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Measuring same-sex sexual behaviour: the influence of the male social environment

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Same-sex sexual behaviour (SSB) is attracting increasing research attention, but quantifying and interpreting SSB present unique challenges. Chief among these are (1) partitioning the expression of SSB into separate influences from interacting partners, (2) distinguishing between same-sex behaviour, same-sex preference and same-sex orientation and testing for correlations between them, and (3) evaluating how the social environment modulates the expression of SSB. We used sexually mature male *Drosophila melanogaster* in staged encounters to address these aims. The expression of SSB was not consistent across choice and no-choice experimental trials, indicating that a tendency to display SSB when females are absent does not correlate with greater same-sex preference when a choice is available. The expression of SSB was sensitive to the social experience of males. Experience with other males and experience with females both decreased the incidence of male SSB, suggesting that both avoidance learning and mating experience mitigate its expression. SSB in *D. melanogaster* appears to be a highly labile trait susceptible to varied influences from the social environment. We suggest that SSB expressed in different social contexts probably represents different physiological origins, which is a potentially important consideration in studies examining its genetic architecture and evolutionary maintenance.

Same-sex sexual behaviour (SSB) in nonhuman animals is attracting increasing research attention (Bailey & Zuk 2009). Researchers in the fields of animal behaviour and genetics have observed sexual behaviours directed at partners of the same sex for many years, but detailed dissections of its neural and hormonal basis, its behavioural functions and its evolutionary maintenance have been concentrated in the last decade (e.g. Adkins-Regan 2002; Gavrilets & Rice 2006; MacFarlane et al. 2007; Levan et al. 2009; Abbassi & Burley 2012). Despite increasing interest in studying SSB as a distinct behavioural trait, there is limited information about the merits or drawbacks of different ways of quantifying it. In this study our goal was to examine different ways of quantifying SSB in a model organism, *Drosophila melanogaster*, with an emphasis on testing how variation in the social environment influences the expression of SSB in males.

Many of the challenges of measuring SSB parallel issues that arise when quantifying female choice for sexually selected male traits. An expansive literature developed around measuring female choice after seminal sexual selection models were published in the early 1980s (Lande 1981; Kirkpatrick 1982). Some key issues focused on technical details, such as whether preferences are best measured in a choice versus no-choice setting (Wagner 1998) and how to quantify the precise nature of the relationship between female attraction to male trait values and variation in those trait values (Ritchie 1996). In addition, female choice can be partitioned into constituent components, such as preference, discrimination and responsiveness (Bailey 2008). Studies have also emphasized the importance of context dependence and flexibility in female choice (Widemo & Sæther 2005; Cotton et al. 2006). All of these features capture distinct influences that combine to shape female choice and, by extension, the operation of sexual selection.

Issues that arise when measuring SSB are often similar, but reflect different goals of SSB research. A notable challenge is distinguishing between same-sex behaviour, same-sex preferences and same-sex orientation. Formal definitions of these are laid out in Bailey & Zuk (2009), but briefly, these traits differ in the degree to which individuals exhibit behavioural consistency. Same-sex sexual behaviour describes the action of engaging in courtship or mating behaviour, normally directed to opposite-sex conspecifics, with a conspecific of the same sex, and the definition is limited to the specific action of the acting individual. Same-sex preference, however, can only be inferred when other, opposite-sex, options are available. An animal exerts a same-sex preference if it chooses to engage in sexual behaviour with an individual of the same sex even when a conspecific of the opposite sex is available. Preference

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thus implies active choice. Orientation, however, implies a consistent preference over a long period of an animal's life. It is crucial to distinguish between these different types of traits when studying SSB, but methods employed to measure SSB can sometimes limit our ability to make these distinctions. For instance, an animal placed with an individual of the same sex may exhibit SSB, but in the absence of an alternative choice no preference can be inferred. Conflation of SSB with preference and orientation has afflicted studies in the past and can lead to controversy (Levine 2008), so it is important to develop rigorous frameworks for measuring these different traits.

SSB is an interacting phenotype (Moore et al. 1997), which generates additional considerations when quantifying it. In other words, SSB is an emergent property of two interacting individuals, so it would be useful to be able to partition different individuals' contributions to the expression of SSB. For example, an animal might display SSB because it has a genetic predisposition to do so. However, the ultimate expression of the behaviour might depend on characteristics of the interacting partner, for example that individual's aggressive behaviour or dominance status, activity level, degree of sexual differentiation, etc. An associated challenge is that sexual behaviour by definition depends on social context. Identifying and quantifying broader social influences on the expression of SSB would also help clarify its behavioural functions and possible adaptive value.

We used different strains of *Drosophila melanogaster* to address three primary goals: (1) to test the effects of interacting, same-sex individuals on the expression of SSB, (2) to test whether SSB is consistently exhibited in no-choice and choice contexts and (3) to evaluate the impact of variation in the male social environment during maturation on SSB. *Drosophila melanogaster* has a long history of involvement in research on the behavioural genetics of courtship and reproductive behaviour (Greenspan & Ferveur 2000), but the majority of studies that have documented SSB appear to have done so in knock-out laboratory mutants (e.g. Svetec & Ferveur 2005; Grosjean et al. 2008). Nevertheless, SSB occurs in wild-type flies. Immature males appear to attract SSB because they lack sex-specific cuticular hydrocarbons that identify them as male (McRobert & Tompkins 1983; Curcillo & Tompkins 1987). There is no consensus on whether such immature males derive a benefit from being the recipients of such same-sex interactions, with some studies suggesting a later advantage during opposite-sex matings (McRobert & Tompkins 1988), and other studies finding no such benefit and suggesting they develop to be more aggressive (Dukas 2010). Mature *D. melanogaster* males also exhibit SSB (Dukas 2010). Social experience is known to alter many aspects of opposite-sex mating behaviour in *D. melanogaster* (Dukas 2005; Krupp et al. 2008; Ödeen & Moray 2008), and there is evidence that social isolation increases levels of SSB in several drosophilids (Kim & Ehrman 1998; Svetec & Ferveur 2005). Given these previous findings, we developed assays to address three aims: (1) to partition individual contributions to the expression of SSB in male–male pairs of *D. melanogaster* and quantify variation in different strains, (2) to compare no-choice trials that assess SSB with choice trials that assess same-sex preference, and (3) to evaluate how variation in the male social environment affects the expression of SSB.

METHODS

Fly Strains and Maintenance

Males used in trials were from standard inbred, wild-type Canton-S or Oregon laboratory strains (obtained from S. F. Goodwin, University of Oxford, and C. P. Kyriacou, University of Leicester, respectively). For ease of identification, we usually measured their SSB with a strain carrying the yellow body colour mutant (the y strain we used is on wild-type background originally isolated from a natural population: Hmr^2; obtained from the Bloomington Stock Center, Bloomington, IN, U.S.A.; FlyBase ID: FBal0144828; Hutter & Ashburner 1987). The wild type provided the experimental 'focal' flies, while the yellow-body males were used as standardized and easily identified 'model' males. We also used an ebony-bodied mutant (eAFA), as an alternative model strain but did not proceed with this strain owing to low activity levels in preliminary tests.

With the exception of one experiment designed to compare levels of SSB in trials using only wild-type flies, we always used yellow-body males as models, so pleiotropic effects of the mutation on mating behaviour (Bastock 1956) were unlikely to confound comparisons between different focal fly strains or treatments. Thus, for these experiments, all focal males were tested against the same strain of yellow-body model, so that differences in focal fly SSB could be attributed to differences in the focal treatment or strain. All flies were kept on a standard cornmeal/agar medium, seeded with yeast. Maintenance fly stocks were kept in the laboratory at 18 °C on a continuous light cycle in medium (25 × 95 mm) vials at a density of approximately 50 adults. Experimental fly stocks were kept at 23 °C on a 12:12 h light:dark cycle in large (29 × 95 mm) vials. Virgins were collected from uncrowded vials under light CO_2 anaesthesia 1–12 h posteclosion, whereupon they were kept in small (16 × 95 mm) vials. Males used in experimental trials were maintained either individually or in groups of four depending on their treatment group.

Behavioural Observations

All behavioural observations were performed between 21.4 and 25.0 °C during the flies' daylight hours. Focal and model males were introduced into a 16 × 95 mm observation tube using an aspirator, and five pairs were observed at a time. Each pair was observed for a total of three 1 min observations, spaced evenly over 15 min. Age has strong effects on the expression of SSB in *D. melanogaster* (Curcillo & Tompkins 1987; McRobert & Tompkins 1988). Males and females of both wild-type and yellow-body strains express sex-specific cuticular hydrocarbons (CHCs; Averhoff & Richardson 1974; Jackson et al. 1981), but males lack these CHCs immediately after eclosion which substantially increases their attractiveness to other males (Curcillo & Tompkins 1987). We were only interested in SSB that occurred when males were identifiable as males to potential interacting partners, so we only used focal males between 6 and 8 days old and yellow-body model males between 3 and 5 days old.

During each observation, the presence or absence of courtship behaviour (Table 1) was noted for the focal male and the model male separately. For some trials Canton-S males were paired with other Canton-S males, and the presence or absence of courtship behaviour was noted without regard to which male was exhibiting it, since individual males could not be visually tracked.

The courtship repertoire of *D. melanogaster* is well established (Greenspan & Ferveur 2000), and in the case of opposite-sex pairs involves a series of behaviours performed by a male and directed at a female. A male begins by orienting head-first towards a female, and then follows her if she moves around. He may tap her body with one of his front feet and lick her genitals. Males further court females by extending one wing and vibrating it to produce a low-frequency courtship song. Finally, a male may curl his abdomen under his body towards a female's posterior and then physically mount her and transfer sperm. During observations, we scored these behaviours as: Orienting, Following, Tapping, Licking, Singing and Abdomen curling (Table 1). Annotated video recordings of each behaviour are included in the Supplementary Material.