Conservation of *fruitless* role as master regulator of male courtship behaviour from cockroaches to flies

Elke Clynen[1,3†], Laura Ciudad[2,4†], Xavier Bellés[2], Maria-Dolors Piulachs[2*]

[†] Both authors contributed similarly to the work

Affiliations:

[1] Research Group Functional Genomics and Proteomics, K.U.Leuven, Naamsestraat 59, 3000 Leuven, Belgium

[2] Institute of Evolutionary Biology (CSIC-UPF), Passeig Marítim de la Barceloneta 37, 08003 Barcelona, Spain

[3] BIOMED Research Institute, Hasselt University, Agoralaan building C, 3590 Diepenbeek, Belgium

[4] Present address: Epigenetic and Cancer Biology Group (ICO-IDIBELL), Gran Via s/n km 2.7, 08907 L'Hospitalet de Llobregat (Barcelona), Spain

[*]**Corresponding author:** mdolors.piulachs@ibe.upf-csic.es,

Tel 34 93 230 96 48

Fax 34 93 221 10 11

Abstract In *Drosophila melanogaster*, male courtship behaviour is regulated by the *fruitless* gene. In *D. melanogaster*, *fruitless* encodes a set of putative transcription factors that are sex-specifically spliced. Male-specific variants are necessary and sufficient to elicit male courtship behaviour. *Fruitless* sequences have been reported in other insect species, but there are no data available on their functional role. In the present work, we cloned and sequenced *fruitless* in the German cockroach, *Blattella germanica*, and we studied its expression in male brain and testes. *B. germanica fruitless* encodes a 350 amino acid protein with BTB and Zinc finger domains typical of *fruitless* sequences. Upon RNAi-mediated knockdown of fruitless in *B. germanica* males no longer courtship, thus implying that *fruitless* is necessary for male sexual behaviour in our cockroach model. This suggests that the role of *fruitless* as master regulator of male sexual behaviour has been conserved along insect evolution, at least from cockroaches to flies.

Keywords *Blattella germanica* . courtship behaviour . *Drosophila* . *fruitless* . insect . RNAi

Introduction

Mating in animals, from insects to humans, is often preceded by elaborate courtship by the male. The genetic and molecular mechanisms underlying this complex sex-specific behaviour are well studied in the fruit fly *Drosophila melanogaster*. In a male fruit fly the sequential stereotyped behaviours that lead to copulation are very characteristic. Briefly, the male orients himself towards the female, taps her with his forelegs, extends

and vibrates one wing to sing a courtship song, licks her genitalia and finally attempts copulation (Hall 1994). Thanks to the advanced genetic tools available for *D. melanogaster*, more than 30 genes that affect some behavioural step in male courtship have been identified, although the gene *fruitless* (*fru*) appears to be the master regulator. Male flies lacking the *fru* gene are sterile, given that the last courtship steps, from singing to copulation, are abnormal or absent (Villella and Hall 2008).

In *D. melanogaster*, *fru* encodes a set of putative transcription factors containing a common BTB (Broad complex, Tramtrack, Bric à brac) N-terminal domain involved in protein-protein interactions, and one of four possible C-terminal Zinc finger DNA binding domains (A, B, C, and D). A number of transcripts are generated through the use of four promoters (P1-P4) and alternative splicing at both the 5' and 3' ends. Only those transcripts generated by the most distal promoter P1 are spliced in a sex-specific way: in males by default and in females by the control of the sex-determination proteins Transformer (Tra) and Tra2 (Ito et al. 1996; Ryner et al. 1996). Male-specific *fru* transcripts appear to be necessary and sufficient for male sexual behaviour, which was demonstrated by activating the *fru* gene in neural cells in the female fly's brain and sensory organs, resulting in masculinised females, directing at other females a sexual display resembling that of their male counterparts (Demir and Dickson 2005).

In *D. melanogaster*, male-specific *fru* transcripts are expressed in *ca.* 2% of neurons in the male central nervous system, which are organized into 21 distinct clusters that are (all) interconnected in a circuit that is directly and specifically involved in male sexual behaviour. This circuit includes central, sensory and motor components. Among the sensory neurons are olfactory neurons that may be specialized for detection of female sex pheromones, whereas the motor neurons innervate the penis and ejaculatory bulb. Recent reports (Cachero et al. 2010; Yu et al. 2010) have described

significant differences between male and female *D. melanogaster* brains, not only anatomical but also in terms of wiring, which could explain, at least in part, the differences observed in sexual behaviour.

D. melanogaster is a much modified species within the evolutionary history of insects, and functional genomics data obtained in this fly can not always be extrapolated to other species, especially to less modified ones. In this respect, our goal was to study the possible role of *fru* in male courtship behaviour in a phylogenetically basal insect, the German cockroach *Blattella germanica*. In *B. germanica*, courtship behaviour is also robust and easy to study. In an encounter, the male touches the female with the antennae, raises the wings upward and then it turns around 180° thus exposing the tergal gland to the female. The secretion of these glands stimulates the female to mount the male and feed, and while the female feeds on the tergal gland, the male pushes the abdomen under the female and clasps her genitalia with his left phallomere to accomplish genital connection (Roth and Willis 1952). This innate courtship behaviour is very different with respect to that of *D. melanogaster* commented above, and the question that arises is, are such evolutionary divergent male courtship behaviour of flies and cockroaches governed by the same master regulator *fru*?

Materials and Methods

Insects

Staged males of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1ºC and 60–70% relative humidity, and fed on dog chow (Panlab 125) and water *ad libitum*. All dissections and treatments were carried out on carbon dioxide anaesthetized specimens.

Cloning and sequencing

Total RNA was isolated from whole male body using the General Elute Mammalian Total RNA kit (Sigma, Madrid, Spain). Degenerate primers based on conserved *fru* sequences from *Tribolium castaneum, Apis mellifera*, *Nasonia vitripennis*, *Chorthippus brunneus*, *D. melanogaster* and *Drosophila pseudoosbscura* were used to obtain a *B. germanica* orthologue cDNA fragment by PCR amplification. The primers were as follows: Forward, 5'-TGYYTNMGNTGGAAYAAYCAYCC-3'; and Reverse, 5'-TCNGCNGTYTTNARRAACATNGG-3'. The PCR parameters were: 95°C (180s), 45 cycles of 95°C (30s), 50°C (30s), 72°C (40s) and a final extension of 72ºC (300s). The amplified fragment (303 base pairs) was subcloned into the pSTBlue™-1 vector (Novagen, Madison, WI, USA) and sequenced. By BLAST analysis, the sequence was confirmed to correspond to *fru*. The sequence was completed by 3'- and 5'-RACE (rapid amplification of cDNA ends) experiments (Invitrogen, Paisley, UK), according to the manufacturer's instructions. The PCR products were analyzed by agarose gel electrophoresis, subcloned into the pSTBlue™-1 vector and sequenced. For 3'-RACE, forward primers were as follows: Forward 1, 5'-CCCATCCTCATCCGATAATATTT-3'; and nested Forward 2, 5'-GTATGAAGGAGAAGTTAATGTTAAGCC-3'. For 5'-RACE, reverse primers were as follows: Reverse 1, 5'-GCTTGATAGGAGGTGGTGATGG-3'; and nested Reverse 2, 5'-CTTCTTCGATCTCGGGGTGTTATGG-3'.

PCR studies

Total RNA was isolated from brain and testes from adult males at different ages using the GenElute Mammalian Total RNA kit (Sigma). Samples represent a pool of 5 specimens. An aliquot of 200 ng from each RNA extraction was DNAse treated

(Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and random hexamers (Promega). Genomic DNA was removed by treatment with RNasefree DNase I (Invitrogen). To measure mRNA levels, quantitative real-time PCR (qRT-PCR) determinations were carried out using the cDNA products as templates, and SYBR Premix Ex Taq™ (iQ SYBR Green supermix, Bio-Rad, Hercules, CA, USA). The iQ5 optical system software version 2.0 was used for detection (Bio-Rad), and the sequences of the primer pairs for each of the specific RNA transcripts were designed using the "Primer Express" software (Applied Biosystems, Foster City, CA, USA). The PCR primer sequences used to amplify the *fru* transcript were: Forward, 5'-GTGGTGCAGGTGATCGGTTT-3'; and Reverse, 5'-GCTTGATAGGAGGTGGTGATGGG-3'. Determinations were carried out in triplicate and normalized to the internal control of BgActin-5c (Accession number AJ862721) mRNA for each sample. The amplification protocol used for all genes was: initial denaturalization at 95ºC for 3 min followed by an amplification program for 45 cycles of 10 sec at 95ºC and 60 sec at 60ºC, with a final melting curve analysis at 95ºC for 60 sec, 60ºC for 60 sec and 81 cycles of 55ºC for 30 sec. Genomic control and no template were used as negative controls in duplicate. Statistical analysis of gene expression values was carried out using the REST 2008 program (Pfaffl et al. 2002). This program calculates changes in gene expression between two groups, control and treated, using the corresponding distributions of *Ct* values as input. The program makes no assumptions about the distributions, evaluating the significance of the derived results by Pair-Wise Fixed Reallocation Randomization Test_ tool in REST (Pfaffl et al. 2002).

Courtship and mating behaviour

Individual adult males of chosen ages (from freshly emerged to 10-day-old), were placed in a cylindrical glass jar (4.5 x 8.0 cm) with two 5-day-old virgin females, and observed for 30 min, during which we recorded whether the presence of the females elicited courtship behaviour (wing raising).

RNAi experiments

To obtain a dsRNA to target the mRNA of *B. germanica fruitless* (dsBgFru), a 302 bp fragment located between nucleotide 56 and 358 (Fig. 1A) was amplified by PCR and subcloned into the pSTBlue^{TM}-1 vector. As control dsRNA (dsMock), we used a 92 bp noncoding sequence from the pSTBlue^{TM}-1 vector. dsRNA synthesis was performed as described earlier (Ciudad et al. 2006). dsRNA was re-suspended in diethyl pyrocarbonate-treated water, and 1 µl of the solution (at the concentration of 1 µg/µl of dsBgFru or dsMock) was injected into the abdomen of freshly emerged male fifth instar nymphs. To analyze the RNAi effect on target transcript levels, brain and testes of 5-day-old adult males that had been treated with dsBgFru or dsMock (n = 8, respectively) were dissected and processed individually as described above. RNAi effects in terms of courtship behaviour were studied at day 9 of the adult stage. Two 5-day-old virgin females were added to the jar, as in the experiments to observe courtship and mating behaviour (see the previous section), and the specimens were observed during 1 h. After that, the three specimens were left together in the same jar, and the presence of sperm in the spermathecae was examined in the two females 10 days later.

Results and discussion

Molecular cloning of *fru* in *B. germanica*

Cloning of *fru* cDNAs in *B. germanica* was accomplished by a RT-PCR approach using degenerate primers designed on the basis of conserved motifs of known *fru* sequences. Using cDNA from 5- to 7-day-old adult males of *B. germanica* as a template, a 303 bp PCR fragment was obtained, which was highly similar to the equivalent region in known insect *fru* sequences. 5'-RACE and 3'-RACE experiments using the same template gave a full-length cDNA of 1175 bp. In the sequence (accession number FN429764), a putative start codon is preceded by in-frame stop codons, thus suggesting that we obtained a full-length open reading frame. A database BLAST search indicated that it encoded a *B. germanica* orthologue of *fru*, which we called BgFru. It encodes a 350 amino acid protein, with a BTB and C_2H_2 Zinc finger domain (Fig. 1a), organised as in other *fru* sequences.

In *D. melanogaster*, male-specific Fru sequences have a *ca.* 110-amino acid N-terminal extension upstream of the BTB domain, which contain a stretch of 12 histidines alternating with neutral residues followed by a proline-rich stretch (Demir and Dickson 2005). However, this extension is absent in BgFru. The question whether *fru* sex-specific splicing occurs in hemimetabolan insects remains unanswered. Transcripts showing the typically male N-terminal extension have been reported in *D. melanogaster* (Demir and Dickson 2005), in another Diptera, the mosquito *Anopheles gambiae* (Gailey et al. 2006), and in the Hymenoptera *N. vitripennis* (Bertossa et al. 2009), the three species being holometabolan. Conversely, detailed studies on *fru* variants carried out in males of three Orthoptera species of the genus *Chorthippu*s, which is hemimetabolan, revealed only two different transcripts differing in the 5' region, but having the coding sequences identical and starting immediately upstream the BTB domain, thus without having the *ca.* 110 amino acid extension typical of male-specific *fru* of *D. melanogaster*, *A. gambiae* and *N. vitripennis* (Ustinova and Mayer 2006).

Instead, the authors found a number of intraspecific *fru* paralogous sequences, very slightly divergent each other by single nucleotide substitutions, and they proposed that in *Chorthippu*s spp. different functions of *fru* are accomplished by different paralogues, whereas in *D. melanogaster* they are regulated by alternative transcripts of the *fru* gene (Ustinova and Mayer 2006). We did not find variants of *fru* in *B. germanica* males, and our exhaustive 5'- and 3'-RACE experiments gave only the transcript described above, which is clearly expressed in males, as reported in the next section.

Expression of *fru* and courtship behaviour in males of *B. germanica*

Expression of *fru* was studied by qRT-PCR in testes and brain tissues of adult males from emergence to day 10. In testes, *fru* mRNA levels oscillate between *ca.* 50 to *ca.* 120 copies of *fru* per 1000 copies of BgActin-5c, but they do not show regular and dramatic fluctuations (Fig. 1b). Conversely, the mRNA expression pattern in brain tissues showed low values (*ca.* 25 copies of *fru* per 1000 copies of BgActin-5c) until day 4 of adult life, they then increased on day 5 (*ca.* 50 copies), and peaked on day 6 (*ca.* 100 copies) (Fig. 1c).

Interestingly, the expression pattern of *fru* in male brains has a correspondence with the temporal development of courtship capabilities. The study of courtship behaviour from adult emergence until day 10 (Fig. 1d) showed that males do not respond at all to the presence of mature females during the first 4 days of adult life. On day 5, 61% of the tested males responded to the presence of females by raising the wings and thus exposing the tergal glands. The percentage of response increased on day 6 (74%), slightly decreased on days 7 and 8 (70 and 67%, respectively), and increased again on days 9 and 10 (>80%) (Fig. 1d). The time of response of the males that responded by raising the wings after adding the two females to the jar was between 3

and 11 min. The coincidence between the peak of expression in the brain and the onset of courtship suggests that *fru* has a role in this behaviour.

RNAi of *fru* abolishes courtship behaviour in *B. germanica*

The role of *fru* in male *B. germanica* was studied by RNAi. For this purpose, freshly emerged fifth instar nymphs were treated with 1 µg of dsBgFru (n = 22) or dsMock (n = 29). Both experimental groups moulted to sixth instar nymphs 6 days later, and then to adults 8 days later. On day 6 of adult life we measured the mRNA levels of BgFru in testes and brain tissues of both groups, and found that transcript levels were significantly lower in dsBgFru-treated specimens with respect to those treated with dsMock (Fig. 2). On day 9 of the adult stage, when males are fully responsive to the presence of females (Fig. 1d), courtship behaviour was examined in dsBgFru and dsMock groups. Results (Table 1) indicated that *ca.* 80% of the 29 dsMock-treated males touched the antennae of one or the other female partners and raised the wings after 12 min of interaction, as average. Conversely, none of the 22 dsBgFru-treated males exhibited any wing raising behaviour, in spite that they had a number of physical contacts with the two partner mature females (Table 1).

Ten days later the females used in the experiments were dissected to assess the presence of spermatozoids in the spermathecae. Only 2 out of the 44 females used in the experiments with dsBgFru-treated males had formed an ootheca (in *B. germanica*, the formation of unviable oothecae by virgin females in not unusual), and none of these 44 females had spermatozoids in the spermathecae. Conversely, all the 56 females that had been in contact with dsMock-treated males formed an ootheca and had spermatozoids in the spermathecae, thus indicating that all them achieved mating.

This is the first functional evidence for a role of *fru* in sex-specific behaviour outside dipterans, and indicates that *fru* regulates courtship in males also in *B. germanica*, as it occurs in *D. melanogaster*, in spite of the divergent courtship behaviours selected by these two species along evolution. The fact that *fru* regulates male courtship in cockroaches, which is a considerably basal insect group from a phylogenetical point of view, suggest that this function might be one of the most ancestral of *fru*.

Inspired by the formidable success of forward genetics in the identification of key regulators of morphogenetic processes, a number of authors predicted that single genes might have the role of master regulators of complex innate behaviours (Dulac 2005; Lorenz 1981). Several years ago, *fru* was proven to be one of such genes, in this case regulating male courtship behaviour, in the evolutionarily much modified dipteran *D. melanogaster* (Demir and Dickson 2005). Our present results on a cockroach model show that *fru* not only plays such a role of master regulator of a complex innate behaviour, but also that this role has been conserved along insect evolution, at least from the phylogenetically basal order Blattaria to the most distal Diptera.

Acknowledgements Financial support from the Ministry of Education and Science, Spain (projects BFU2008-00484 to M-D. Piulachs and CGL2008-03517/BOS to X. Bellés), Generalitat de Catalunya (2005 SGR 00053) is gratefully acknowledged. L. Ciudad received a pre-doctoral research grant (I3P) from CSIC and E. Clynen received a travel grant from the Fund for Scientific Research (FWO)-Flanders (Belgium). Thanks are also due to Elena Torres, who received a JAE-intro (CSIC) grant, for helping in the experimental work.

References

Bertossa RC, van de Zande L, Beukeboom LW (2009) The *Fruitless* gene in *Nasonia* displays complex sex-specific splicing and contains new zinc finger domains. Mol Biol Evol 26:1557-1569

Cachero S, Ostrovsky AD, Yu JY, Dickson BJ, Jefferis GS (2010) Sexual dimorphism in the fly brain. Curr Biol 20:1589-1601

Ciudad L, Piulachs MD, Belles X (2006) Systemic RNAi of the cockroach vitellogenin receptor results in a phenotype similar to that of the *Drosophila* yolkless mutant. FEBS J 273:325-335

Demir E, Dickson BJ (2005) *fruitless* splicing specifies male courtship behavior in *Drosophila*. Cell 121:785-794

Dulac C (2005) Sex and the single splice. Cell 121:664-666

Gailey DA, Billeter JC, Liu JH, Bauzon F, Allendorfer JB, Goodwin SF (2006) Functional conservation of the *fruitless* male sex-determination gene across 250 Myr of insect evolution. Mol Biol Evol 23:633-643

Hall JC (1994) The mating of a fly. Science 264:1702-1714

Ito H, Fujitani K, Usui K, Shimizu-Nishikawa K, Tanaka S, Yamamoto D (1996) Sexual orientation in *Drosophila* is altered by the satori mutation in the sex-determination gene *fruitless* that encodes a zinc finger protein with a BTB domain. Proc Natl Acad Sci U S A 93:9687-9692

Lorenz K (1981) The Foundations of Ethology: The principal ideas and discoveries in animal behavior. Springer, New York

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36

Roth LM, Willis ER (1952) A study of cockroach behaviour. Amer Mid Nat 47: 65-129

Ryner LC, Goodwin SF, Castrillon DH, Anand A, Villella A, Baker BS, Hall JC, Taylor BJ, Wasserman SA (1996) Control of male sexual behavior and sexual orientation in *Drosophila* by the fruitless gene. Cell 87:1079-1089

Ustinova J, Mayer F (2006) Alternative starts of transcription, several paralogues, and almost-fixed interspecific differences of the gene *fruitless* in a hemimetabolous insect. J Mol Evol 63:788-800

Villella A, Hall JC (2008) Neurogenetics of courtship and mating in *Drosophila*. Adv Genet 62:67-184

Yu JY, Kanai MI, Demir E, Jefferis GS, Dickson BJ (2010) Cellular organization of the neural circuit that drives *Drosophila* courtship behavior. Curr Biol 20:1602-1614

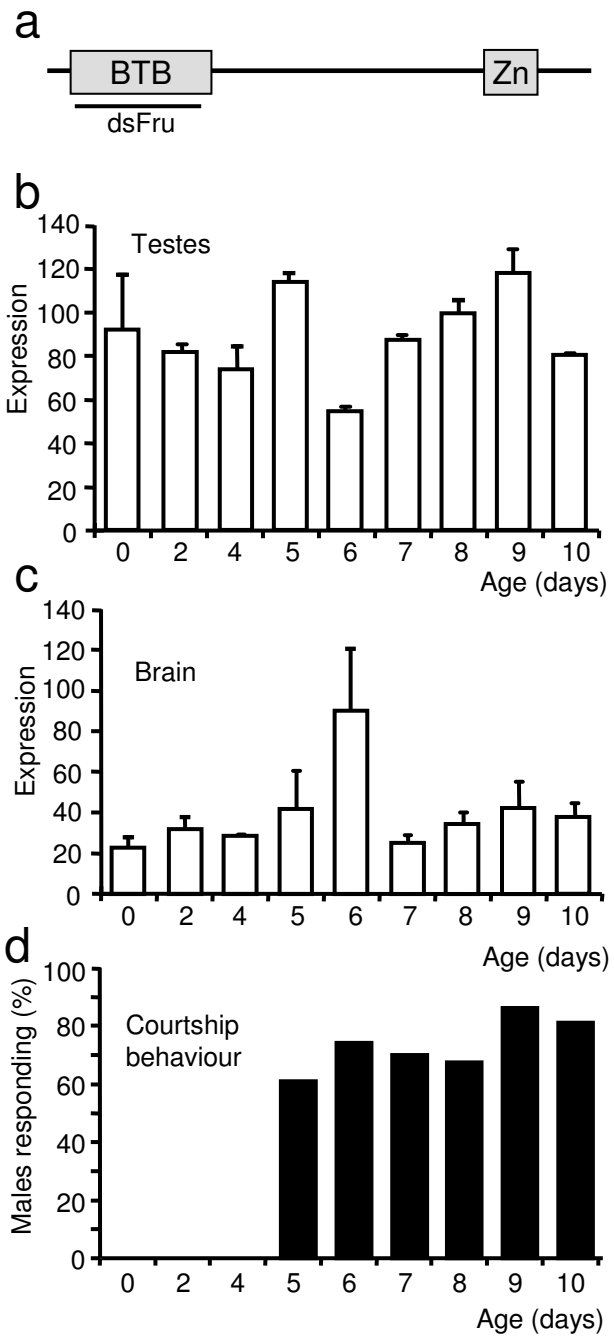
Table 1. Effects of treatments with dsBgFru and dsMock on male courtship behaviour and mating in *Blattella germanica*. Treatments were carried out in penultimate nymphal instar and effects were studied on day 9 of adult life.

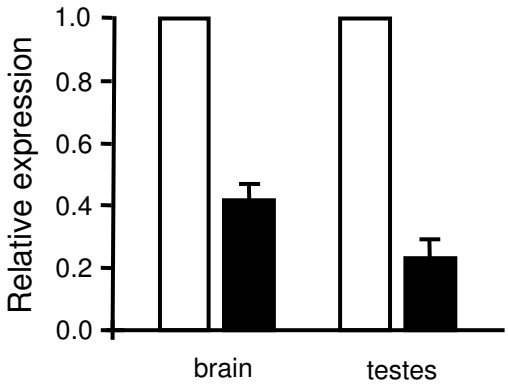
Treatment	N	Males exhibiting courtship	Time elapsed until courtship (min ± SEM)	Othecae formed by the female partners	Presence of spermatozoids in the spermathecae of the female partners
dsMock	29	23 (79%)	12.0 ± 1.7	58 females, 58 oothecae	Present in the 58 females used
dsBgFru	22	0 (0%)	--	44 females, 2 oothecae	Absent in the 44 females used

Figure Legends

Fig. 1 Fruitless in *Blattella germanica*. (a) Sequence organization showing the BTB and the Zn finger domains; below the BTB domain is indicated the region encompassing the dsBgFru designed for the experiments of RNAi. (b-c) Expression of *fruitless* mRNA in testes (b) and brain (c) tissues of adult males measured by qRT-PCR; in both cases data represent 3 biological replicates (mean ± SEM), and are indicated as copies of *fruitless* mRNA per 1000 copies of BgActin-5c. (d) Percentage of males of different adult ages that exhibited courtship behaviour after being exposed to two mature females (n = 20-24).

Fig. 2 RNAi of *fruitless* in *Blattella germanica*. Expression of *fruitless* mRNA in testes and brain tissues from 5-day-old adult males. qRT-PCR data was normalized against BgActin-5c expression, and data represent values of dsBgFru-treated specimens (black columns) normalized with respect to dsMock expression levels (white columns), and indicated as the mean ± SEM (n = 3-4). According to REST software (Pfaffl et al. 2002), the expression in brain and testes is down-regulated by a factor of 0.42 and 0.23, respectively.


a
BTB
Zn
dsFru
b
Testes
Expression
140
120
100
80
60
40
20
0
0 2 4 5 6 7 8 9 10
Age (days)
c
Brain
Expression
140
120
100
80
60
40
20
0
0 2 4 5 6 7 8 9 10
Age (days)
d
Courtship behaviour
Males responding (%)
100
80
60
40
20
0
0 2 4 5 6 7 8 9 10
Age (days)


Relative expression
1.0
0.8
0.6
0.4
0.2
0.0
brain
testes