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Courtship in Two Morphotypes of the *Anastrepha fraterculus* (Diptera: Tephritidae) Cryptic Species Complex and their Implications for Understanding Mate Recognition

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Abstract

Ritualized courtship behaviors are used to recognize potential mates and behavioral patterns are inevitably different among populations that demonstrate reproductive incompatibility. We characterized and compared the courtship behaviors of two morphotypes of the cryptic species complex *Anastrepha fraterculus*: Brazil-1 morphotype and Brazil-3 morphotype. Courtship behaviors were filmed to analyze the behavioral sequences of these two morphotypes during homotypic crossings. The behavioral units *Alignment* (AL) and *Abdominal movements* (AB and AB-call) were newly recognized in the courtship ethogram of *Anastrepha fraterculus* males. The two morphotypes show distinct behavioral sequences leading up to copulation. Some behaviors were repeated frequently during the courtship process, while others were more restricted to the final moments of courtship. The three behavioral units that contributed most to copulation success were *Contact*, *Alignment,* and *Arrowhead* 1 in the Brazil-1 morphotype and *Alignment*, *Arrowhead* 1, and *Fanning* in the Brazil-3 morphotype. Some behavioral routines differed across the two morphotypes. Significant differences were also noted between the frequencies of the behavioral units displayed during courtship in the two morphotypes. The relationships between the pre-zygotic incompatibilities of the Brazil-1 and Brazil-3 morphotypes and the differences between the courtship behaviors of their males are discussed. Our results indicate that behavioral isolation is involved in the process of pre-zygotic reproductive isolation of Brazil-1 and Brazil-3 morphotypes.

Keywords Sexual selection · pre-zygotic isolation · speciation · fruit flies · partner recognition

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Introduction

Courtship behaviors involve interactions between male and female stimuli and responses (Butlin and Ritchie 1994). Courtship signals generally involve visual displays, chemical and auditory signals – or combinations of all three (Greenspan and Ferveur 2000; Wicker-Thomas 2007). Courtship may vary between isolated populations and mediate mate recognition and choice (Cobb and Jallon 1990). Geographically isolated populations may show divergence in traits, resulting from divergent selection that can result in the emergence of new species (Schluter 2001). However, geographically isolated populations do not always mate assortatively (Aluja et al. 2009) and therefore show in-complete intrinsic isolation (Rull et al. 2010).

Anastrepha fraterculus is widely distributed from northern Mexico to southern South America (Stone 1942; Aluja 1994; Hernández-Ortiz et al. 2015). One of the earliest identifications of morphological differences within *A. fraterculus* was reported by Stone (1942). Since then, genetic molecular and morphometric studies indicate that the fruit fly *A. fraterculus* constitutes a cryptic species complex (Steck 1991; Selivon and Perondini 1998; Smith-Caldas et al. 2001; Selivon et al. 2004; Hernández-Ortiz et al. 2004; Barr et al. 2005; Selivon et al. 2005; Silva and Barr 2008; Cáceres et al. 2009; Hernández-Ortiz et al. 2012; Dias et al. 2016; Canal et al. 2015). A morphometric analysis of eleven populations resulted in the definition of three taxonomic units: Mexican, Andean and Brazilian (including populations from Brazil and Argentina) (Hernández-Ortiz et al. 2004). Subsequently, Hernández-Ortiz et al. (2012) analyzed 32 Neotropical populations and described seven morphotypes (Peruvian, Colombian-Andean, Mexican, Venezuelan, and Brazil-1, Brazil-2, and Brazil-3); more recently, an eighth morphotype (Ecuadorian) was added (Hernández-Ortiz et al. 2015). Similarly, phylogenetic analyses showed distinct clades within *Anastrepha obliqua* organized in six groups, suggesting the need for a taxonomic revision (Ruiz-Arce et al. 2012).

Morphological differences do not always represent reproductive incompatibility between species, as evidenced by other fruit fly species, such as *Rhagolethis sp.* (Rull et al. 2012). Analyses of reproductive compatibility between distinct morphotypes of the cryptic species complex *Anastrepha fraterculus* have shown homotypic mating preferences (Cáceres et al. 2009; Vera et al. 2006; Rull et al. 2013; Devescovi et al. 2014). Recently, Roriz et al. (2017) evidenced pre- and post-zygotic incompatibility between allopatric morphotypes Brazil-1 and Brazil-3 suggesting that differences in courtship behavior have important functions in reproductive isolation of morphotypes. Male signaling traits and female preferences generate strong selective pressures that reduce heterotypic matings (Liou and Price 1994; McPeek and Gavrilets 2006).

Sex pheromones have a role in mate recognition (Antony and Jallon 1982; Cobb and Jallon 1990; Giglio and Dyer 2013). Qualitative differences in the pheromone of strains of *A. fraterculus* are likely emergent properties of speciation within that cryptic species complex (Lima et al. 2001; Cáceres et al. 2009). Analysis of the pheromone composition of the seven allopatric morphotypes of that complex revealed significant variation, even among populations classified as belonging to the same morphotype (Břízová et al. 2013). Consequently, these variations in composition are not necessarily directly related to mating compatibilities. Cuticular hydrocarbons are another class of compounds that can mediate mate recognition and appear to differ between *A. fraterculus* strains (Vaníčková et al. 2014). The Mexican and Colombian morphotypes differ from the Peruvian and Brazil-1 groups in relation to their cuticular hydrocarbons (CH) but differ little within morphotypes (Vaníčková et al. 2015c). Similarly, based on its CH profile, the Andean morphotype is distinct from the Brazil-1 and Brazil-3 morphotypes, (Vaníčková et al. 2015b). Other pre-zygotic factors, such as courtship behavior, are probably involved in conspecific recognition within of the *A. fraterculus*.

Courtship in *A. fraterculus* occurs within a polygynous system in which the males delimit territories to attract females (Malavasi et al. 1983; Segura et al. 2007), and females select among lekking males (Aluja et al. 1999; Whittier and Kaneshiro 1995). Differential copulation rates among participating males is consistent with the hypothesis that mate choice is an important component of these mating systems (Whittier et al. 1994). *Anastrepha* sexual behavior is complex, heterogeneous, and demonstrates high phenotypic plasticity (Aluja 1994; Aluja et al. 1999; Gomez-Cendra et al. 2011), and *A. fraterculus* courtship involves both long and short distance behavioral interactions (Calcagno and Vilardi 2001). Long-distance male signals characterized by the emission of a pheromone droplet from the tip of the male abdomen together with continual rapid up and down vibrational movements of its wings, which appears to serve as visual and auditory stimuli (and also facilitates pheromone dispersal). Male and female short distance signals before and during copulation also exist. For example, during copulation, the male holds the female between his three pairs of legs sometimes touching the female's head with his proboscis. *Anastrepha fraterculus* courtship has only been analyzed for Brazil-1 morphotype (Calcagno and Vilardi 2001; Gomez-Cendra et al.2011; Dias et al. 2016). Gomez-Cendra et al. (2011) compared successful to unsuccessful courtships, describing 15 behavioral units, organized in three categories: pheromone emission (Call 0, Call 1 and Call 2), wing positioning (Relax, Transversal, Enation, Hamation, Arrowhead 1, Arrowhead 2, Fanning and Spin), and body movements (Stationary, Mobile, Oscillation, Fight, Attempt and Contact).

In light of the existence of wide variability and complexity in courtship behavior, it has been assumed that there are behavioral differences between the distinct morphotypes of this cryptic species complex. The identification of variation in sexual behavior is important to characterize behavioral isolation in cases of mating incompatibility. We therefore sought to analyze and compare the courtship behaviors of Brazil-1 and Brazil-3 morphotypes, focusing on male behavior.

Material and Methods

Biological Material

Anastrepha fraterculus fruit flies were obtained from infested guava fruits (*Psydium guajava*) collected in the field near the towns of Parnamirim in Rio Grande do Norte State (05° 54′ 57“S × 35°15’46”W) (Brazil-3) and Bento Gonçalves, Rio Grande do Sul State (29° 10′ 15“ S and 51° 31’ 08” W) (Brazil-1), both in Brazil. Guava fruits in Brazil are normally infested by various species of fruit flies of the genus *Anastrepha*; a pure population of each morphotype of *Anastrepha fraterculus* was obtained utilizing the protocol described by Roriz et al. (2015).

Random samples of the isolated females were preserved in 70% alcohol and identified by Dr. Vicente Hernández-Ortiz of the Instituto de Ecología A.C. México (personal communication), a specialist in that group.

The fly populations were maintained in the Laboratório de Ecologia Comportamental de Insetos (LECI) at the Biological Institute (UFBA) in Salvador, Bahia State, Brazil, using methodologies adapted from the entomology laboratories of FAO/IAEA (Seibersdorf, Áustria) (Vera et al. 2006) and CENA (USP, São Paulo, Brazil).

The colonies were kept at 25 ± 1 °C, relative humidity 70 ± 10%, under a 12 h photoperiod. After emergence, water and a diet based on hydrolyzed protein and sugar (1:3) were furnished ad libitum. The individuals used in all of the experiments were between 17 and 20 days old.

Recording Courtship

Courtships were videotaped in a glass box (9 × 7 × 9 cm) containing a pitanga (*Eugenia uniflora*) leaf as the substrate for sexual interactions (Briceño et al. 2007). Females were introduced 5 min after a male initiated calling behavior. Insect behavior was filmed for up to 30 min. (using a Geovision- GV-BX 220D-3, 2 M camera with a variable focus lens) at 30 frames/s, positioning the camera frontally to the glass container, with artificial illumination. The images were captured using Eagle Vision Pro version 4. software, and the videos saved in AVI format using Geo Vision 800. All recordings were made between 07:00 and 12:00. Ten independent groups of each morphotype that showed successful mating were analyzed.

Video Analyses

Only the male that mated successfully was filmed in each session. The behavioral sequences of the two morphotypes were recorded during preliminary analyses, in which 10 videos of each morphotype were observed and all male activities were closely followed and identified on a second-to-second basis to construct a courtship ethogram in tabular form. Male activities were characterized according to definitions established in the literature, supplementing them with any new details that could be added to their behavioral descriptions; these new behavioral units were also introduced into the courtship ethogram.

After defining the courtship ethogram, 10 new video recordings of each morphotype were analyzed to determine the sequential behaviors associated with successful male courtship. The analyses of the behavioral sequences of the morphotypes were performed using EthoSeq software (Japyassú et al. 2006), which extracts probabilistic behavioral sequences (tree-generated sequences, or TGSs) from observational data. The flow of behavioral units of the males in each morphotype were transformed into a first order transition matrix that was then used to construct maximum probability trees. EthoSeq software produces a hierarchical representation (TGSs) with all behavioral categories in the matrix placed as nodes in a branching diagram, whose base is called the root. This analysis uncovers much of the hidden behavioral structure within the data. We used here the tree whose root indicated copulation success, ending in the *Mating* unit (the diversity of paths leading to mating). The DiTree maximizes the sum of the probabilities of transitions between all of the behavioral units (i.e., the contained routines were the most probable within the data set) (Japyassú et al. 2006). Using the data from the most frequent behavioral routines, a canonical discriminant analysis was performed to detect behavioral dyads (the EthoSeq behavioral routines) that contributed most to the differentiation of the morphotypes. The behavioral routines were included in the analyses using the *stepwise* mode, with $F = 0.05$ to enter and $F = 0.10$ to remove the discriminant functions utilized in Wilk's Lambda method. When necessary, Fisher's Exact Test was used to compare frequencies of behavioral routines between the morphotypes. The total frequencies of the behavioral units in each morphotype were also analyzed, the lenght of the behavioral sequences and the total courtship times of the morphotypes were evaluated using unpaired t-tests. The total duration of courtship (t) was considered to be the time between the introduction of the female into the glass cage with the male and the moment copulation (mating) was initiated. The lenght of the behavioral sequence was defined as the sum of all the behavioral units executed in each courtship event. The frequency of each behavioral unit was compared between the morphotypes of *A. fraterculus* using the Generalized Linear Model (GLM), assuming that the data followed a Poisson distribution, and using the Log as a link connection. The statistical analyses were performed using R studio free software, except for the discriminant analysis, which was executed using STATISTICA 7.1 software (Stat soft. 1984–2005).

Results

The ethogram of the courtship produced was compatible with that reported by Gomez-Cendra et al. (2011) and Calcagno and Vilardi (2001), with some minor alterations and insertions. The male courtship behavioral units included: grooming; pheromone emission; body movements; movement, positioning, or signaling with wings; abdominal movements; agonistic behavior; near copula male/female interactions; copulation (Table 1). The *Alignment* behavioral unit (AL), previously described for *Ceratitis capitata* (Diptera-Tephitidae) (Briceño and Eberhad 2002), was observed in *A. fraterculus*; *Abdomen movement* (AB and AB-call) units are described here for the first time.

Some behavioral units were merged to facilitate descriptions of the behavioral sequences. Due to the angle at which the video images were captured, distinctions between *Calling*-1 (CALL 1) and *Calling*-2 (CALL 2) were often difficult to establish. To address this problem, these two behavioral units were consolidated into a single unit of *Calling* (CALL). We observed that the *Stationary* unit always occurred with the wings being held in the *Relax* (RE) position or at 45° (ATR), and these two categories were therefore grouped into the *Stationary* (ST) unit. Similarly, the *Mobile* behavioral unit always occurred with the wings held at 45°. Some behavioral units were executed concomitantly with pheromone emission (*Calling*-CALL) or performed on their own, and were therefore segregated as distinct units (AB/AB-call; HA/HA-call; EN/EN-call; OC/OC-call; TR/TR-call) (Table 1). As such, the courtship ethogram constructed here consisted of 26 behavioral units, including the behavioral routines resulting in successful mating (*Attempt*-AT followed by *Mating*-MT).

Table 1 Descriptions of the behavioral units executed by *Anastrepha fraterculus* males during courtship

Stage	Behavioral categories	Abbreviations and descriptions of the behavioral units
Start	Pheromone emission	**(CALL)** *Calling*: pheromone emission with a bright and translucent drop of pheromone visible by the eversion of the anal membrane of the male (Call 1) and/or pheromone emission with visible exposure of the prominent lateral pleural glands (Call 2) (Gomez-Cendra et al. 2011; with modifications)
Courtship development	Movement, positioning, or signaling with wings	**(FA-call)** *Fanning*: male, walking or stopped, performs rapid and strong wing vibrations, continuous as well as intermittent, while emitting pheromone (Call 1 and/or Call 2) (Gomez-Cendra et al. 2011)
		(EN) *Enantion*: slow and simultaneous back and forth motions of the wings. The angle between each wing and the body varies between 0° and 90° (Robacker and Hart 1985).
		(EN-call): *Enantion* realized simultaneously with pheromone emission visible by the eversion of the anal membrane of the male (Call 1) and/or pheromone emission with visible exposure of the prominent lateral pleural glands (Call 2).
		(HA) *Hamation*: The flies, usually stopped, performs alternate wing movements, with the angle between each wing and the body varying between 0° and 90° (Robacker and Hart 1985; with modifications)
		(HA-call): *Hamation* undertaken simultaneously with pheromone emission visible by the eversion of the anal membrane of the male (Call 1) and/or pheromone emission with visible exposure of the prominent lateral pleural glands (Call 2).
		(TR) *Transversal*: The flies, usually still, holds its wings transversal (180°) to the body axis (Gomez-Cendra et al. 2011)
		(TR-call): transversal position of the wings with concomitant pheromone emission visible by the eversion of the anal membrane of the male (Call 1) and/or pheromone emission with visible exposure of the prominent lateral pleural glands (Call 2).
		(AH 1-call) *Arrowhead*-1: male holds its wings pointing backwards, maintaining them rigid and close to its body while emitting pheromone with visible exposure of the prominent lateral pleural glands (Call 2) and moves the abdomen (AB-call). The insect may move its proboscis (or not) up and down (Gomez-Cendra et al. 2011; with modification).
		(AH 2-call) *Arrowhead*-2: male moves its wings quickly and alternately, stroking them against the lateral pheromone glands for emission (Call 2); the maximum angle between the wing and the body is 45°; can occur (or not) with up and down movements of the proboscis (Gomez-Cendra et al. 2011, with modification).
	Body movements	**(OC)** *Oscillation*: the fly moves its legs laterally, walking, creating arcs with the movement of its body from side to side. At each lateral oscillation the insect executes wing movements of *hamation* (HA) or *enantion* (EN)

Table 1 (continued)

Stage	Behavioral categories	Abbreviations and descriptions of the behavioral units
		(Gomez-Cendra et al. 2011, modified).
		(OC-call): *Oscillation* undertaken simultaneously with pheromone emission visible by the eversion of the anal membrane of the male (Call 1) and/or pheromone emission with visible exposure of the prominent lateral pleural glands (Call 2).
Courtship development		**(SP-call)** *Spin*: the fly turns 360° on its own axis, emission pheromone visible the eversion of the anal membrane of the male (Call 1) and/or pheromone emission with visible exposure of the prominent lateral pleural glands (Call 2) and performing *Fanning* (Gomez-Cendra et al. 2011)
		(GF-call) *Graceful:* the fly liberates its sexual pheromone (CALL), performing *Fanning* (FA-call), and simultaneously executing arching rapid movements of its body (Aluja et al. 1999).
		(FL) *Flying:* the fly flies, flaps its wings and moves to a landing field.
		(MO) *Mobile*: When the male walks by moving his legs synchronously, and usually keeps his wings in an almost transversal position to 45° (ATR) (Gomez-Cendra et al. 2011; with modification).
		(ST) *Stationary*: the fly remains stationary with its wings in a relaxed position (RE), near the body - in a normal position, resting on its body (Gomez-Cendra et al. 2011) - or held almost transversal (ATR) - wings with median angle of 45° in relation to its body; proximal portion of the wing positioned downward, distal portion upward (Gomez-Cendra et al. 2011; with modification).
		(AT) *Attempt*: the male fly jumps onto the female and attempts to copulate, raising the ovipositor of the female with its rear legs while, sometimes, touching the head of the female with his proboscis, making forward and backward movements (Gomez-Cendra et al. 2011; with modification).
	Abdominal movements	**(AB)** *Abdominal movements*: up and down movements of the abdomen in relation to its normal position. Wings generally in an almost transversal position to 45° (ATR), although the wings can also be maintained in a transversal position (TR) or relaxed (*Relax*-RE).
		(AB-call): abdominal movements executed simultaneously with pheromone emission visible by the eversion of the anal membrane of the male (Call 1) and/or pheromone emission with visible exposure of the prominent lateral pleural glands (Call 2)
		(ML) *Marking* leaf: male touches the leaf with his inverted anal membrane, marking the substrate with pheromone (Sivinski et al. 1994)
	Grooming	**(GR)** *Grooming:* the fly remains still and performs body cleaning movements, stroking the legs on the wings, head, thorax, abdomen, proboscis, antennae, and ovipositor.
	Agonistic behavior	

Table 1 (continued)

Stage	Behavioral categories	Abbreviations and descriptions of the behavioral units
		(FI) *Fight*: aggressive interactions, between the males, or between males and females where the fly pushes back the other one or strikes the other with its head (Gomez-Cendra et al. 2011).
End of the courtship	Near copula male/female interactions	**(AL)** *Alignment*: the male and female remain still and position themselves directly facing one another at short distances, looking directly at their potential partner.
		(CO) *Contact*: male touches the female with its anterior legs before *attempt*; or touching proboscis between male and female before *attempt*; or touching antennae between male and female (Morgante et al. 1980 modified).
	Copulation	**(MT)** *Mating*: the male fly holds the female, positioning its three pairs of legs on the body of the female, introduces its copulatory organ into the female, while sometimes touching the head of the female with its proboscis, making forward and backward movements.

Behavioral Sequences

A total of 458 courtship routines (transition between behavioral units) were observed and analyzed, 275 were performed by Brazil-1 and 183 were performed by Brazil-3 morphotype. The courtship behavior sequences of the two morphotypes showed different patterns. Within the behavioral routines that resulted in successful copulation (MT-*mating*), the copulation attempt (*Attempt*) behavioral unit in the Brazil-1 morphotype was preceded by *Contact* (CO-31.58%), *Alignment* (AL-25%), *Arrowhead*-1 (AH1-call 21.5%), *Transversal* with pheromone emission (TR-call 5.26%), *Fight* (FI- 5.26%), or *Abdominal movements* with pheromone emission (ABcall 5.26%) (Fig. 1a). In the Brazil-3 morphotype, copulation attempts were preceded by *Alignment* (AL-30.77%), *Arrowhead*-1 (AH1-call 23.08%), *Fanning* (FA-call 15.38%), *Contac*t (CO- 7.69%), *Flying* (FL7.69%), *Mobile* (MO-7.69%), or *Calling* (CALL- 7.69%) (Fig. 1b). The behavioral units *Alignment* (AL), *Arrowhead* 1 (AH1-call), and *Contact* (CO) contributed in both morphotypes to the occurrence of *Attempt* (AT). The unit *Contact* contributed more to the occurrence of copulation in the Brazil-1 morphotype than in the Brazil-3 morphotype (which demonstrated *Fanning* as having the third greatest percent occurrence).

The three behavioral units that preceded the behavioral routine AT> MT and presented a higher percentage of occurrence in the Brazil-1 morphotype were AL, AH-call and CO, and in Brazil-3 were AL, AH-call and FA-call (Fig. 2). The frequency of execution of the behavioral routine CO > AT in the Brazil-1 morphotype and FA-call> AT in Brazil-3 morphotype contributed to the difference between them (Fisher's Exact Test, $P = 0.046$). Various behavioral units preceded *Alignment* in the Brazil-1 morphotype (HA-call, FA-call, AH2-call, MO, CALL, EN, FL, TR, HA or ST) but only three preceded *Alignment* (ST, AB or LI) in the Brazil-3 morphotype. The behavioral unit *Fanning* (FA-call) was preceded by various behavioral units in the Brazil-3 morphotype (EN-call, ML, TR-call, FI or OC) (Fig. 1b).

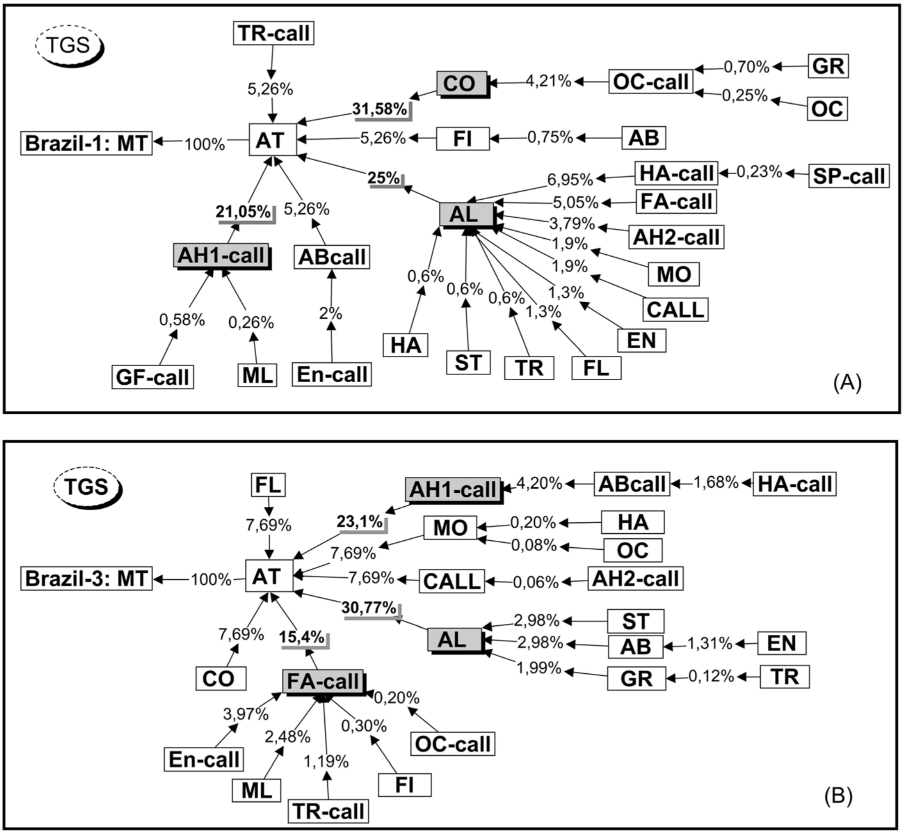

Fig. 1 Probabilistic behavioral courtship tree-generated sequences (TGSs) that resulted in successful copulation (ended in MT) in (**a**) Brazil-1 and (**b**) Brazil-3. The arrows in the TGSs represent the behavioral routines, with the respective percentages of occurrence of each specific routine. The percentages are the probabilities of each path. As the path recedes from the occurrence of copulation (MT), the smaller are the percentages of the behavioral routines: the probability of CO/AT/MT is 31,6%, while that of OC-call/CO/AT/MT is 4,2% (A). The abbreviations of the behavioral units are described in Table 1

Discriminant analyses were performed with the behavioral frequencies of 16 routines generated by Ethoseq (Wilks' Lambda = 0.03422; $F_{8.11} = 38.81$; $p < 0.00001$). The behavioral routines (included in the model) that most contributed to the separation of the two groups were: AB>MO and ML > FA-call, more frequent in Brazil-3, and MO > EN, FA-call>HA-call, MO > EN-call>ABcall, and FA-call>TR-call, more frequent in Brazil-1 (Table 2). The distance between the two groups (Brazil-1 and Brazil-3) for the behavioral routines tested in the discriminant analysis was MD = 101.61.

Brazil-3 morphotype did not perform the behaviors *Spin* (SP-call) or *Graceful* (GR-call), and likewise showed only a single occurrence of *Arrowhead*-2 (AH2-call) behavior. During the behavioral repertoires of courtship, some behaviors showed high frequencies of occurrence while others were rarer. The behavioral unit that occurred with the highest frequency in the courtship behavior sequences of Brazil-1 morphotype was *Fanning* (FA-call = 330), followed by *Mobile* (MO = 306) and *Hamation*-call (HA-call = 300). The Brazil-3 morphotype showed the greatest frequency of *Mobile* (MO =

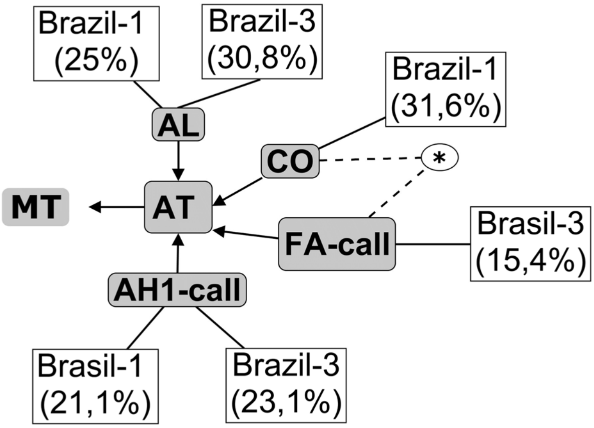

Fig. 2 Probabilistic tree (TGS) created by EthoSeq showing only the three most frequent behavioral units that preceded the behavioral routine AT> MT. * Indicate difference in the in the frequency of execution between the morphotypes (Fisher's Exact Test, $P = 0.046$)

278) behavior, followed by *Fanning* (FA-call = 154) and then *Flying* (FL = 148) (Fig. 3). The frequencies of some of the behavioral units differed between the two morphotypes: AB-call ($\chi^2 = 16.35$, d.f. = 1, $P < 0.0001$), AB ($\chi^2 = 36.41$, d.f. = 1,

Table 2 Total frequencies of the behavioral routines in the TGSs (Figs. 1 and 2) considered in the canonic and discriminant analyses

$N = 20$	Total frequency: TGS		Variables that were included	
Behavioral routines	Brazil-1	Brazil-3	Wilks'Lambda	P
FA-call > ML	3	27	0.049	0.053
AB > MO	14	63	0.237	<0.001
MO > EN	85	59	0.096	<0.001
FA-call > HA-call	127	9	0.145	<0.001
MO > EN-call > ABcall	23	15	0.054	0.030
FA-call > TR-call	20	15	0.070	0.006
ML > FA-call	4	24	0.065	0.009
EN > MO	54	27	0.042	0.139
Behavioral routines	Total Frequency:TGS		Variables that were not included	
	Brazil-1	Brazil-3	Wilks'Lambda	P
HA-call > FA-call	105	6	0.034	0.999
HA-call > ABcall	100	56	0.032	0.477
MO > VO	53	62	0.034	0.841
EN > AB	30	54	0.034	0.685
FA-call > EN-call	58	44	0.033	0.608
ABcall > EN-call	51	24	0.034	0.768
EN-call > FA-call	45	40	0.032	0.466
ABcall > HA-call	76	46	0.034	0.823

Of the 16 behavioral routines analysed in the model, eigth participated in the discriminant analysis (variables that were included). The abbreviations of the behavioral units are described in detail in Table 1

$P < 0.0001$), AH1.call ($\chi^2 = 35.9$, d.f. = 1, $P < 0.0001$), AH2.call ($\chi^2 = 39.4$, d.f. = 1, $P < 0.0001$), AL ($\chi^2 = 5.18$, d.f. = 1, $P = 0.0227$), CALL ($\chi^2 = 18.86$, d.f. = 1, $P < 0.0001$),CO ($\chi^2 = 14.69$, d.f. = 1, $P = 0.0001$), EN-call ($\chi^2 = 26.48$, d.f. = 1, $P < 0.0001$), FA-call ($\chi^2 = 74.05$, d.f. = 1, $P < 0.0001$), HA ($\chi^2 = 5.3018$, d.f. = 1, $P = 0.02$), HA-call ($\chi^2 = 143.62$, d.f. = 1, $P < 0.0001$), ST ($\chi^2 = 28.21$, d.f. = 1, $P < 0.0001$), ML ($\chi^2 = 31.81$, d.f. = 1, $P < 0.0001$), TR-call ($\chi^2 = 12.984$, d.f. = 1, $P = 0.0003$) (Fig. 3).

There was no significant difference between the durations of the courtships executed by the distinct morphotypes ($T = 1.45$; $p = 0.162$), nor were there differences in the length (number of units) of their behavioral sequences ($T = 0.85$; $p = 0.40$).

Discussion

In the probabilistic tree (TGS) created by EthoSeq, the behavioral routines that most contributed to the divergence between the morphotypes were CO > AT, more frequent in Brazil-1 and FA-call> AT in Brazil-3. The males exhibited diverse variations of behavioral routines during their courtship repertoire until acceptance by the female. Some behaviors, however, occurred with greater frequencies near the end of courtship, when the female accepted the male, while others occurred more uniformly throughout the courtship period. With the exception of the behavioral units *Alignment* (AL), *Contact* (CO) and *Arrowhead* (AH1-call), the two populations differed in terms of the other behaviors leading up to the behavioral routine *Attempt* at copulation with success (AT>MT). The behavioral unit that most contributed to the occurrence of

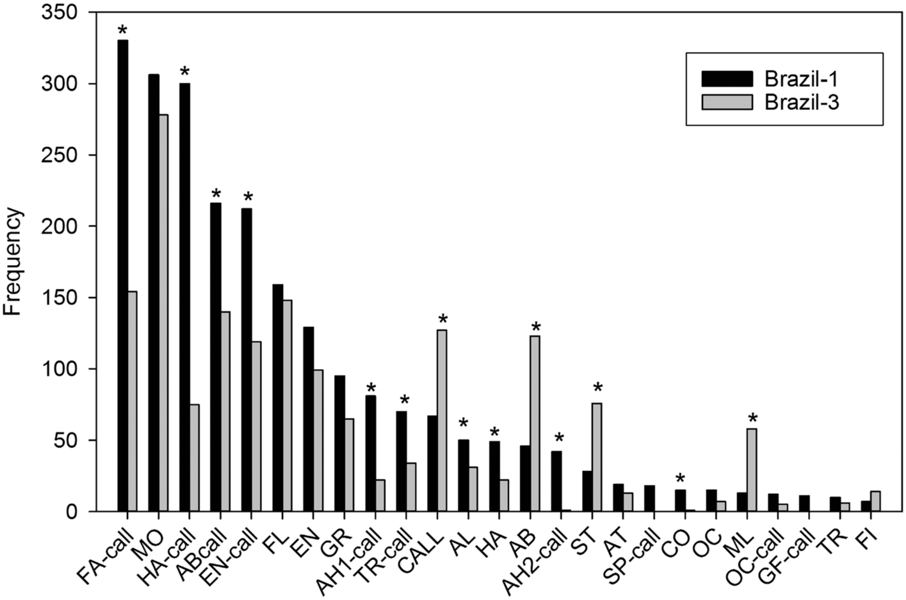

Fig. 3 Total frequencies of the behavioral units in all of the courtship sequences of Brazil-1 and Brazil-3 Bars followed by the symbol "*" indicate statistical significance ($P < 0.05$, Generalized Linear Model, Tukey post-hoc). The abbreviations of the behavioral units are described in Table 1

copulation in the Brazil-1 population was *Contact*; *Alignment* most contributed in Brazil-3. The behavioral units *Transversal* with pheromone emission (TR-call), *Fight* (FI), and *Abdominal movements* with pheromone emission (AB-call) preceded *Attempt* only in the Brazil-1 population; the behavioral units *Fanning* (FA-call), *Flying* (FL), *Mobile* (MO), and *Calling* (CALL), on the other hand, preceded copulation attempts only in the Brazil-3 population. The percentages of the contributions of behavioral units to the occurrence of copulation were significantly different between the two populations.

Mating incompatibility has been shown among morphotypes of *A. fraterculus* (Cáceres et al. 2009; Vera et al. 2006; Rull et al. 2013; Devescovi et al. 2014), inclusive of the two morphotypes studied here (Roriz et al. 2017). There appear to be certain behavioral units that can favor male copulation success (Calcagno and Vilardi 2001; Briceño and Eberhad 2002; Gomez-Cendra et al. 2011) and there are some behavioral routines in the courtship sequence that are more closely related to that success. Gomez-Cendra et al. (2011) analyzed a single population of *A. fraterculus* from Argentina and found that the behavioral units *Fanning*, *Arrowhead* (1 and 2), *Calling* (1 and 2), and *Attempt* were executed for significantly longer periods in successful courtship than in courtship without success. These results indicated that copulation success can be correlated with differences in the durations of different activities. In the population Brazil-1, 6 behavioral units were consistently observed in routines that resulted in successful mating (AT>MT); the Brazil-3 population showed 7 such behavioral units. Three of these behavioral units were similar in successful copulation attempts in both populations: *Alignment* (AL), *Contact* (CO), and *Arrowhead*-1 (AH1-call). *Alignment* and *Contact* represent behavioral units that demonstrated interactions between the partners (Briceño and Eberhad 2002; Briceño et al. 2007). In *Ceratitis capitata,* mating success is favored when the male aligns frontally with the female and the distance between the pair diminishes (Briceño et al. 2007). Male characteristics are evaluated by the females during courtship, such as the angle and distance between the aristae, the size of the male in *C. capitata* (Briceño and Eberhad 2002; Briceño et al. 2007), or the size of the male's head in *Drosophila heteroneura* (Boake et al. 1997). The positions of the two flies, looking directly at one another, would allow a more accurate evaluation of the suitability of the male (by the female). According to Calcagno and Vilardi (2001), the *Arrowhead* unit always occurs when the female is near the male. In *A. suspensa* the behavioral unit *Arrowhead* was classified as a posture of defense against intrusion and postulated to reflect the territorial fitness of the male (Dodson 1982). These behavioral units therefore appear to represent moments when the female directly evaluates the male.

In *D. melanogaster,* males recognize potential mates of the same species during courtship execution (Deepa 2013) with their sensory system (i.e., males have a Gr32 chemical receptor on their front legs). Something similar may be happening with *A. fraterculus* during the execution of the Contact behavioral unit. Both females and males of diverse species of *Drosophila* produce cuticular hydrocarbons that appear to act as sexual signals that can influence male courtship behavior (Cobb and Jallon 1990; Lacaille et al. 2007; Takahash et al. 2011; Bontonou and Wicker-Thomas 2014). Analyses of the hydrocarbons of morphotypes of the *A. fraterculus* complex observed differences between sexually mature males and females (Vaníčková et al. 2012, 2015a, b, c). The cuticular hydrocarbons of Brazil-1 and Brazil-3 are apparently similar to one another (Vaníčková et al. 2015b) and do not appear to be useful in distinguishing potential reproductive mates, although the two populations tend not to mate with one another (Roriz et al. 2017). We observed successful mating without the execution of the behavioral unit Contact, indicating that other behavioral units or interactions among males and females may be involved in recognizing and choosing mates.

To be able to execute *Alignment* with a female of the Brazil-3 population, Brazil-3 males executed three behavioral units beforehand while remaining stationary (ST, AB or LI). Similarly, the female of *D. silvestres* stimulates the male to remain stationary during the final sequence of courtship (Boake and Hoikkala 1995). Females of the Brazil-1 population aligned themselves with males that executed various behaviors including body movements and movements of their wings, as well as stationary behaviors such as *Stationary* (ST). These differences between the populations may be related to behavioral responses stimulated by their respective females. It is known, for instance, that the decision of *Drosophila* males to advance during the courtship process is influenced by signals they receive from the female (Boake and Hoikkala 1995; Hoikkala and Welbergen 1995). These courtship stimuli are sometimes related to chemical compounds produced by the females (Antony and Jallon 1982). Additionally, the behavioral responses of the female to male behavior can also act as stimuli for continuing or finalizing courtship and therefore result in sexual isolation between closely related species (Yamada et al. 2008).

That the behavioral units *Spin* and *Graceful* were not detected in the Brazil-3 population may reflect intrinsic characteristics of the courtship behavior of that population, although a larger sampling of that population will be required in light of the low frequency of occurrence of these units. The populations likewise differed in terms of the frequencies of their behavioral units of the courtship. Similarly, Dias et al. (2016) observed significant differences in the frequencies of courtship behavioral units among *A. fraterculus* populations belonging to the same Brazilian morphotype.

Some behaviors are often repeated during the courtship ritual and others occur only at specific moments, generally at the conclusion of the courtship ritual (Spieth 1974). The behavioral units *Fanning*, *Hamation,* and *Enation* with pheromone emission (FA-call, HA-call, EN-call) within the signaling category using wings, the *Mobile* and *Flying* units within the category of body movements, and the *Abdominal movements* units with pheromone emission (AB-call) all had high frequencies in the courtship sequences of both populations studied. Other units, however, such as *Fight*, *Transversal*, *Graceful,* and *Oscillation* with pheromone emission, showed low frequencies during courtship. Behavioral units which occurred simultaneously with pheromone emission were generally frequent.

The two populations also differed from one another in terms of the frequencies of some behavioral routines. Within the routines that participate in the model that discriminated the two groups, the routine FA-call>HA-call was more frequently executed by Brazil-1 and AB>MO was more frequently executed by Brazil-3. These routines may represent specific characteristics of the courtship behavior of each population.

Males of the Brazil-1 population devoted more time to their courtship routine and showed longer behavioral sequences. Long courtship periods with long behavioral sequences or short courtship times with shorter behavioral sequences may reflect female preferences. Briceño and Eberhard (1998) reported that the short courtship periods of certain lineages of *Ceratitis capitata* that were observed under laboratory conditions were presumably advantageous to males living under extremely crowded conditions, as they reduced the probability of their courtship being interrupted by other individuals.

Divergence of behavioral patterns can arise as a result of adaptations to specific environmental conditions (Paterson 1985). The Brazil-1 morphotype is more commonly encountered in plateau landscapes, while the Brazil-3 morphotype is more common in coastal regions (Selivon et al. 2004; Selivon et al. 2005; Vaníčková et al. 2015a). Manni et al. (2015) reported that genetic differences between populations of *A. fraterculus* in Brazil were more influenced by altitude than by the geographic distances between those groups.

Ryan and Rand (1993) suggested that nonrandom mating patterns could be the result of the link between the courtship signals and receptors. The low observed compatibility of mating between Brazil-1 and Brazil-3 (Roriz et al. 2017) could reflect behavioral divergences during courtship. Females of certain populations in a cryptic species complex could be more discriminating and exacting, as could the males, which would influence interactions between those distinct populations (Crowder et al. 2010). Behavioral factors, linked to specific preferences of the females, could collaborate in the recognition of conspecifics. As such, the observed differences in the courtship behaviors of Brazil-1 and Brazil-3 apparently relate to the different mate recognition processes across the two populations.

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