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Flexible memory controls sperm competition responses to male Drosophila melanogaster

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Abstract

Males of many species use social cues to predict sperm competition and tailor their reproductive strategies, such as ejaculate or behavioural investment, accordingly. Whilst these plastic strategies are widespread, the underlying mechanisms remain largely unknown. Plastic behaviour requires individuals to learn and memorise cues associated with environmental change before using this experience to modify behaviour. Drosophila melanogaster respond to an increase in sperm competition threat by extending mating duration after exposure to a rival male. This behaviour shows lag times between environmental change and behavioural response suggestive of acquisition and loss of memory. Considering olfaction is important for a male's ability to assess the sperm competition environment, we hypothesised that an olfactory learning and memory pathway may play a key role in controlling this plastic behaviour. We assessed the role of genes and brain structures known to be involved in learning and memory. We show that sperm competition responses depend on anaesthesia sensitive memory, specifically the genes rut and amn. We also show that the γ lobes of the mushroom bodies are integral to the control of plastic mating behaviour. These results reveal the genetic and neural properties required for reacting to changes in the sperm competition environment.

Keywords

Sperm competition, Learning, Memory, Behavioural plasticity

Introduction

Plastic responses to sperm competition (SC) are widespread and can include behavioural [1] or ejaculate changes [2] to match investment to the prevailing competitive threat. Given these responses can directly impact their fitness [3, 4], males need to accurately assess changes in their social environment to predict both current and future SC. The SC

environment can fluctuate rapidly [5] and this may require males to integrate multiple components of information from separate cues [6]. This might be a cognitively challenging process, and indeed there has been a suggestion that responses to SC are implicated in the evolution of quantity estimation [7]. However, the neural and cellular mechanisms controlling how males respond to SC are largely unknown. It has been suggested that novel behaviours either utilize switches between existing neural circuits or the development of new neural circuits [8], therefore understanding which pathway is used could have implications for the evolvability of behavioural plasticity generally.

Here, we use a behavioural response to increased SC in male Drosophila melanogaster to investigate underlying genetic and neural mechanisms. If exposed to rivals, D. melanogaster males increase their mating duration [9] and transfer more sperm and seminal fluid to the female [10], leading to fitness benefits through increased paternity share and reduction in female remating [9]. For males to extend mating duration requires at least two cues that include olfactory, auditory or tactile elements [11]. Timing seems crucial in this system, as males need 24 hour exposure to a rival in order to respond [3] and once a rival is removed, males continue to respond for 12 hours [12]. This suggests that males use time as a way of determining whether the current environment accurately reflects the general level of SC threat, requiring males to “remember” their recent competitive environment. The time period males continue to respond to a rival after its removal suggests extended mating duration relies on one of two distinct long term memory pathways, either a form of anaesthesia sensitive memory (ASM) or anaesthesia resistant memory (ARM) (Supplementary figure S1). These two forms of memory are suggested to be distinct at the molecular level though behaviourally they are indistinguishable [13]. ASM requires protein synthesis and develops from consolidation of short term memory and medium term memory, whereas ARM does not require protein synthesis to form [14].

The mechanism controlling plastic mating duration has drawn some attention [15], however, inconsistencies have arisen in the identification of cues involved in controlling

plastic mating duration. In contrast to the combined cues described by Bretman et al [11], Kim et al [15] reported that only one cue, vision, was needed for males to extend mating duration. Indeed it has been claimed that the only stimulus required is moving red eyes [15], though this is contested as further work was unable to replicate this result, and showed that males will not necessarily respond to heterospecific rivals with red eyes [16]. The cues important for behaviour to accurately react to environmental change will directly affect the mechanistic processes controlling behaviour [17]. In light of the uncertainty between cues needed for extended mating behaviour, and to test whether the behaviour is indeed a function of long term memory as we have predicted [12], we aimed to assess neural mechanisms controlling plastic mating behaviour. We first established whether extended mating duration was due to ASM or ARM through the application of anaesthesia. We then tested the role of well-studied learning and memory associated genes and established whether the mushroom bodies (MB, a brain region required for olfactory learning [18]) are needed to achieve a sperm competition response.

Materials and Methods

Fly rearing and strains

Unless otherwise stated, experiments were conducted in a 25℃ humidified room with a 12 hours light: 12 hours dark cycle, using plastic vials (75x25mm) with 7 ml standard sugar-yeast-agar medium [19]. All wild type flies were the Dahomey strain as in our previous studies [3, 9, 11, 20]. Wild type larvae were raised at a standard density of 100 per vial. At eclosion, flies were collected and sexed using ice anaesthesia, and stored 10 per vial. Females were supplemented with live yeast granules.

All transgenic stocks were raised in vials, using 5 females and 5 males to create progeny. dnc^{1} (FBst0006020), amn^{EP346}, rut^{2080};UAS-rutZ (FBst0009405), UAS-Nf1 (FBst0010201), UAS-shibire (BDst0044222), elav-GAL4 (FBst0008765) and 1471-GAL4

(FBst0009465) were obtained from Bloomington Stock centre. OK107-GAL4, NP3061-GAL4, NP1131-GAL4 were obtained from Kyoto stock centre. Radish-RNAi (FBst0463293) were obtained from Vienna Stock centre.

Mating duration

For all tests, comparisons were made between focal males of the same genotype held singly or exposed to a wild type rival, identified with a wing clip as in previous experiments [12], for 3 days. A wild-type Dahomey control was run alongside every transgenic experiment. Focal males were aspirated into a vial containing a single virgin Dahomey female and mating duration was recorded for all matings within 3 hours.

Cold shock

Cold anaesthesia abolishes ASM but leaves ARM intact [21]. We therefore used this approach to assess the importance of ASM versus ARM when reacting to rival males. Wild type focal males were transferred to a vial in ice for 2 minutes after being exposed to a rival or held singly for 3 days. Flies were then allowed 30 minutes to acclimatise to 25℃ in isolation before being placed with a female and the latency to mating and duration of mating scored.

Assessment of genetic control

In D. melanogaster, the ability to associate two independent cues through olfaction is partly determined by the genes dunce (dnc), rutabaga (rut), and amnesiac (amn) that act to control cAMP formation and form anaesthesia-sensitive memory (ASM) [18, 22]. Additionally, the expression of dnc is altered by exposure to a rival male [23]. We used a dnc knockout, and rut and amn knockdown driven in the whole brain by elav-Gal4, to investigate the importance

of ASM in extended mating duration (Supplementary figure S1). All of these gene are involved in short term memory, so mating duration was analysed immediately after removal of rival males.

To assess whether there was any role for ARM, we also knocked down Radish (rsh), the only gene known to be directly responsible for ARM [21], throughout the nervous system using elav-GAL4 [24]. As rsh is specifically involved in controlling a type of long term memory (Supplementary figure S1), and we have previously shown that males can continue to react to rivals for 12 hours [12], evaluation of the effect of knockdowns on mating duration were performed at 0, 6 and 12h after removal of rivals.

Assessment of neural control

The mushroom bodies (MBs) are integral to the formation of associative memory [18]. They are involved in sensing olfactory information [25] and are the location of stimuli convergence controlled by rut [18]. As extended mating duration involves (though does not require) olfactory cues [11], we hypothesised that the MBs would be important in responding to rivals. To test this, we rescued wildtype rut function in the previously used rut knockouts using Gal4 drivers specific to different lobes of the MBs: OK107-Gal4 (all the lobes), NP3061-Gal4 (α/β lobes) and NP1131-Gal4 (γ lobes and a subset of α'/β' neurons) [26] and 1471-Gal4 (mainly in the γ lobes [26] and to a minor extent in the α/β lobes [27]) (Supplementary figure S2, also see Supplementary figure S3 for GFP verification). We also blocked neuronal transmission in these same lobes using UAS-shibire^{ts1} (UAS-shi^{ts1}), a dominant temperature sensitive transgene that at restrictive temperatures (31℃) blocks vesicle recycling [28] (Supplementary figure S2). The role of α/β lobes in controlling extended mating duration was also established through the use of NF1 knockdowns driven by elav-Gal4. NF1 encodes a ras GTPase activating protein that is required for memory via rut activation in the α/β lobes only [29]. Any reduction in mating duration in NF1 knockdowns would therefore implicate α/β lobes in the control of the behaviour.

For all experiments each focal male was exposed to rivals for 3 days before mating duration was assayed immediately. In experiments that utilised UAS-shibire, males were heat shocked three times at 32℃ for 12 hours every 24 hours over the 72 hours males were kept with rivals. This prevented transmission of information in lobes associated with Gal-4 drivers but reduced negative effects of constant heat shock. We found no negative effects of these heat shocks on the ability of males to mate, as shown by there being no difference in successful matings by the worst performing line (NP3061;shi^{ts1} 51/80 matings in 2 hours) compared to the Dahomey wild type (61/80, $\chi^2_1 = 2.411$, $p = 0.121$). Prior to undergoing the mating assay males were again heat shocked for 30 minutes. Matings were performed at 32℃ to abolish neural transmission during the output phase of the behaviour.

Statistical analysis

Statistical analysis was performed using SPSSv14 and R 3.3.1 [30]. Extended mating duration assays were analysed by pairwise comparisons of flies of the same genotype either kept single or with rivals. As the key comparisons are always within genotype, this gives an internal control for genetic background and off target effects [11]. The cold shock data were normally distributed and analysed using a linear model with rival exposure and anaesthesia treatment as fixed factors. Otherwise, pairwise comparisons were made using Mann-Whitney U or t-tests (depending on the normality of the data).

Results

Anaesthesia sensitive memory controls extended mating behaviour

When anaesthetised with cold shock males fail to increase mating duration after exposure to a rival male ($t_{67} = 0.135$, $p = 0.135$; Figure 1a). Males that had not undergone anaesthesia still significantly increased mating duration ($t_{74} = 1.033$, $p = 0.002$) and there were no latent effects of anaesthesia as males kept singly compared between anaesthesia treatments

showed no effect of cold shock (t_{77} = 0.330, p = 0.146). Knock down of ARM controlling rsh also had no effect on the mating duration response at any of the time periods investigated (0 hours: Z = -2.259, N = 49, p = 0.024; at 6 hours: Z = -3.998, N = 68 p < 0.001; at 12 hours: Z = -3.526, N = 63 p < 0.001; Figure 1b). To confirm ASM was responsible for controlling SC responses we perturbed cAMP synthesis using widely used learning and memory mutants. Both amn (t_{53} = -0.883, p = 0.381; Figure 1c) and rut (Z = -0.960, N = 44, p = 0.337; Figure 1c) knock-downs abolished extended mating duration. However, dnc knockout did not significantly affect extended mating duration (t_{41} = -2.565, p = 0.014; Figure 1c). Taken together these experiments suggest that males rely on ASM controlled by the cAMP learning and memory pathway rather than ARM to control plastic mating duration.

The γ lobes of the MBs are integral to extended mating duration

Rescuing rut expression in all lobes of the MBs rescued a male's ability to increase mating duration after exposure to a rival (OK107;rut+ t_{54} = -2.580, p = 0.013; Figure 2a). Stopping neural transmission in the same lobes with UAS-shi^{ts1} abolished extended mating behaviour (OK107;shi^{ts1} Z = -1.619, N = 68, p = 0.105; Figure 2b). When rut was rescued in the α/β lobes, extended mating duration was not rescued (NP3061;rut+ Z = -0.309, N = 55, p = 0.757; Figure 2a) and expression of UAS-shi^{ts1} had no effect on extended mating duration when driven in the same lobes (NP3061;shi^{ts1} Z = -2.901, N = 51, p = 0.004; Figure 2b). Nf1 knockdown males also continued to significantly increase mating duration after exposure to rivals (Z = -2.449, N = 56 p = 0.014; Figure 2c). Extended mating duration behaviour was restored when rut was rescued in γ lobes and a subset of α'/β' neurons (NP1131;rut+ t_{59} = -3.737, p < 0.001; Figure 2a) and in the γ lobes (1471;rut+ Z = -4.315, N = 72, p < 0.001; Figure 2a). This pattern was not directly mirrored when preventing transmission in the same lobes using shi^{ts1}. Removing transmission only in the γ lobes had no effect (1471;shi^{ts1} t_{60} = -3.516, p = 0.001; Figure 2b). However, abolishing neural transmission in a combination of α'/β' and γ lobes abolished extended mating duration (NP1131;shi^{ts1} t_{56} = -0.672, p = 0.504;

Figure 2b). This suggests that responses to the SC environment do not rely on the α/β MB lobes, but instead uses a combination of α'/β' and γ lobes to control extended mating behaviour.

Discussion

We show that the ability to alter mating duration in response to rival males requires a form of long term, anaesthesia sensitive memory in D. melanogaster males. Moreover, these mechanisms are localised to the MBs, in contradiction to a previous report [15] and reiterating the key role of olfactory cues in this context [6]. Interestingly, although dnc and Nf1 are both differentially expressed when males are exposed to rivals [23], we found these were not necessary to produce the response. This cautions about making functional inferences from changes in gene expression. Our work also implies that this sophisticated response utilizes circuitry required for simple associative learning, and hence is likely a type of activational plasticity [8].

Although we show that the MBs are important in this SC response, the specific brain area required is not simple to define, as we have shown that blocking both α'/β' and γ lobes is necessary to abolish extended mating duration. Considering at least two cues are needed to extend mating duration [11], and that visual, tactile, gustatory and olfactory inputs can be processed by the MBs in bees [31], multiple lobes may work together to regulate SC responses. Indeed, γ lobes in the Drosophila MBs are required for both olfactory [32] and gustatory [33] learning, raising the possibility that a combination of sensory inputs could interact in the γ lobes to achieve extended mating duration. However, the γ lobes are commonly viewed as controlling short term memory [22]. This is at odds with the 12 hours males continue to respond to rivals when separated [12], which is more in line with memory associated with the α/β lobes [34]. Control through γ lobes may allow for both a short-term response to transient increases in the SC environment, and a longer response after greater exposure to rivals. This does occur, as males held with a rival for 24 hours only extend

mating duration up to 1 hour after the rival is removed, whereas males exposed for 36 hours continue to do so for 12 hours [12]. There is also evidence γ lobes can form a long term memory trace distinct from those formed in the α/β lobes [27, 35]. This dynamic control of behaviour may therefore underlie the ability of males to use the length of time they are exposed to a rival to estimate the risk of SC in a capricious social environment [7, 12]. It is worth noting that the sperm competition response comprises both alteration of mating duration and ejaculate, which may become uncoupled after constant rival exposure [36]. Although the mechanisms outlined above control mating duration, they may not necessarily control ejaculate composition. In future, it will therefore be important to measure ejaculate directly to understand whether the same pathways and brain regions control ejaculate composition as well as mating duration.

Similar examples of ecologically relevant memory are referred to as “Tailor made memory”, defined as the properties and temporal dynamics of acquisition, consolidation and retrieval of memory after learning specific for an ecological context [37, 38]. For example, parasitoid wasps (Cotesia glomerata) differ in the spatial memory pathway (ARM or ASM) used to remember different species of host depending on the size of oviposition reward with which the host is associated [39]. As ARM is less costly than ASM [40] this means the wasp only invests in long term memory when the reward is large. It has been suggested that the 24 hour lag time seen before D. melanogaster males extend mating duration occurs so males can confirm that a competitive threat is sustained [12]. After this initial investment, the maintenance time of this behaviour relies on exposure time [12]. This mirrors memory development in the wasp, in that long lasting behavioural change is only initiated if a threat [12] or reward [39] is substantial. In D. melanogaster, memory developed by the γ lobes is a more malleable form of memory than that which is developed in the α/β lobes after training [41], in that it controls both relatively short and long memory periods [34]. This should increase the ability of a male to react to rapid changes in the SC environment through short term memory and also guard against reversion of behaviour when SC threat within a locality

is still high but the immediate cue of rival presence has been removed. In comparison, memory developed through α/β lobes is 'all or nothing' long term memory that may become mal-adaptive in a fast-changing environment.

Although specifically focussing on SC, our study could give us insights into the evolution of plastic behaviour generally. Plastic behaviour requires individuals to learn and memorise cues associated with environmental change before using this experience to subsequently modify behaviour [17]. This has been theorised to be controlled by switches between neural circuits already present after development (activational plasticity [8]), or require the development of new neural circuits to control a new behaviour (developmental plasticity [8, 42]). Based on our study, plastic behaviour can be controlled by a generalised mechanism also required in other behaviours. For example, the nervous circuitry we have shown to help control plastic mating duration also plays a role in appetitive and aversive learning [43-45]. Plasticity in mating duration could therefore have co-opted neural circuitry involved in simple associative learning, and changed its context. This suggests this response is an example of "activational" behavioural plasticity [42], as it uses neural circuits already present in adulthood to facilitate behavioural change. Considering the cost of neural development to control this behaviour be spread across multiple behaviours (including general learning and courtship conditioning [44]) this may hint that the neural cost of controlling plastic mating duration is minimal. From an evolutionary perspective, behaviours that "piggyback" an existing neural circuit or "share" development of a behavioural mechanism may evolve much more easily than behaviours that require the development of novel control mechanisms. Future studies could integrate neurogenomic changes with neuronal mechanics to understand how generalised cognitive processes can be tailored to specific contexts.

Competing interests

The authors declare no competing interests.

Author Contributions

JR and AB conceived and designed the study. JR carried out all experimental work with the help of KW (for dnc and amn experiments). JR and AB analysed the data. JR and AB drafted the manuscript. All authors gave final approval for publication.

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Data Availability

For review the datasets are included as supplementary material but will be available in the open access Dryad repository upon acceptance.

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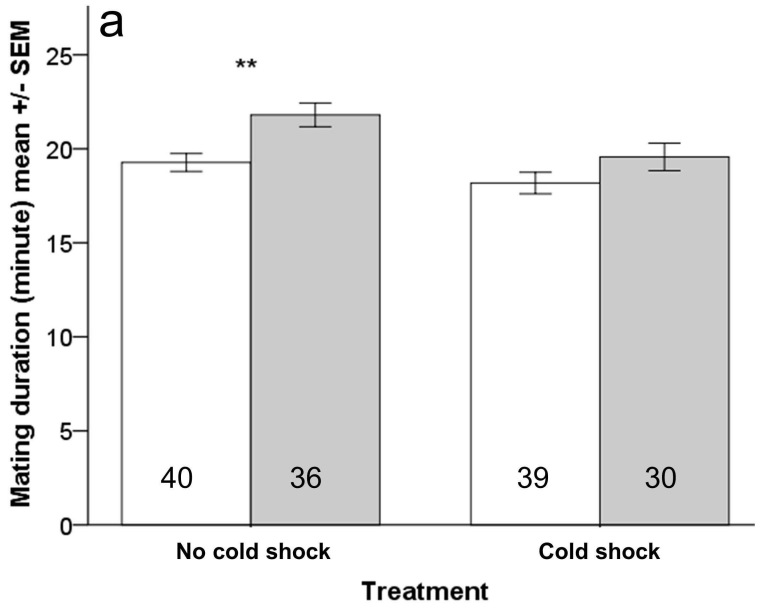
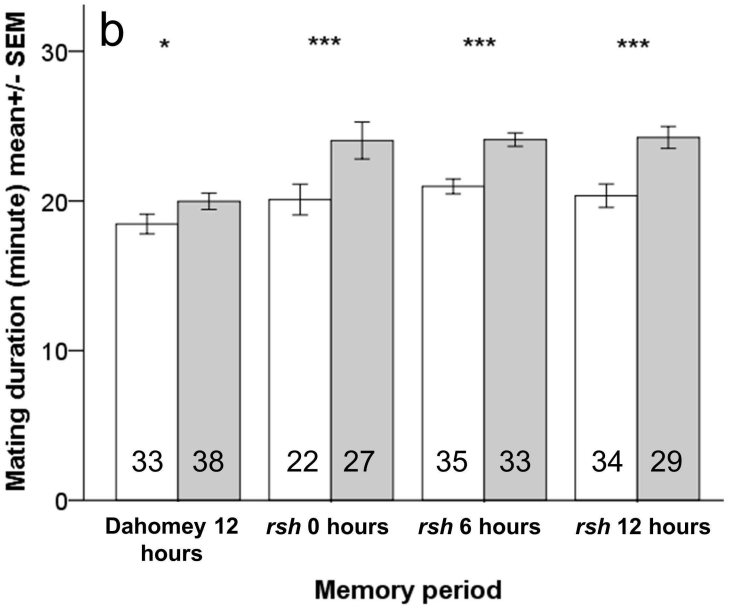
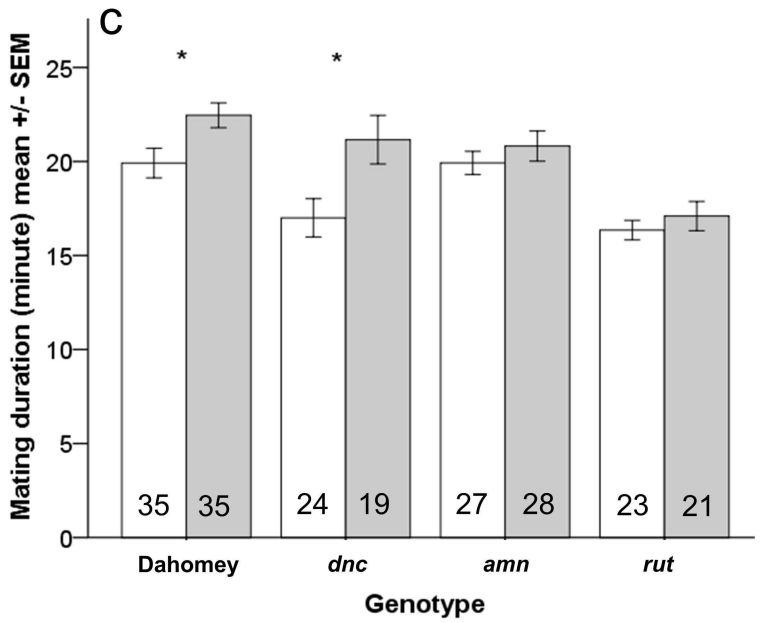
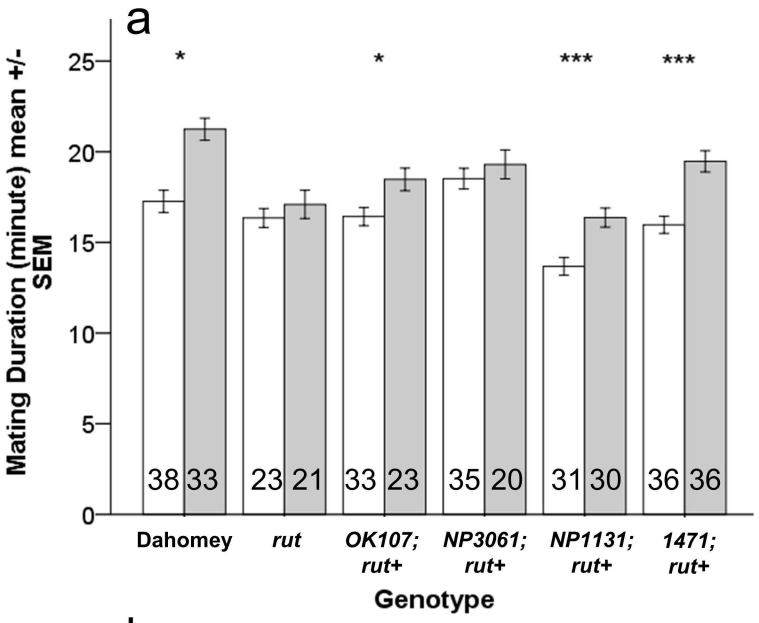
Figure 1: Investigation of anaesthesia sensitive memory (ASM) and anaesthesia resistant memory (ARM) pathways in the behavioural response to rivals, measured as mating duration (mean +/- S.E.M.) for males kept singly (white bars) and males kept with rivals (grey bars). a) To test ASM, males either did or did not undergo cold shock, whereby males were placed on ice for 2 minutes half an hour prior to the mating assay. b) ARM was tested using knockdown of rsh at 0, 6 or 12h after removal of rivals from the rival-exposed treatments. A wild type control (Dahomey) was also measured for the longest memory period. c) ASM was further tested via interruption of the cAMP pathway using dnc knock out or rut or amn knock down. Samples sizes are given within each bar. * indicates a significant difference between paired treatments (* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$).

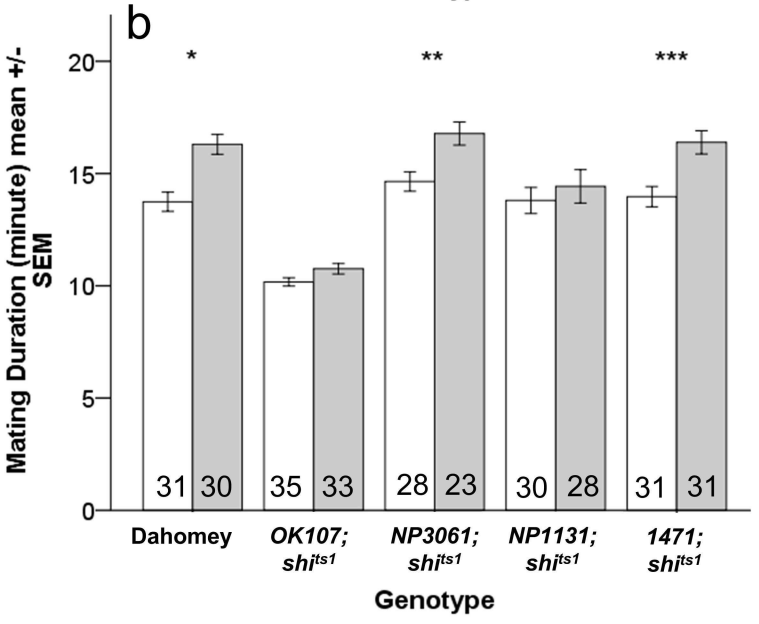
Figure 2: Investigation of the role of the Mushroom bodies (MB) in the behavioural response to rivals, measured as mating duration (mean +/- S.E.M.) for males kept singly (white bars) and males kept with rivals (grey bars). MB lobe-specific Gal4 drivers OK107-Gal4 (all lobes), NP3061-Gal4 (α/β lobes), NP1131-Gal4 (γ lobes and a subset of α'/β' neurons) and 1471-Gal4 (γ lobes) were used to a) rescue rut expression or b) prevent neural transmission using shi^{ts1} expression. c) α/β lobes were further investigated using pan-neuronal knock-down of NF1 driven by the elav-Gal4. Error bars represent standard error. Samples sizes are given within each bar.* indicates a significant difference between paired treatments (* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$).

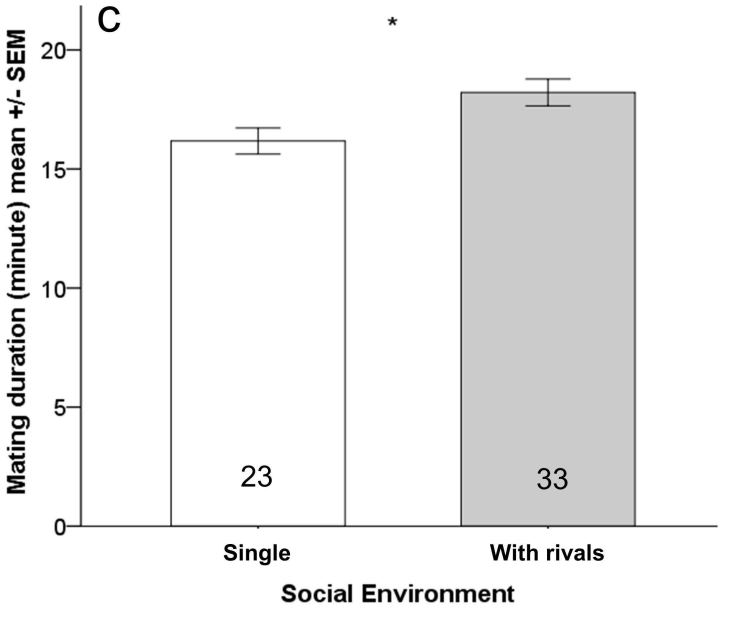

a
Mating duration (minute) mean +/- SEM
25
20
15
10
5
0
**
40
36
39
30
No cold shock
Cold shock
Treatment


b
Mating duration (minute) mean+/- SEM
30
20
10
0
*
***
***
***
33
38
22
27
35
33
34
29
Dahomey 12 hours
rsh 0 hours
rsh 6 hours
rsh 12 hours
Memory period


c
Mating duration (minute) mean +/- SEM
25
20
15
10
5
0
*
*
35
35
24
19
27
28
23
21
Dahomey
dnc
amn
rut
Genotype


a
Mating Duration (minute) mean +/- SEM
25
20
15
10
5
0
*
*
***
***
38 33
23 21
33 23
35 20
31 30
36 36
Dahomey
rut
OK107; rut+
NP3061; rut+
NP1131; rut+
1471; rut+
Genotype


b
Mating Duration (minute) mean +/- SEM
20
15
10
5
0
*
**
***
31 30
35 33
28 23
30 28
31 31
Dahomey
OK107; shits1
NP3061; shits1
NP1131; shits1
1471; shits1
Genotype


c
Mating duration (minute) mean +/- SEM
20
15
10
5
0
*
23
33
Single
With rivals
Social Environment