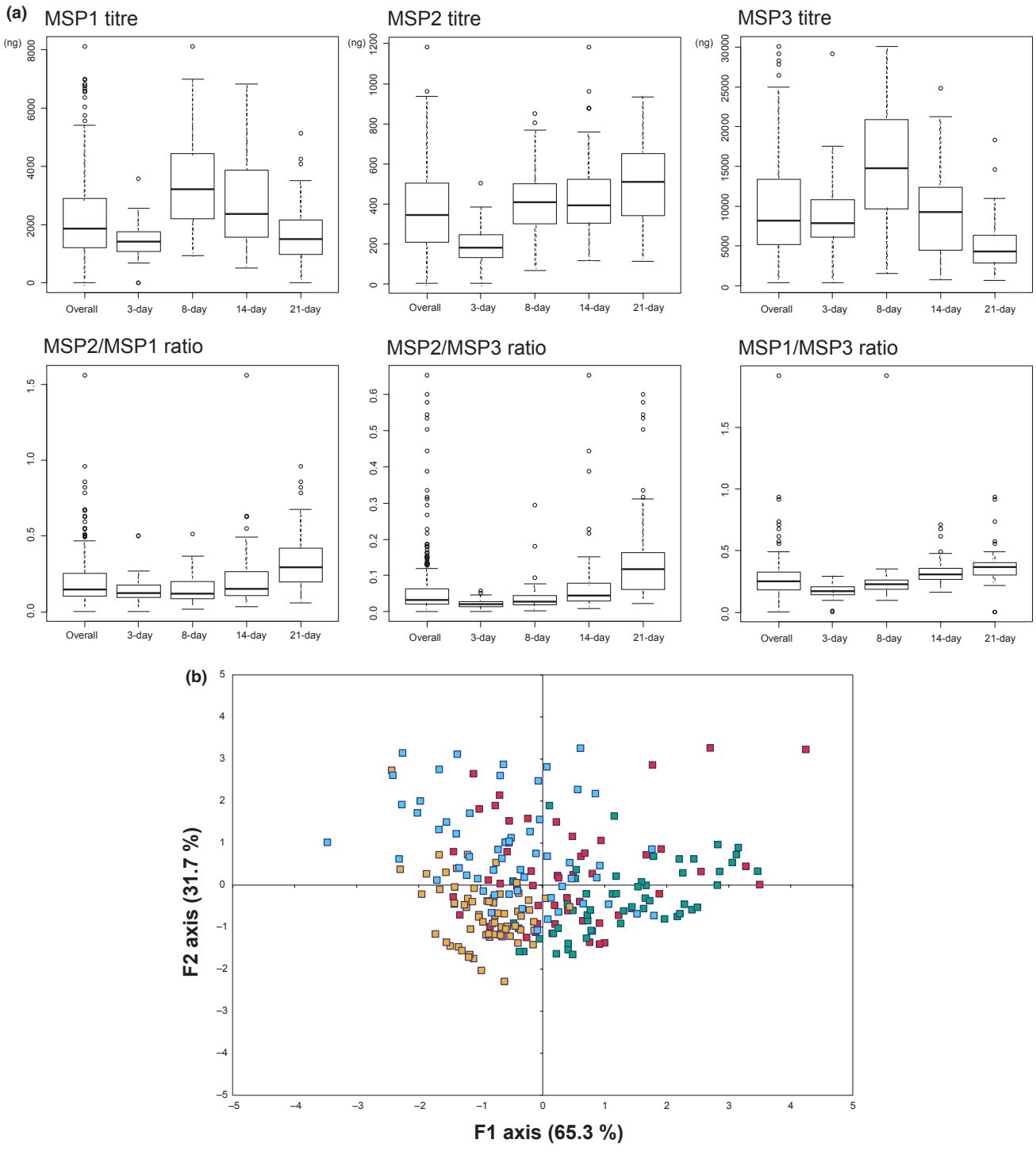

Figure 2 Male sex pheromone composition is a reliable indicator of male age (n = 230 males from 3- to 21-day old). (a) Boxplots of MSP titres and ratios overall, and across, age classes. MSP titres and ratios each varied significantly across age classes (Kruskal–Wallis rank sum tests with X above 51.7, d.f. = 3, p below $3.5.10^{-11}$ for each MSP titre and ratio). (b) Discriminant factorial analysis on MSP titres for males sampled at 3 (orange), 8 (blue), 14 (red) or 21-day old (green). Projection of (MSP1/MSP3) and (MSP2/MSP3) ratios on axis 1 were 0.86 and 0.66, respectively, and projection of (MSP2/MSP1) ratio on axis 2 was −0.81 (for projection of MSP titres, see main text).

(n = 50 individuals, linear regression between MSP residuals after correction for RMR, $P < 0.00001$, $R^2 = 0.32$), but not with MSP2 titre (n = 50 individuals, $P = 0.07$, $R^2 = 0.07$), while RMR itself did not affect MSP production (n = 50 individuals, $P = 0.64$, $R^2 = 0.004$).

Age affects male mating success

Based on the observation that MSP composition varies with male age, behavioural experiments were performed to test whether females discriminate between males of different age. Due to a life expectancy

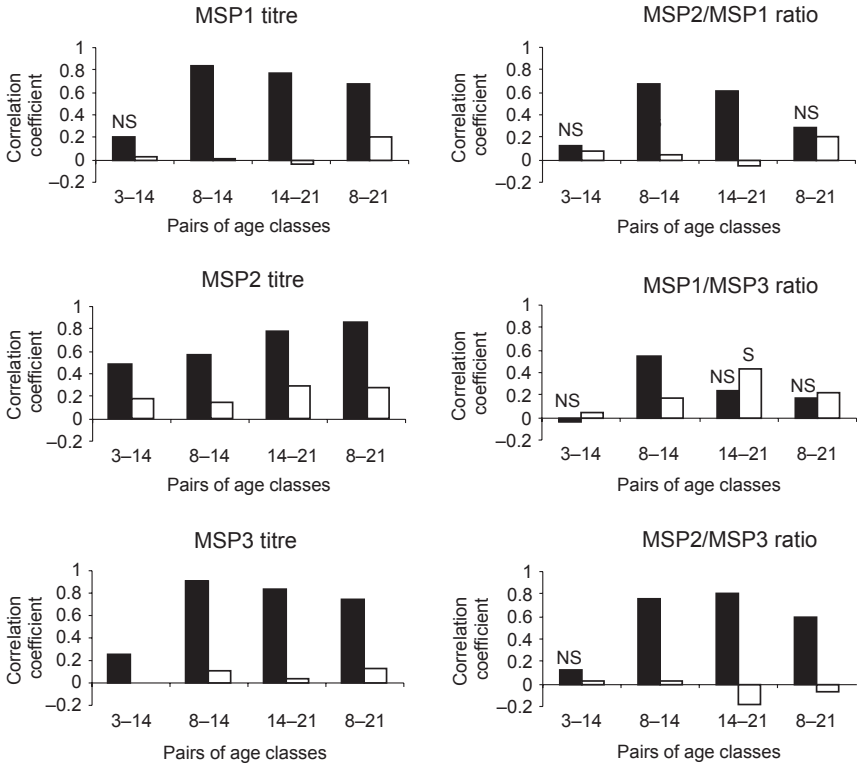

Figure 3 Male sex pheromone composition is a reliable indicator of male identity. Comparison of the Pearson product-moment correlation coefficient for MSP composition between (i) the hindwings from single individuals sampled at 2 age classes (mean $r = 0.53$, $P < 0.05$ for all comparisons except if not significant (NS, in black), and between (ii) the hindwings of two random males of corresponding pairs of age classes (mean $r = 0.08$, NS except if significant (S, in white). On the *X*-axis, the correlation coefficients obtained between 3-day vs. 14-day-old males, 8 vs. 14, 14 vs. 21, and 8 vs. 21 are represented, from top to down and left to right, for MSP1 titre, MSP2 titre, MSP3 titre, MSP2/MSP1 ratio, MSP1/MSP3 ratio and MSP2/MSP3 ratio.

Table 1 Heritabilities for pheromonal and androconial traits, after log-transformations of MSP ratios

Trait	Repeatability (SE)	H^2	R^2 (♣)
MSP1 titre	> 0.99 (♦)	0.36***	0.35
MSP2 titre	> 0.99 (♦)	0.00	0.27
MSP3 titre	> 0.99 (♦)	0.38***	0.35
(MSP2/MSP1) ratio	> 0.99 (♦)	0.00	0.25
(MSP1/MSP3) ratio	> 0.99 (♦)	0.00	0.25
(MSP2/MSP3) ratio	> 0.99 (♦)	0.00	0.24
Area of fore- and hind-wings	0.89 (0.002)	0.45***	0.38
Area of androconial spot 1 (†)	0.77 (0.003)	0.21**	0.29
Area of androconial spot 2 (†)	0.81 (0.003)	0.57***	0.42
Length of androconial hairpencil 1 (†)	0.93 (0.001)	0.23**	0.29
Length of androconial hairpencil 2 (†)	0.75 (0.001)	0.22**	0.29

(♦) Repeatability provided by the gas chromatograph. Significance based on 10000 permutations: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (♣) R^2 is the coefficient of determination and allows assessing the goodness of model fit ($0 < R^2 < 1$). (†) Area of androconial structures remained significantly heritable when corrected for their correlation with wings area.

of the wet season form in the field of a few weeks, *B. anynana* females may encounter many males during their lifetime, enabling them to choose among males and mate several times (Brakefield & Reitsma 1991).

Mating experiments (two replicates) involved the competition among large groups of 3-, 14- and 28-day-old males (±max. 2 days) under semi-natural conditions allowing free flight and a full expression of courtship behaviour in a spacious tropical greenhouse (Joron & Brakefield 2003). This resulted in a high mating rate of females (overall, 87%) whilst ensuring a relatively high competition for mates between males. There was evidence of differential male mating success in both trials (I-II; G-test for the 3 days : 14 day: 28 days comparison, X = 36.05, d.f. = 2, $P < 0.001$; Fig. S1A), and there was no heterogeneity across trials (Pearson's chi-squared test; X = 0.02, d.f. = 2, $P = 0.99$; Table 2). In each trial, 28-day-old males achieved significantly fewer matings than either 3-day or 14-day old males (chi-squared test for either the 3-day : 28-day or the 14-day : 28-day comparisons = 27.22, d.f. = 1, $P < 0.001$). When mating success was standardised by the relative male survival between age classes, the difference in mating success for the pooled data was higher between 14-day and both 3-day and 28-day males (chi-squared test for the 14-day : (3-day + 28-day) comparison, X = 24.4, d.f. = 1, $P < 0.001$), than between 3-day and 28-day old males (Chi-squared test for the 3-day : 28-day comparison, X = 4.8, d.f. = 1, $P = 0.03$). However, this experiment was biased in two ways. First, this set up does not allow us to distinguish between female preference and male eagerness to mate which has been suggested to increase in older males including in *B. anynana* (Fischer *et al.* 2008). Secondly, recaptures of males of the three groups at the end of each experiment indicated declining survivorship with increasing age (I–II; Chi-squared test for the 3-day: 14-day : 28-day comparison, X = 47.3, d.f. = 2, $P < 0.001$ Fig. S1B). Such a differential survivorship can have different implications for relative fitness across groups, depending on whether males die before or after reproduction. Thus, if 14-day-old males die before reproduction, mid-aged males are relatively rare and would therefore seem to have a higher mating success whereas if 14-day-old males tend to die after reproduction, young, 3-day-old, males would rather have a relatively higher mating success.

MSP composition directly influences male mating success

To distinguish between these scenarios, two groups of 3-day old males were treated with a synthetic pheromone perfume mimicking the

Table 2 Female mating and male recapture in greenhouse experiments for stock 3-day, 14-day and 28-day old male groups

	Experiment		Total
Females	I (n = 90)	II (n = 80)	I+II (n = 170)
Mated with			
3-day	29	22	51
14-day	23	17	40
28-day	3	2	5
Males	I (n = 120)	II (n = 120)	I+II (n = 240)
Recapture number (Fig. S1B)			
3-day	32	30	62
14-day	10	12	22
28-day	5	5	10
Relative loss of males			
3-day	1	1	1
14-day	3.2	2.5	2.8
28-day	6.4	6	6.2
Mating success of 3-, 14-, 28-day-old males standardised by the relative disappearance of males (female data)			
3-day	29	22	51
14-day	73.6	42.5	112.7
28-day	19.2	12	31
Mating success of 3-, 14-, 28-day-old males relative to that of 14-day males (set at 1) (female data; Fig. S1A)			
3-day	0.39	0.51	0.45
14-day	1	1	1
28-day	0.26	0.28	0.28

For females, the figures are the numbers of recaptured females that mated with each male group. For males, the figures are the absolute numbers after recapture for each male group (see also Fig. S1).

olfactory profile of either young or mid-aged males (following the method of Nieberding *et al.* 2008). Thus, male competitive ability and other traits related to age were standardised among competing groups of males. Specifically, we tested for an effect of age-related changes in the ratios of MSP on female mating preference and thereby male mating success. The 'mid-aged' synthetic perfume differed from the 'young' perfume mainly by a 100% relative increase of MSP2 titre, although MSP1 and MSP3 titres also differed slightly between the two perfumes and corresponding age classes (Table S1). For mating experiments (four replicates), we released groups of 4- to 5-day-old males scented with a 10-male equivalent of either the 'young' or the 'mid-aged' synthetic perfume, and let them compete for mating with 3-day old virgin females for 3 h (Table 3). Male activity, measured as RMR, did not differ significantly among male groups (Wilcox rank sum test, n = 40 per replicate, $P > 0.23$ for each of four replicates). There was no heterogeneity among trials (Fisher's Exact Test for Count Data; $P > 0.14$ between pairs of trials), and the pooled data sets for the first three trials showed a significantly higher mating success of 'mid-aged' perfumed males compared to 'young' perfumed males (G = 5.77, d.f. = 1, $P = 0.017$). Trial IV was excluded from the analysis because it started to rain heavily during the course of the experiment, and we suspect that the correlated change in temperature, humidity and air pressure influenced the behaviour of the butterflies (decreasing female selectivity and/or increasing male eagerness), as suggested by the higher number of double matings (n = 8 double 'young' and 'mid-aged' matings in trial IV compared to n = 0 to 2 in previous trials). Males perfumed with the 'young' synthetic extract achieved only 31% of all matings.

DISCUSSION

A sex pheromone revealing male age

The aim of the present study was to identify which specific information is extracted from the MSP when *B. anynana* females select a mating partner. We showed that the MSP composition changes during male lifetime and that females display a preference for mid-aged males over younger males. Although other traits in addition to pheromone profiles vary with age in butterflies (e.g. wing ultraviolet reflectance; Papke *et al.* 2006), and although *B. anynana* females rely on additional visual and behavioural traits for selecting a mate (e.g. ultraviolet reflectance of wings pattern elements; Costanzo & Monteiro 2007; Prudic *et al.* 2011), we demonstrate here that variation only in a single olfactory signal is sufficient to extract detailed information about male age, at least sufficient to assess a 10-day difference in this long-lived butterfly species.

In *B. anynana*, mid-aged males have higher mating success than younger males (this study; Fischer *et al.* 2008). This pattern has been observed in many long-lived species; older males might demonstrate their superior genetic quality simply by surviving longer in the environment (Brooks & Kemp 2001). In this regard, we think that the mating success of 28-day-old males was lower than that of 14-day-old males in our first behavioural experiment, despite increased MSP2 titre, because we are not able to produce *B. anynana* individuals in the lab that are as fit as, and survive as long as, the species in the field (Brakefield & Reitsma 1991).

Table 3 Female mating in competition experiments for male groups perfumed with either a 'young' or a 'mid-aged' perfume. The figures are the numbers of recaptured females that mated with each male group (four replicates I–IV) (see also Table S1)

Female matings	I	II	III	IV	I-II-III-IV	I-II-III
'young' perfume	2	5	4	6	17	11
'mid-aged' perfume	9	8	9	4	30	26
« young » + « mid-aged »	2	0	0	8	10	2
Unmated	7	6	7	1	21	20
G test 'young' vs. 'mid-aged'	G, 1 d.f., =3.77, P = 0.05	G, 1 d.f., =0.69, P = 0.40	G, 1 d.f., =1.9, P = 0.16	G, 1 d.f., =0.22, P = 0.63	G, 1 d.f., =2.96, P = 0.08	G, 1 d.f., =5.77, P = 0.016

Importantly, our data together with previous courtship analysis in *B. anynana*, support that female choosiness, rather than male activity, is responsible for the outcome of the behavioural experiments. Although one finds occasionally more than one male simultaneously courting a female, *B. anynana* males do not compete between each other such that only the winner would have access to the female (Nieberding *et al.* 2008). *B. anynana* males are also not territorial. Male–male competition could act via the MSP composition itself, reducing the courtship activity of other males (e.g. Hirai *et al.* 1978). In contrast to Hirai *et al.*'s study (Hirai *et al.* 1978) however, in our experiments perfumed males were surrounded simultaneously by the same olfactory environment and a difference in MSP perception between males cannot thus be responsible for the difference in mating success. Thus, we infer here that intrasexual competition among males may be rather limited in this species, whereas females actively exclude or accept males as mating partners based on their perception of MSP.

A sex pheromone revealing male individuality

Males produce consistently more, or less, of each component throughout their lifetime, and the corresponding MSP ratios display similar stability (Fig. 3). Similar results were observed recently in social vertebrates (Mardon *et al.* 2010; Whittaker *et al.* 2010). MSP composition in *B. anynana* thus conveys information on male individuality and provides the opportunity for interindividual recognition. Our population-level analysis does not exclude the possibility that some males may smell as if they were either younger or older than their real age, as the absolute amounts of MSP are sometimes similar between males of different age classes. Therefore, our analysis does not exclude the presence of some potential cheaters in the population.

Moreover, MSP composition may be a honest signal of male quality. MSP2 titre is indeed an indicator of male age and is not linked to wing or body size, or physiological activity. The evolution of MSP2 as a signal of male quality is therefore probably not constrained by selection on these other traits. This is not the case for MSP1 and MSP3. However, we cannot exclude the possibility that MSP1 and MSP3 are part of the active signal since they affect male mating success as well (Costanzo & Monteiro 2007) and they participate as a ratio, which could influence female behaviour.

A specific part of the signal explains female preference

In our study, the specific part of the signal relevant to female choice was identified. Mid-aged and young males differ most significantly in MSP composition via a 100% relative increase (*c.* 220 ng; Table S1) in one component (MSP2; first axis of the FDA; Fig. 3), and a corresponding change of MSP ratios. MSP1 and MSP3 titres also differed with age but the amplitude of the changes was limited (from 13.9 to 19.8% for MSP1 titre, and from 85.2 to 76.5% for MSP3 titre; Table S1). In addition, we have shown recently that the lower mating success of inbred *B. anynana* males is associated with a reduction of MSP2 titre (Joron & Brakefield 2003; Van Bergen, E., Zwaan, B.J., Brakefield, P.M. & Nieberding, C.M., unpublished data). Increasing MSP2 titre is thus likely to be the part of the MSP signal most closely associated to female preference. It is noteworthy that differences in mate perception in other insects can be induced by a similar range of variation as the one observed for MSP2 titre (Schlyter & Birgersson 1989). Together, these data emphasise the biological importance of fine-scale variation in titres of pheromonal components.

Evolution of the MSP composition under sexual selection

Our results allow us to make predictions about the evolution of the male signal. We found that the specific component of the olfactory signal preferred by *B. anynana* females, namely increasing MSP2 titre, displays high phenotypic variability but no significant heritability, while MSP1 and MSP3 titres showed moderate heritabilities (at the age of 8 days). Thus, there cannot be any directional change in MSP2 titre unless there is first evolutionary change in the direction of female preference, because there is no additive genetic variance left for increasing MSP2 titre.

Furthermore, directional mate choice should deplete the sexually selected trait of additive genetic variance. However, this theoretical expectation contrasts with repeated empirical findings of substantial genetic variance in sexually selected traits. The 'lek paradox' refers to the presence of genetic variability despite strong directional sexual selection for a trait (Kotiaho *et al.* 2008). Here we show that the part of the sex pheromone under direct sexual preference by females (MSP2 titre and related MSP ratios) is actually depleted in additive genetic variance. In contrast, the other parts of the MSP (MSP1 and MSP3 titres) and the morphological structures producing the pheromone retain significant genetic variance since they may not be targeted by strong sexual selection (Van Homrigh *et al.* 2007; McGuigan *et al.* 2008). This suggests that there may actually be no 'lek paradox' to resolve once the precise feature(s) of sexual signals of importance to females are identified. Our results are in agreement with the predicted depletion of additive genetic variance under strong directional sexual selection (Cotton & Pomiankowski 2007; Kotiaho *et al.* 2008).

ACKNOWLEDGEMENTS

We thank H. de Vos, P. Beldade and B. Zwaan for help with the experimental design and preliminary experiments, G. San Martin and S. Van Dongen for statistical help regarding the heritability experiment, R. Libois for statistical help regarding the FDA analysis, G. San Martin for pictures in Fig. 1, H. de Vos, K. Koops, M. Lavrijzen and N. Wurzer for technical help and host plant cultivation, C. Desmet, M. de Jong, N. Schtickzelle and H. van Dyck for fruitful suggestions on a earlier draft of the manuscript. We are grateful to the Hortus Botanicus Leiden for access to the greenhouse. Finance and support were provided by the European Union (Marie Curie International European Fellowship to C.N. FP6-2005-Mobility-5 nr039083), by the Belgian 'Fonds National de la Recherche Scientifique' (mandat 'Chargée de recherches FNRS' honorifique to C.N. and grant FRFC 2.4600.10), by UCL (grant 'Action de Recherche Concertée ARC 10/15-031), and by the Academy of Finland (grant 125970 to M.S.). E.H. and E.W. are grateful for the financial support from EU (Objective 2 the region of South Forest Counties) and Länsstyrelsen i Västernorrlands län. This is publication BRC234 from the BDIV Research Centre.

AUTHORSHIP

All authors agreed to submission of the manuscript. The lead author is responsible for the integrity of the entire manuscript and other authors are responsible for the integrity and accuracy of methods or data they contributed. All authors made a substantial intellectual contribution to the manuscript.

AUTHORS CONTRIBUTIONS

CMN and PMB designed the study. CMN and CEA designed the heritability experiment. CMN performed all experiments except the following ones: KF provided the samples for the heritability estimates, MS provided the RMR, dry thorax and abdomen masses, MS and CMN performed the behavioural experiments for which the pheromone had been manipulated, EAW and EH identified the chemical structure of MSP3 and provided the synthetic components of MSP1, MSP2 and MSP3. CMN and PMB wrote the first draft and all authors contributed substantially to revisions.

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Editor, Greg Grether
Manuscript received 16 November 2011
First decision made 16 December 2011
Manuscript accepted 26 January 2012