



Exploring the influence of individual courtship behaviors on male mating success in a blow fly

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Abstract Male courtship behavior and displays influence female mating decisions, and therefore affect mating success in a diverse range of organisms. While there is substantial evidence confirming that females prefer males who invest more in courtship, less attention has been paid to the relative importance of individual behaviors, and the discrete sequences of courtship that result in mating success. The small hairy maggot blow fly, *Chrysomya varipes*, performs stereotyped courtship behaviors, involving orienting, tapping, waving, arching, wing vibration and mounting. This study aimed to quantify male investment in specific courtship behaviors, and compare courtship investment and behavioral transitions between males who gained mating success (successful males) and those that did not (unsuccessful males). Our results show that mating success was influenced by the behaviors orienting, tapping, arching and mounting. Behavioral transitions revealed a distinct pattern of behaviors leading to a mating attempt, and some differences were observed between successful and unsuccessful males. Overall, our findings suggest that female mating decisions were based on differences in specific male courtship behaviors. This detailed observational study has quantified multiple courtship behaviors for the first time in *C. varipes*, and highlights the importance of considering multiple behavioral traits when exploring the influence of male courtship on mating success.

Keywords *Chrysomya varipes* · courtship behavior · mate choice · mate preference · sexual selection

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Introduction

Male courtship displays consist of specific signals conveyed and perceived by potential mates, and are characterized by repetitive or stereotyped behaviors that are directed at the female prior to mating (Andersson 1994). Courtship displays can play a crucial role in both female mating decisions and mate preferences, and, therefore, the evolution of male courtship behavior can be driven by female mate choice (Andersson 1994; Darwin 1859, 1871). There is substantial evidence confirming that males with higher courtship intensity, or a higher investment in the time spent courting, are preferred by females in a wide range of taxa (reviewed by Beyers et al. 2010), including birds (Patricelli et al. 2002), mammals (Longpre et al. 2011), fishes (Karino 1995), amphibians (Vinnedge and Verrell 1998) and insects (Callander et al. 2012; Shamble et al. 2009). However, while female preference for male courtship investment has received extensive empirical attention, almost no attention has been paid to the relative importance of individual behavioral components of courtship, and the discrete sequences of courtship behaviors that result in a higher probability of mating success (but see Chelbi et al. (2012) and Lasbleiz et al. (2006)). Female choice may act on multiple behavioral traits, and selection may operate on different traits either in concert or antagonistically (Candolin 2003). In addition, courtship displays are often complex, involving multiple components, each of which may provide different signals. Therefore, in order to gain a greater understanding of the relationship between male courtship behavior and female mating decisions, there is a need for studies that quantify multiple courtship behaviors. Disentangling these aspects of courtship behavior could provide key insights into indicators of male quality and advance our understanding of the associations between display traits and female preferences, thus improving our capacity to predict the influence of male phenotype on mating success.

There are three main hypotheses that explain the use of multiple male secondary sexual characteristics in mate choice: 1) the multiple messages hypothesis, which proposes that different traits reflect different qualities and evaluated together provide a more accurate assessment of mate quality (Johnstone 1996; Møller and Pomiankowski 1993); 2) the back-up signal hypothesis, which proposes that multiple traits reflect the same aspect of male quality to protect against signal redundancy (Johnstone 1996; Møller and Pomiankowski 1993); and 3) the species recognition hypothesis, which proposes that different traits are required to ensure effective species recognition (Pfennig 1998). In addition, hypotheses relating to the use of multiple signals may not necessarily be mutually exclusive; for example, a study in the eland, *Tragelaphus oryx*, found that male display traits provided simultaneous support for the multiple messages hypothesis and for the back-up signal hypothesis (Bro-Jørgensen and Dabelsteen 2008).

Empirical studies have begun to explore the use, function and interaction of multiple male secondary sexual characteristics (Hebets et al. 2011; Jones et al. 2014; Kekäläinen et al. 2010; Kodric-Brown and Nicoletto 2001; Lehtonen et al. 2007; Patricelli et al. 2003). However, these types of investigations have almost invariably focused on examining the combination of male ornamental traits and courtship displays (in terms of overall investment) on male mating success and female mating preferences. Very few studies have attempted to divide courtship behavior into its multiple components, and examine the influence of individual courtship behaviors on mating success (but see

Bray and Hamilton 2007; Chelbi et al. 2012). Owing to the paucity of work on the use and importance of specific courtship behaviors for mating success, there is a need for studies that thoroughly analyze the importance of specific courtship components in a wider range of species. Such research will enhance our understanding of the complex relationship between female mate choice and male courtship, as the interpretation of courtship behaviors by behavioral ecologists is often oversimplified (Johnstone 1996).

Studies in invertebrates, such as fruit flies *Drosophila* spp., and wolf spiders *Schizocosa* spp., are unraveling the signaling function of multiple courtship behavioral components (see Gleason et al. 2012; Hebets 2005). For example, studies of *Drosophila* spp. have investigated the function of individual courtship behaviors, and analyzed the relevance of the temporal structure of behavioral sequences (Liimatainen and Hoikkala 1998; Markow 1987; Markow and Hanson 1981; Saarikettu et al. 2005). Markow (1987) found slight differences in the temporal patterns of male courtship between successful and unsuccessful males; most notably that successful males had reduced locomotion and movement compared with unsuccessful males. However, no particular behavior was identified as an absolute predictor of mating success (Markow 1987). Manning (1967) suggested that courtship behaviors deliver different stimuli that, in combination, gradually raise the receptivity of a female until a certain threshold is attained and the female accepts the courting male. Therefore, it may be that a combined effect of multiple behaviors is required for mating success in *Drosophila* (rather than individual behaviors being crucial). In addition, there is evidence that the use of multiple signals in *Drosophila* (including the exchange of visual, acoustic, olfactory, gustatory and tactile signals) informs each fly about the identity of the potential mate, thereby providing support for the species recognition hypothesis (Greenspan and Ferveur 2000; Lasbleiz et al. 2006; Ritchie et al. 1999). Furthermore, these signals have also been correlated with male condition and genetic quality (Hoikkala et al. 1998), thereby also supporting the multiple messages hypothesis. However, further research is required to test the generality of these findings, and more thoroughly explore the hypotheses regarding the use of multiple behavioral traits in *Drosophila* and other species.

The small hairy maggot blow fly, *Chrysomya varipes*, provides an excellent model for a detailed examination of male courtship behavior, and the importance of individual behaviors. *Chrysomya varipes* is an obligate inhabitant of vertebrate carrion. After forming leks, males typically engage in courtship behavior on vegetation adjacent to a carcass. The work presented in this paper builds upon data from a recent study in which we described courtship behavior in *C. varipes* for the first time. We reported that courtship consists of a number of discrete behaviors, including: orienting, tapping, waving, wing vibration, arching and mounting, and found that mating success was significantly influenced by investment in courtship behavior (defined as the total time spent courting a female) (see Jones et al. 2014). However, the relative importance of individual behaviors, and the sequence of behaviors leading to copulation, were not explored and remain unknown. The aims of this study were to: 1) characterize male courtship behavior through the analysis of stereotyped pre-copulatory behaviors; 2) determine which behaviors were most important in determining mating success in this species; and 3) compare the temporal patterns of courtship behaviors between males that gained mating success (successful males) and males that did not (unsuccessful males). We predict that female mating decisions will be based on differences in specific

male courtship behaviors, and that investment in the different behaviors will influence male mating success. Addressing the aforementioned aims will also allow us to begin evaluating hypotheses relating to the use of multiple traits (i.e. multiple messages, back-up signals and species recognition).

Methods

Fly collection, culturing and mating experiments

Flies were trapped in January 2013 at the University of Wollongong Australia, and then kept in a constant temperature room ($23\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$) with a 12:12 h light/dark cycle. Flies were allocated a constant supply of raw sugar and water, as well as approximately 100 g of kangaroo mince which provided a food source and oviposition medium (Luddenham Pet Meats, Sydney, Australia). After eggs were laid, an additional 200 g of meat was provided and the meat and eggs were removed and placed in a plastic rearing container (130×190 mm and 70 mm high). The bottom of the container was covered in wheaten chaff as a pupation substrate, and upon pupation the container was placed in a larger cage (300×500 mm and 250 mm high) to permit free movement of adult flies after eclosion. To ensure that all flies used in experiments were virgins, the flies were sexed within 24 h of emergence, and males and females were kept in separate cages.

Mating trials commenced when the F1 generation of flies were aged $12 (\pm 2)$ days. Two flies were randomly selected and placed in mixed-sex pairs into a sealed transparent petri dish (85×15 mm), containing approximately 0.5 g of kangaroo mince positioned centrally. The behavior of flies was recorded with Samsung SCB-2000P digital cameras and a CCTV recording system under fluorescent lights and constant temperature conditions ($23\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$). Eight behavioral trials ran concurrently, with each trial recorded using a separate video camera. The experimental arenas were surrounded with cardboard barriers to ensure that flies in different trials were unable to see each other. Behavioral trials ran until mating occurred, or for a maximum of 20 min. All trials took place between four and eleven hours since lights on, and between one and seven hours until lights out, over three consecutive days in January, 2013 (i.e. trials were conducted between 1000 and 1700). The experimental trials described here are the same as those previously published by Jones et al. (2014), but the present study uses new behavioral analysis methods to address completely different aims.

Behavioral analysis

Analysis was performed for trials in which courtship behavior occurred ($n = 73$) using the behavioral analysis software package EthoLog 2.2 (Otoni 2000). Male courtship behavior was described and quantified from the time of courtship initiation until successful mating or cessation of the trial at 20 min (whichever occurred first). This approach was used because the focus of this study was in analyzing courtship duration and the behaviors in this period (time from first contact until mating), and is consistent with other studies of courtship behavior (Birge et al. 2010; Dyakonova and Krushinsky 2008).

There were six distinct courtship behaviors, and these were categorized as: orienting, tapping, waving, arching, wing vibration and mounting (Jones et al. 2014). Orienting occurred when the male followed and circled around a female. Tapping occurred when the male touched the female using his forelegs. Waving occurred when the male raised straightened forelegs (one or both legs, usually alternating). Arching was when a male stood directly in front of a female with straightened forelegs and an arched back. Arching was often accompanied by rocking backwards and forwards and an occasional slow wing flick. Wing vibration was rapid wing beating and also usually (but not always) occurred while the male was arched and standing directly in front of a female.

Mounting involved a male attempting to mate with the female by jumping onto her back. Males that mounted females were either rejected (pushed away by the female) or accepted (not pushed away by the female). Acceptance was followed by copulation (engagement of the male and female genitalia). At the end of a 20-min trial, males that achieved copulation were deemed to have gained mating success and were categorized as ‘successful males’, while males that did not achieve copulation were categorized as ‘unsuccessful males’. (see video footage of behaviors in Online Resource 1).

The behaviors orienting, wing vibration and arching were recorded as ‘state’ events as defined by the program EthoLog, and quantified as the proportion of time spent by the male in that behavior (quantified from the time of courtship initiation until mating, or completion of the trial). The behaviors orienting, wing vibration and arching will hereafter be referred to in terms of relative duration. Tapping, waving and mounting were recorded as discrete events, and quantified as the number of taps, waves or mountings per min (averaged from the time of courtship initiation until mating, or completion of the trial). The behaviors tapping, waving and mounting will hereafter be referred to in terms of occurrence per unit time (i.e. frequency).

Statistical analysis

Descriptive statistics, including means, standard deviations and range (minimum – maximum value), were calculated for each behavior. In addition, to determine the degree of variability in courtship behaviors, the coefficient of variation (CV) was quantified. The CV was calculated by dividing the standard deviation by the mean. Pairwise correlations were conducted to determine if there were significant associations between individual courtship behaviors. These correlations were conducted on the full set of data ($n = 73$) as well as separately for successful males ($n = 25$) or unsuccessful males ($n = 48$). All correlations were tested for significance using nonparametric methods (Spearman’s rank correlation), as not all behaviors satisfied assumptions of normality.

To determine if there were differences in courtship behaviors between successful and unsuccessful males, *t*-tests were conducted for each courtship behavior, courtship initiation latency, and for time spent on non-courtship behaviors. To determine which male courtship behaviors were predictive of mating success, a generalized linear regression model (GLM) with binomial errors and a logit link was used. Male mating success (successful or unsuccessful) was the response variable, and the predictor variables were: proportion of time spent orienting, wing vibrating, arching, and frequency of taps, waves and mountings. Data were analyzed using JMP V11 (SAS Institute, USA). The appropriate diagnostic procedures were implemented, including

residual inspection and testing for overdispersion, and all assumptions of GLM analysis were satisfied. Statistical significance was accepted when $p < 0.05$.

To complement significance testing, we also calculated effect sizes with confidence intervals for the GLM. These were determined in order to gauge the magnitude of effect (Hebets et al. 2011; Nakagawa and Cuthill 2007). For all of our models we calculated the statistic ϕ (phi) from the chi-square values generated for each of the predictor variable in our models. We calculated ϕ and its confidence intervals (CI) using freely available software (es calculator; Wilson (2001)).

To test for temporal associations between courtship behavioral patterns, we used a first order Markov chain model (where the probability of a given act depends on the identity of the act immediately preceding it) (Colgan 1978; Gottman and Roy 1990). Sequential data for both successful and unsuccessful males were used to construct two separate transition matrices, by tabulating all instances in which one behavior led to another (excluding non-courtship behaviors, following Chelbi et al. (2012)). Each matrix was compared to a randomly distributed matrix, using likelihood-ratio tests (G statistics) to evaluate whether certain transitions were more likely to occur than others (Chen et al. 2002). Williams correction was performed as part of the analysis. Freeman-Tukey deviates (cell-wise examinations) were used to identify which cells were statistically more likely to precede other behaviors. In addition, conditional probabilities were calculated as the total number of each transition from one behavior to the subsequent behavior, divided by the total number of the antecedent behavior in question (Saarikettu et al. 2005). These matrices, Freeman-Tukey deviates, conditional probabilities, as well as direct observations of courtship pairs, were used to construct a flow diagram of courtship behaviors for successful males and unsuccessful males (Bray and Hamilton 2007; Chelbi et al. 2012; Chen et al. 2002). These analyses were all performed using freely available Java applets (<http://caspar.bgsu.edu/~software/java>).

Results

Variation and correlations of courtship behavior in *C. varipes*

Males varied considerably in the time that they invested in each courtship behavior, with wing vibration and mounting having the largest amount of variation (Table 1). In total, 25 of the 73 (33%) male-female pairs achieved a successful mating. Both successful and unsuccessful males invested the largest amount of time during the mating trial in orienting themselves. Orienting and tapping were displayed by every male, while waving, wing vibration, arching and mounting were not always observed (indicated by % occurrence in Table 1).

Pairwise correlations between behaviors revealed a number of significant associations (Table 2). When pooled, orienting was positively correlated with all other behaviors except for wing vibration. In addition, waving and arching were each positively correlated with mounting, and arching and wing vibration were positively correlated with each other. All other correlations were not significant. There were several key differences when comparing correlations of behaviors for successful versus unsuccessful males. Notably, all of the associations between orienting and other behaviors were not significant in the successful mating group (Table 2).

Table 1 Descriptive statistics highlighting the variation in courtship behaviors for male *C. varipes* that were either successful (S, $n = 25$) or unsuccessful (U, $n = 48$) at mating. Occurrence refers to the percentage of males that engaged in each behavior. Significant differences between successful and unsuccessful males are indicated by an asterisk (*)

Behavior	Units	Mean ± standard deviation	Range (min-max)	CV	Occurrence %	t-test between S and U	
						t	DF p
Orienting	S	17.2 ± 14.8	1.8–55.7	0.86	100	0.85	36
	U	13.5 ± 9.8	2.2–39.7	0.72	100		
Tapping	S	2.2 ± 1.3	0.04–5.7	0.61	100	3.0	38
	U	1.3 ± 0.8	0.01–4.2	0.61	100		
Waving	S	0.4 ± 0.5	0–1.6	1.25	64	-1.5	50
	U	0.6 ± 0.7	0–3.4	1.24	77		
Wing vibration	S	3.4 ± 6.9	0–24.3	2.01	40	2.0	27
	U	0.5 ± 1.9	0–13.0	3.55	33		
Arching	S	10.1 ± 10.4	3.0–52.7	1.03	100	3.4	26
	U	2.8 ± 3.3	0–17.8	1.15	81		
Mounting	S	0.1 ± 0.7	0–2.4	2.38	24	1.5	28
	U	0.04 ± 0.1	0–0.7	3.24	15		
Non-courtship behaviors	S	74.4 ± 18.8	28.8–95.1	0.25	100	-2.9	33
	U	85.4 ± 10.9	50.0–98.2	0.13	100		

CV = coefficient of variation; S = successful mating, $N = 48$; U = unsuccessful mating, $N = 25$

Courtship behavior and mating success

Successful males invested more time in the behaviors arching, tapping and mounting compared to unsuccessful males (Table 1). The differences were not significant for the behaviors orienting, waving and wing vibration. However, when an outlier was removed the difference for wing vibration became significant, whereby successful males invested more in this behavior (*t*-test: *t* = 2.2, *DF* = 25, *p* = 0.03). There was no significant difference between courtship latency between successful and unsuccessful males (*t* test: *t* = -1.1, *DF* = 42, *p* = 0.27). This result is consistent when one outlier from each of the groups was removed (*t* test: *t* = -1.3, *DF* = 41, *p* = 0.20).

When pooled, male courtship behaviors had a significant effect on mating success (GLM: $\chi^2 = 51.08$; *df* = 6 *p* = <0.0001, $\phi = 0.84$). Mating success was dependent on time invested in orienting and arching, and frequency of taps and mounting (Table 3). Mounting occurred more often in the trials of successful males (Table 3, Fig. 1b). The frequency of tapping and the proportion of time spent orienting were also higher for successful males (Table 3, Fig. 1a and b). Arching was always present in the trials of successful males (with successful males spending at least 16 s or 3% of the time arching), and time invested in arching had a highly significant effect on mating success (Table 3). The magnitude of the difference in time invested in arching for successful and unsuccessful males is shown in Fig. 1a. Mating success was not influenced by the number of waves or by time invested in wing vibration. Unsuccessful males spent more time on non-

Table 2 Pairwise correlations between male courtship behaviors in the small hairy maggot blow fly. Data are presented for the total data set as well as individually for those who were successful and unsuccessful at mating

Total data set (<i>N</i> = 73)	Tapping	Waving	Wing vibration	Arching	Mounting
Orienting	0.39 (<0.001)	0.35 (0.003)	0.06 (0.640)	0.31 (0.007)	0.36 (0.002)
Tapping	—	-0.04 (0.739)	-0.03 (0.815)	0.21 (0.068)	0.19 (0.115)
Waving	—	—	0.06 (0.627)	0.03 (0.829)	0.23 (0.047)
Wing vibration	—	—	—	0.24 (0.044)	0.19 (0.109)
Arching	—	—	—	—	0.45 (<0.001)
Successful males (<i>N</i> = 25)	Tapping	Waving	Wing vibration	Arching	Mounting
Orienting	0.30 (0.152)	0.12 (0.569)	0.10 (0.632)	0.31 (0.130)	0.30 (0.142)
Tapping	—	0.003 (0.988)	-0.316 (0.124)	0.04 (0.861)	0.33 (0.113)
Waving	—	—	0.23 (0.264)	0.12 (0.561)	0.04 (0.866)
Wing vibration	—	—	—	0.11 (0.594)	0.01 (0.944)
Arching	—	—	—	—	0.50 (0.010)
Unsuccessful males (<i>N</i> = 48)	Tapping	Waving	Wing vibration	Arching	Mounting
Orienting	0.38 (0.008)	0.49 (<0.001)	0.01 (0.937)	0.33 (0.021)	0.29 (0.049)
Tapping	—	0.01 (0.937)	0.04 (0.778)	-0.02 (0.893)	0.13 (0.378)
Waving	—	—	0.04 (0.780)	0.22 (0.134)	0.28 (0.058)
Wing vibration	—	—	—	0.25 (0.092)	0.26 (0.073)
Arching	—	—	—	—	0.51 (<0.001)

Data presented as Spearman’s *r* (*P* value). Significant correlations are shown in bold

Table 3 Generalized Linear Regression Model indicating the effect of courtship behaviors on mating success in the small hairy maggot blow fly

Behavior	χ^2	<i>P</i>	ϕ	CI
Orienting	5.51	0.019*	0.27	0.047–0.48
Tapping	12.37	<0.001**	0.41	0.19–0.59
Waving	0.95	0.33	0.13	–0.14–0.38
Arching	34.59	<0.001**	0.74	0.56–0.85
Wing vibration	1.40	0.24	0.23	–0.15–0.56
Mounting	5.87	0.0154*	0.65	0.15–0.88

ϕ = phi coefficient (effect size); CI = confidence interval; **P* < 0.05; ***P* < 0.001

courtship behaviors, while successful males spent more time on courtship (*t*-test: *t* = –2.9, DF = 35, *p* = 0.007).

Sequence of behaviors during courtship

The flow diagrams (Fig. 1) were derived from the transition matrices (Table 4 and Table 5), and suggest the following simplified model of *C. varipes* courtship behaviors. Male flies initiated courtship by orienting themselves. The male then alternated between orienting and tapping (male tapped the female, usually on the abdomen or legs) and waving (slowly raised his forelegs). Following orienting, the male then stood directly in front of the female (arching). During arching, the male often slowly raised and lowered his wings. Arching could also lead to wing vibration, where the male rapidly vibrated his wings (which then led back to arching). In successful males, arching only progressed to wing vibration or

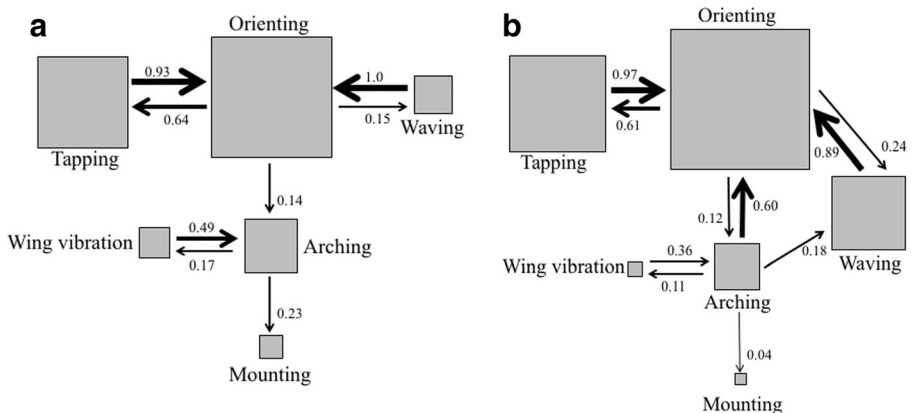


Fig. 1 Flow diagrams of courtship behaviors in *C. varipes* derived from the analysis of (a) 25 males that mated successfully; and (b) 48 males that were unsuccessful at mating. The figures represent a first-order Markov chain analysis of transition matrices, and the sequences of behaviors are depicted when transitions occur significantly more than predicted by chance. The sizes of the boxes represent the relative frequency of occurrence of a particular behavior. The size of the arrows and the numbers beside the arrows indicate the conditional probability for each transition (or the degree to which particular transitions are over-represented). See attached files ‘Fig1a.JPG’ and ‘Fig1b.JPG’

Table 4 Transition matrix summarizing how each courtship behavior (in the rows) was followed by a subsequent behavior (columns) in the small hairy maggot blow fly for 25 males that mated successfully. The numbers represent the conditional probability of a particular transition relative to the number of males analyzed (i.e. occurrence of event divided by n). By comparing these values with a null distribution from a model of independence, we identified those transitions that occur more or less frequently than those predicted by chance. The significant transitions are indicated by an asterisk ($p < 0.05$) and these transitions are represented in Fig. 1a

Preceding behavior	Following behavior						
	Orienting	Tapping	Waving	Wing vibration	Arching	Mounting	Σ
Orienting		11.1*	2.6*	0.8	2.4*	0.4	17.3
Tapping	11.2*		0	0	0.9	0	12.1
Waving	2.6*	0		0	0	0	2.6
Wing vibration	0.8	0	0		0.7*	0	1.5
Arching	1.6	0.9	0	0.7*		0.9*	4.1
Mounting	0.3*	0	0	0	0		0.3
Σ	16.5	12.0	2.6	1.5	4.0	1.3	37.9

mounting (Fig. 1a and Table 4). By comparison, in unsuccessful males, although arching also led to wing vibration and mounting, more often it reverted to orienting, or progressed to the behavior waving (Fig. 1b). Thus, mounting featured more prominently in the post-arching behavior of successful males than of unsuccessful males. Overall, the two flow diagrams highlighting the differences between successful and unsuccessful males (Fig. 1a and b) are very similar, with only subtle differences with respect to the sequence and frequency of transitions.

Table 5 Transition matrix summarizing how each courtship behavior (in the rows) was followed by a subsequent behavior (columns) in the small hairy maggot blow fly for 48 males that were unsuccessful at mating. The numbers represent the conditional probability of a particular transition relative to the number of males analyzed (i.e. occurrence of event divided by n). The significant transitions are indicated by an asterisk ($p < 0.05$) and these transitions are represented in Fig. 1b

Preceding behavior	Following behavior						
	Orienting	Tapping	Waving	Wing vibration	Arching	Mounting	Σ
Orienting		13.1*	5.0*	0.4	2.6*	0.3	21.4
Tapping	13.2*		0.02	0	0.3	0	13.5
Waving	5.1*	0.02		0	0.6	0	5.7
Wing vibration	0.5	0	0		0.3*	0	0.8
Arching	2.2*	0.3	0.6*	0.4*		0.2*	3.7
Mounting	0.4	0	0	0	0.04		0.4
Σ	21.4	13.4	5.6	0.8	3.8	0.5	45.5

Discussion

The results of our study demonstrate that variation in relative amounts and sequences of courtship behavior influenced male mating success in *C. varipes*. In particular, mating success was significantly influenced by the relative duration of orienting and arching, and by the frequency of tapping and mounting. While the sequence of behavioral events appeared to follow a pattern, there were some subtle differences observed between successful and unsuccessful males in the progression of behavioral sequences leading to mating. The progression from arching to mounting was particularly characteristic of successful males, while unsuccessful males more often displayed orienting or waving after arching, rather than mounting. In addition, the differences between courtship behaviors between individual flies were highly variable with respect to their duration and frequency. Overall, our findings support our prediction that female mating decisions are based on differences in the male's courtship behaviors. In addition, this study has provided the first step towards understanding the role played by these multiple behavioral traits in female mate choice.

Orienting was performed by every courting male; it comprised a very large proportion of male courtship behavior and time spent orienting had a significant effect on mating success. *Drosophila melanogaster* displays a similar behavior (also termed orienting) and in these flies orienting is used by the male to initiate courtship (Greenspan and Ferveur 2000). Similarly, this appears to be the case in *C. varipes*. However, in *D. melanogaster*, orientation is characterized by the male facing the female, whereas orientation in *C. varipes* was often accompanied by a distinct movement by the male around the female, which is not seen in *D. melanogaster* (Greenspan and Ferveur 2000). However, males from other species of *Drosophila* that possess male-specific wing spots, such as *D. suzukii* and *D. biarmipes*, engage in a circling behavior around the female in order to increase the visibility of their ornamented spotted wings (Kopp and True 2002; Mazzoni et al. 2013; Revadi et al. 2015). Thus, similarly, orienting behavior in *C. varipes* may be to improve visibility of their ornamented forelegs. There were positive associations between orienting and all other courtship behaviors in unsuccessful males (except for wing vibration), but no associations in successful males. The reason for this difference is unknown, but it may be that orienting is uninformative in regard to male quality (or at least not directly reflecting quality). The positive associations between orienting and other courtship behaviors in unsuccessful males may be a reflection of a male's motivation to mate, rather than male quality *per se* (Guevara-Fiore et al. 2010).

In between periods of orienting, males proceeded to the behaviors tapping and waving. Male tapping behavior (where the male touched the female using his forelegs) was present in every trial in which males displayed courtship behaviors, with contact most often made on the female's legs or abdomen. The frequency of taps performed by a male significantly influenced his mating success. The most likely explanation for this association is that tapping is a tactile signal, as is seen in many other insects including *Drosophila* (Colyott et al. 2016; Giglio and Dyer 2013; Narda 1966). It is also possible that this behavior may facilitate the transfer of pheromone signals, such as cuticular hydrocarbons. Chemical signals are extremely important in the mating behavior of various insect species (Barry et al. 2010; Billingham et al. 2010; Howard and

Blomquist 2005). For example, in *D. melanogaster*, tapping is used to detect chemical substances through contact with gustatory receptors which are predominately located on the tarsi of their forelegs (Bray and Amrein 2003). *Chrysomya varipes* may therefore also use chemical signals in recognizing and even discriminating between potential mates, and these may be transmitted when physical contact is made. However, we cannot disentangle the exact function of this behavior based only on observation; further research is therefore needed to quantify other modalities.

Waving did not significantly influence male mating success, which may indicate that this behavior does not signal male quality to prospective mates. However, analysis of the sequence of behavioral transitions revealed that unsuccessful males sometimes reverted back to waving after arching, which was not the case for successful males. Waving may be used as a signal to gain the initial attention of females, or to re-engage unreceptive females. Once a female is engaged, other behaviors may then be more important for female mating decisions and male mating success.

After males oriented, they also proceeded to the behavior arching. Arching appears to be crucial for mating success as it always preceded mounting, and mating success was highly influenced by this behavior. Arching involved the male and female facing each other for long periods (up to 4 min) with the male occasionally lifting his wings. Arching may be similar to the behavior ‘face-off’ in the flesh fly *Phrosinella aurifacies* (Spofford and Kurczewski 1985) or ‘facing’ in the sand fly *Phlebotomus papatasi* (Chelbi et al. 2012). In *P. papatasi*, Chelbi et al. (2012) stated that this apparent waiting behavior during courtship might indicate that a physiological change needs to occur in either partner prior to mating. In addition, at times it appeared as if contact between the mouthparts was made in *C. varipes*, which might allow for the transfer of substances required to stimulate mating, such as pheromones or nuptial gifts. However, a more detailed behavioral investigation involving close-up video recordings from multiple angles will be required to ascertain the exact nature and function of this behavior. Despite this uncertainty, there appears to be a minimum threshold above which a male must arch in order to mate: males that achieved mating success spent at least 16 s (or 3%) of the trial time arching. This finding supports the notions of Bastock and Manning (1955), who proposed that female receptivity in *D. melanogaster* is enhanced by a gradual summation of courtship, and that once a certain threshold is attained, a female will accept the courting male (Bastock and Manning 1955). However, other than the present study, there is limited evidence for specific individual behavioral traits playing a crucial role in determining mating success (but see Bray and Hamilton (2007) and Budriene and Budrys (2004)).

In between periods of arching, males often proceeded to wing vibration (49% of successful males and 36% of unsuccessful males) (Figs 1a and b, respectively). Occurrence of this behavior did not always lead to mating, and there was no significant effect of wing vibration on mating success. The function of this presumably energetically expensive display is therefore unknown. It has been hypothesized by Bray and Hamilton (2007) that the function of ‘wing-flapping’ in the sand fly *Lutzomyia longipalpis* is to disperse sex pheromones. In addition, wing-flapping produces an auditory signal that is predicted to function in mate recognition (Bray and Hamilton 2007). Similarly, wing vibration in *D. melanogaster* produces an acoustic signal known as their ‘courtship song’ which is important in species recognition (Kyriacou and Hall 1982; Ritchie et al. 1999). A more detailed analysis is required to quantify auditory and

chemical signals produced and/or released by wing vibration in order to detect an effect of wing vibration on mating success in *C. varipes*. In addition, there is likely to be variation in the actual motor performance in terms of vigor and skill between males that cannot be detailed with data only considering the amount of time spent on the action (Beyers et al. 2010). For example, two males may wing vibrate for the same length of time, but one of these males could potentially be beating at a higher frequency, and this may be a more honest indicator of quality (Zahavi 1975). For example, in the olive, fruit fly *Bactrocera oleae*, males who mated successfully had a significantly higher frequency and pulse duration of wing vibration during courtship (Benelli et al. 2012). Further research will therefore be necessary to uncover the exact function of wing vibration.

Although this study has illuminated potential influences of the different courtship behaviors on female mating preferences, the use of these multiple behaviors remains unknown (i.e. it is unknown whether the use of multiple behaviors is for conveying multiple messages, providing back-up signals, or permitting species recognition). Disentangling support for the aforementioned hypotheses is difficult when considering only time invested in a particular action, and also not quantifying the multi-modal aspect of each behavior (e.g. chemical and acoustic signals). To clarify the exact role of each behavior, future studies may need to specifically examine the relationship between courtship behaviors and other reproductive traits, including aspects of male quality such as the fertility and reproductive potential of individual males.

Importantly, while the focus of this study was on female mate choice and male courtship behaviors, it must be recognized that male courtship investment may vary depending on the frequency and intensity of female cues received during the courtship process. Males have been shown to adjust their courtship investment depending on their reception of different female visual, auditory and olfactory signals (Appelt and Sorensen 2007; Bonduriansky 2001; Galán and Price 2000; Guevara-Fiore et al. 2010; Semple and McComb 2000). In addition, specific female behaviors can alter a male's courtship investment. For example, in the eastern fence lizard, *Sceloporus undulatus*, the way in which a female approached or retreated from a male was found to have an important influence on male courtship behavior (Swierk et al. 2013). Swierk et al. (2013) suggested that such behaviors may advertise a female's willingness to mate, which in turn, could increase a male's motivation, and courtship investment in receptive females. The influence of female behavior in *C. varipes* is an area we are currently investigating, and preliminary results suggest that there are indeed associations between female behaviors and male courtship (unpublished data). Therefore, incorporating measures of female behavior in *C. varipes* might show that some of the variation in male behavior, and mating success, is explained by the degree of female receptivity.

Furthermore, studying isolated interactions between single male and female pairs may be difficult to evaluate when lekking species interact in more complex social environments (Fiske et al. 1998; McGhee et al. 2007). Specifically, male behaviors can be influenced by the presence of other males, and male rivalry can play important roles in dictating mating outcomes (Wong and Candolin 2005). From our observations in nature, while males interact and display courtship behaviors towards each other, there is no overt physical male-male combat in this species. Nevertheless, future studies in *C. varipes* may benefit from examining the effect of competition on mating success.

In conclusion, this detailed observational study, the first in blow flies, allowed us to quantify courtship behaviors in *C. varipes*. As predicted, we found evidence for the

importance of individual behaviors on mating success, particularly the behavior ‘arching’ which appears to be critical for mating. We also analyzed the temporal patterns of courtship behaviors, and found there to be a progression of behaviors during courtship that precedes a mating attempt. Additionally, courtship behaviors between individuals were extremely variable with respect to their duration and frequency. An important next step will be to examine and incorporate measures of female behavior, as well as quantifying the relative importance of different sensory cues for mating success, including chemical and acoustic signals. Nevertheless, the present study underscores the importance of considering multiple behavioral traits when attempting to determine the influence of male courtship investment on male mating success.

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