Molecular Genetics of Aggressive Behaviour in *Drosophila melanogaster*

Thesis submitted for the degree
of Doctor of Philosophy at the
University of Leicester

By

Merav Tauber

Department of Genetics,
University of Leicester

July 2009

ABSTRACT

Molecular Genetics of Aggressive Behaviour in *Drosophila melanogaster*

Merav Tauber

Aggression is a key component of the normal repertoire of behaviours in a broad range of animals from insects to mammals. Although the genetic basis for aggression is widely accepted, only a few individual candidate genes have been studied. Recent studies have indicated that *Drosophila melanogaster* can serve as a powerful model system to study the genetics of aggression. The aim of this project was to identify genes associated with aggression by global profiling of the fly transcriptome using DNA expression microarrays. At the core of this study was a behavioural screen in which the aggression of 910 pairs of males was observed and scored. Microarray analysis revealed 350 genes that were differentially expressed between aggressive and non-aggressive flies. Several biological functions such as translation activity, immune response, ion transport, and sensory transduction were significantly over-represented. Analysis of the upstream region of these genes also suggested several shared motifs that might serve as transcription factor binding sites that drive the co-expression of these genes.

One of the top differentially expressed genes was *Dat*, (*dopamine-N-acetyltransferase*), which was upregulated in aggressive flies. *Dat* has two isoforms generated by alternative splicing, *DatA* and *DatB*. QPCR analysis revealed that only *DatB* is upregulated in aggressive flies. In Dat^{lo} mutants that express only *DatB*, aggression is also increased, an effect that can be reverted by over-expressing the *DatA* transgene. Additional experiments over-expressing *DatB* indicate that the two isoforms effectively act in opposite ways to regulate aggression, suggesting that a balance between them is necessary for adaptive levels of aggression.

Another candidate gene was *CG6480*, whose levels were reduced in aggressive flies. The function of this gene is unknown, but it does share a conserved motif called Fascin with its mammalian ortholog *frg1*. Silencing this gene by dsRNAi resulted in flies that show elevated levels of aggression.

ACKNOWLEDGMENTS

I would like to thank my supervisor Professor Bambos Kyriacou for his constant valuable guidance and bright technical suggestions as well as for his friendship, and for giving me the opportunity to complete my PhD course as a part time research technician. This allowed me to enjoy both worlds, having an intriguing working life while still not missing out on my children.

I have not enough words to thank my beloved husband Eran , for his constant support, his encouragement, guidance and technical suggestions through my study years and most of all for bearing working with me, together in the same lab, all these years. I wouldn't have succeeded without him.

I would like to express my gratitude to everyone in the fly groups, who were always happy to help in any way possible. The completion of this thesis would not have been possible without their support. Thanks also to Julien and Guillaume who were Erasmus exchange students under my supervision and assisted with some of the work.

Many thanks to my former boss and dear friend Ila Patel, who was always there for me, helping and guiding me in so many ways through all these years.

A special thank you to my beloved father and late mother who worked hard for many years in order to allow me the opportunity to further my education. Without their love, encouragement and generosity I would never have got this far.

Last but not least, I would like to thank Maya and Noa, my twin daughters, whose good nature, maturity and happy attitude to life allowed me to complete my studies with a bearable amount of guilt. Though I have learnt much about behaviour genetics and biology throughout my study years, observing my daughter's relationship and growing up has been the most beautiful and fascinating lesson of them all.

I would like to acknowledge the Royal Society and the Harry Frank Guggenheim Foundation for funding this project.

CONTENTS

Chapter 1

INTRODUCTION

1.1. General properties of aggression

Aggression is a naturally occurring behaviour that is present in diverse range of animals, from insects to mammals. Various definitions for aggression exist, generally referring to the competitive interactions between members of the same species. Konrad Lorenz who pioneered the field of ethology, defined aggression as "the fighting instinct in beast and man which is directed against members of the same species", in his book *On Aggression* (1963). Lorenz listed three main functions of aggression: (i) balance the distribution of the species, (ii) selection of the strongest and (iii) defence of the young. These functions assume an important adaptive role for aggression, affecting the fitness of individuals.

According to Lorenz, members of the same species compete for the same resources (e.g. food, nesting site) and under some conditions will actively fight for them. Agonistic behaviour (i.e. aggression) also arises from competition for mates, thus serves to select the stronger and fitter individuals for propagation of the species. Lorenz's view on aggression was closely related to Charles Darwin's concept of "survival of the fittest" and was well embedded in the prevailing notion at that time that evolution is driven by adaptations that promote survival of the species (group selection). It should be noted, that although group selection is currently not regarded as a viable mechanism in evolution (Trivers, 2008), the consequences of aggression listed above promote fitness of individuals and therefore fit well with other theories of the evolution of behaviour (Krebs, 1993). Aggression was seen as a mechanism that secures for each individual the minimum territory required to support its existence, and prevents overcrowding; consequently, aggression constituted a force that serves an

important function of "spacing out" individuals or groups in the area they occupy. According to group selection theory, it is favourable for the species if the stronger of two rivals takes possession of the territory or of the desired female. For example, in species where males are territorial, the more aggressive male is more likely to be able to defend and to hold onto a territory. These territories may have certain resources (e.g. food, shelter) desired by the female who wants to reproduce and is looking for a place to lay eggs, nest and rear young - so females will select for males with a territory (aggressive males). Lastly, in species where the young progeny require a relatively long time to develop, aggressive parents may be more effective for protection.

Another important insight of Lorenz into the evolution of aggression was related to the link between aggression and violence. Lorenz noted that fights between individuals of the same species rarely result in serious injury or in death of either combatant. Such fights, in fact, more resemble a tournament than a lethal struggle. Lorenz suggested that in violent interactions, both winner and loser might incur costly injuries. Therefore, it seems that evolution has exerted a strong selective pressure against aggressive behaviour involving physical violence that ends in severe injury. Instead, aggression has evolved in many species as series of ritualised acts that allow opponents to gauge dominance and settle competition without violence (Lorenz, 1963).

1.2. Aggression: the behavioural phenotype

Aggressive behaviour has been extensively studied both in vertebrates and non-vertebrates model organisms. It has long been recognized that animals exhibit few types of aggression: offence (obtaining and protecting resources), defence (self protection from injury by others), and infanticide (Nelson, 2006; Schaik and Janson, 2000). Most of current research and experimental studies of animal aggression mainly focus on offensive behaviour in males.

1.2.1 Aggression and Territoriality

One of the most intriguing aspects of aggression is the relationship between territory and aggressive behaviour. Interestingly, territoriality can serve dual function, either as an instigator of aggression or stabiliser of antagonism to prevent aggression. In

most territorial species, animals fight more during the time of establishing a territory than after territorial boundaries are well defined (Lorenz, 1963). Thus, while un-established disputed territory promotes aggression, established territorial boundaries often increase stability, and consequently reduces hostility and aggression. Another interesting aspect of aggression and territoriality is presented by the familiarity hypothesis, suggesting that territorial animals normally respond less aggressively to neighbours than to strangers. This 'dear enemy effect' has been explained by different threat levels posed by neighbours and strangers that uses vocalized and olfactory cues as signals of recognition between neighbours (reviewed by Ydenberg *et al.*, 1988). However, a recent study challenged the familiarity hypothesis and suggested that in social species, intense competition between neighbours exists, and increased aggression towards neighbours is more common, thus limiting the phenomenon of reduced aggression towards neighbours predominantly to solitary species (Muller and Manser, 2007)

Territorial aggression has been utilized as an experimental tool that has led to new insights into the understanding of agonistic behaviour. In rodents, it has been effectively used in the laboratory by what is known as the *resident- intruder* paradigm (Raab *et al.*, 1986; Hilakivi-Clarke and Lister, 1992; Maruniak *et al.*, 1986). An adult male (resident) is housed singly in a cage (or with a female) for a period of time after which an intruder is introduces into the cage and the behavioural interactions are monitored.

The resident- intruder paradigm was instrumental in exploring the effect of various substances on aggression. In an early study (Yoshimura and Ogawa, 1982), a male mouse housed with a female for five weeks, was injected with an anticholinergic agent; once an intruder male was introduced into the cage the resident mouse showed a significantly reduced aggression in a dose-dependent manner compared to controls. A more recent set of studies examined the effect of steroid hormones like estrogen on aggression, and found that increased levels of estrogen were associated with an elevated number of attacks against intruder (Trainor *et al.*, 2006; Trainor *et al.*, 2007b). Interestingly, it was also demonstrated that the behavioural effects of estrogens on

aggression can be reversed by an environmental signal like day-length, where selective activation of estrogen receptors decreases aggression in long days and increases aggression in short days (Trainor *et al.*, 2007a)

1.2.2 Aggression and dominance hierarchies

In socially organized species, aggression plays an important role in developing the social structure and resolving the rank ordering of members within the group (Krebs, 1993). The formation of a dominance hierarchy and the maintenance of social statues within the group are the key driving force for the display of hostile behaviour. A repertoire of postures and signals have evolved in various species, which stimulate offensive acts or defensive submissive and flight reactions (Krebs, 1993)

Dominance hierarchies are social structures usually formed through pairwise interactions within the group, and often lead to a linear social structure. In a linear hierarchy, one individual dominates all the other individuals in a group; the second dominates all but the first, and so on down to the last individual, who is dominated by all the others. Linear hierarchies are more common in small groups (e.g. $n < 10$), but in larger groups, linearity may be reduced (Jamesone *et al.*, 1999). Hierarchy rank influences important fitness determinants such as access to food, health, mate finding and reproduction (Krebs, 1993).

Dominance aggression and hierarchy formation have been extensively studied in crayfish and lobsters, where conflicts over limited resources often lead to the formation of social dominance hierarchies. This social hierarchy is normally established through aggressive dyadic encounters, resulting in a distinct formation of dominant and subordinate. The winner of a fight often can be predicted from its behaviour during the fight, with the winner displaying more aggressive behaviour patterns like lunges, strikes and tail flips than the loser (Edwards and Herberholz, 2006). Crayfish (and lobster) combatants can estimate the probable winner from these behaviour cues, and also from odour signals excreted in the urine (Breithaupt and Eger, 2002). Furthermore, the aggressive state seems to be dependent on previous agonistic experience, in what is known as the winner-loser effect where winning or losing

experiences are memorised and generate a lasting effect (Chase *et al.*, 1994; Goessmann *et al.*, 2000). Winning enhances further success, while losing decreases successive chances for winning (and increases the likelihood of retreat). It also seems that losing has a longer lasting effect than winning, presumably because it is more essential for an individual to remember its lack of ability to win than to remember previous victories; it is more costly for a subordinate to behave aggressively and initiate a fight (Hock and Huber, 2006) Once the rank order among the members of the group has been established, a significant reduction in aggression is maintained.

1.2.3 Kin selection and the Hamilton rule

The failure of group selection to explain natural phenomena such as altruism or reduced aggression was realised by researchers such as Hamilton and Trivers, arguing that natural selection acts on individuals, and behaviours are selected only if *individual* fitness is increased (Krebs, 1993). One alternative way to explain the evolution of altruism was the concept developed by Hamilton of inclusive fitness, where the evolutionary success of *genes* can be attained by individuals contributing to the fitness of their closely related relatives (kin selection). This model worked particularly well in haplo-diploid systems like the honeybee where worker relatedness is exceptionally high. Hamilton modelled the evolution of altruism as the function of cost and benefit of the behaviour and the relatedness (*r*), and predicted that this mechanism will evolve in either organisms that recognize kin, or in species with limited dispersal, where *r* is maximized. However, limited dispersal also increases competition, which will counteract the evolution of aggression-suppression behaviour. To tease these two factors apart, West *et al.* (2001) studied aggression across species of fig wasps that show various levels of *r* and found that the level of aggression between males was not correlated with *r*, but was correlated with competition for females (as number of females increased, aggression decreased). Thus, although relatedness may still be important (as well as the costs and benefits of altruism/aggression-avoidance), the competition level may drive even closely related siblings into fierce aggression. (West *et al.*, 2001; Saito and Mori, 2005)

1.2.4 Nature versus nurture

In many behavioural traits, including aggression, the relative contribution of nature versus nurture is a matter of continuous debate. The psychoanalytic view asserted by Sigmund Freud, the father of psychoanalysis, sees aggression as an innate personality characteristic common to all humans, and operates as a powerful instinct shared by man with many non-human species (Freud, 1961 pp.58-63). According to this theory, this instinct slowly builds up over time, and if not released in a safe and non-violent way, will reach dangerous levels, when a person can 'explode' and harm himself or others. This theory however does not address the exact nature of aggression nor why some individuals are more aggressive then others. The ethological view shares the concept of aggression as an instinct (Lorenz, 1963; Tinbergen, 1951), however, it adds that the innate fighting instinct does not occur unless somehow invoked by environmental cues. This concept is related to another theory, the frustration – aggression theory (Dollard *et al.*, 1939; Berkowitz, 1989) asserting that "the occurrence of aggressive behaviour always presupposes the existence of frustration", and that the "existence of frustration always leads to some form of aggression." Still, this theory does not explain why not all frustrations result in aggression. The social learning theory claims that aggression is not hereditary but learnt in social contexts and encouraged by direct reward. Children would behave more aggressively after observing a model performing aggressive acts especially if they can directly copy the behaviour (Bandura *et al.*, 1961; Bandura *et al.*, 1963). Yet, this theory does not undermine the hypothesis that aggression is innate, and the two views of aggression are not mutually exclusive. In fact, the importance of the innate element of aggressive behaviour is reinforced by studies in recent years increasingly recognizing the significance of the genetic component of aggression (see below).

1.3. The genetics of aggression

1.3.1 MAOA

The genetic basis of aggression has long been of interest to psychologist, particularly those interested in human aggression and other antisocial behaviours. One of the early studies to have identified a genetic link to aggressive behaviour was that of a family in the Netherlands (Brunner *et al.*, 1993). Five male members of this family were affected by a borderline mental retardation and abnormal behaviour, including impulsive aggression, and had been involved in serious crime including rape, arson and attempted murder. Molecular analysis of the family pedigree (Fig. 1.1) revealed that there was a mutation in an X chromosome-linked gene that encodes the enzyme monoamine oxidase A (*MAOA*) that is involved in regulating the metabolism of serotonin, dopamine, and noradrenaline. A point mutation in the gene changed a glutamine into a stop codon, and resulted in a complete deficiency of the enzyme. The resulting increase in the level of serotonin in the affected men was suggested to cause their aggressive tendency.

Another study associating *MAOA* to violence monitored 500 children from birth to adulthood and showed that a polymorphism in the gene was correlated to the level of the enzyme activity in the brain (Caspi *et al.*, 2002). Individuals with the genotype conferring low levels of MAOA activity were significantly more likely to exhibit high level of aggression and other antisocial behaviour than those with high levels of MAOA, but only if they were also maltreated and abused as young children. In contrast, children with high levels of MAOA activity who were maltreated in childhood did not display antisocial behaviour, demonstrating a clear genotype-environment interaction.

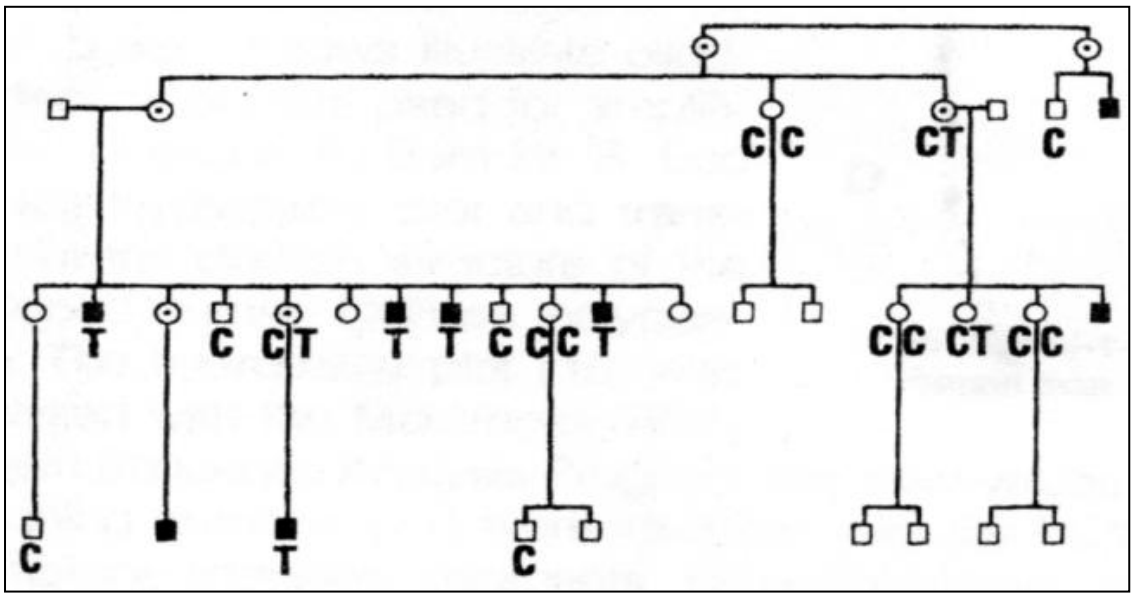


Fig. 1.1. The pedigree of the family with a point mutation in *MAOA*. Males of this family (squares) that carry the X-linked *C* to *T* mutation (filled square) show abnormal behaviour including impulsive aggression. The normal males (empty squares) are homozygous for the allele *C* and show normal behaviour (figure from Brunner *et al.*, 1993).

More evidence for the role of *MAOA* in aggressive behaviour came from several studies in rodents. A study associating *MAOA* with aggression was conducted in transgenic mice carrying a deficiency for the gene (Cases *et al.*, 1995) Mutant mice displayed enhanced aggression that was correlated with high levels of serotonin (concentration increased from up to nine fold), supporting the studies in humans that identified direct consequence of *MAOA* deficiency with agonistic behaviour. Another study with knockout mice lacking the serotonin receptor 5-hydroxytryptamine-1B (5HT1B) also showed evidence of increased aggression and impulsive behaviour (Saudou *et al.*, 1994). When a mutant male mouse was confronted with an intruder in the resident - intruder paradigm, the number of attacks, their intensity and severity were significantly higher compared with wild- type mice.

A common polymorphism in the upstream region of *MAOA* in human populations, involving variable tandem repeats (VNTR), generates two alleles, *MAOA*-L and *MAOA*-H that differ in their expression level (reviewed in Buckholtz and Meyer-Lindenberg, 2008). The difference between the two variants was demonstrated in recent psychological experiments where the aggressiveness of subjects was measured as the amount of hot (spicy) sauce they would administrate to their (fictional) opponents, in response to a financial loss (McDermott *et al.*, 2009). The study revealed that subjects carrying *MAOA*-L behaved more aggressively than *MAOA*-H. The authors suggested that relatively high frequency of *MAOA*-L in population (as much as 30%) is maintained by frequency dependent selection that reflected a mixed strategy, where stable populations are possible, as long as aggressive individuals (*MAOA*-L) do not dominate the population.

1.3.2 Other genes involved in aggression

Another gene implicated in aggression behaviour mice was *Pet-1*, a gene required specifically for the development of serotonergic neurones (Hendricks *et al.*, 2003). In the resident-intruder paradigm, mutant mice lacking the gene presented higher levels of aggression than wild-type. The gene *Nuclear Receptor 2E1* (*Nr2e1*) was also shown to be involved in aggression (Young *et al.*, 2002) and a spontaneous mutation named *fierce* (*frc*) causes mice to display extreme violent behaviour, with mutant males often injuring or killing siblings and intended mates. Interestingly, *frc* females also show increased aggressive behaviour. Remarkably, in a follow up study, it was demonstrated that the pathological aggression in *frc* mice can be rescued by the human *NR2E1* gene (Abrahams *et al.*, 2005)

Attempts to identify natural gene variants (alleles) that contribute to genetic variation in aggression were also made by quantitative trait loci (QTL) mapping (Brodkin *et al.*, 2002). This study used two inbred mouse strains, the NZB/B1NJ (very aggressive) and A/J (non-aggressive). Aggression was assessed in backcross progeny using the resident-intruder paradigm. A set of micro-satellite markers was used to genotype the genome of each mouse at ~20 cM (to identify the parental allele in each locus), and the marker information was correlated with the aggression score. This

genomic scan for loci that contribute to variation in aggression indicated two significant regions (QTLs) named Aggr1 and Aggr2, which included candidate genes such as *diacylglycerol kinase* (*Dagk1*) and the glutamate receptor subunit AMPA3 (*Gria3*).

1.4. The molecular basis of aggression.

1.4.1 Biogenic amines and aggression

Various studies suggest an important role for biogenic amines in the regulation of aggression. Biogenic amines are small organic molecules that function as neurotransmitters or hormones. There are five well-known biogenic amine neurotransmitters divided into two sub-classes: 1) Catecholamines including dopamine (DA), adrenaline[1] and noradrenaline (in vertebrates) or octopamine (in invertebrates). 2) Endolamines including serotonin and histamine. These neurotransmitters are all derived from single amino acids and hence also known as monoamines.

The catecholamines are derived from the common precursor amino acid tyrosine (Fig. 1.2), the endolamine serotonin (5-HT) is derived from tryptophan, whilst histamine is produced from the amino acid histidine. The role of monoamines in synaptic transmission is highly conserved throughout diverse animal groups, and these molecules mediate a variety of functions of the central nervous system; The neurotransmitter noradrenaline is known to be involved in regulating sleep and wakefulness, attention and feeding, as well as serving as a stress hormone (Purves *et al.*, 2001). Octopamine plays a crucial role in the fight-flight reaction, stress and escape. Dopamine (DA) is implicated in motivation, reward and addiction (Purves *et al.*, 2001), and serotonin (5-HT) is known to be involved in regulating mood, sleep, appetite and sexuality. Histamine is known to mediate wakefulness, attention and response to allergic reactions and tissue damage (Purves *et al.*, 2001).

[1] Adrenaline and nor-adrenaline are called epinephrine and nor-epinephrine respectively, in North America.

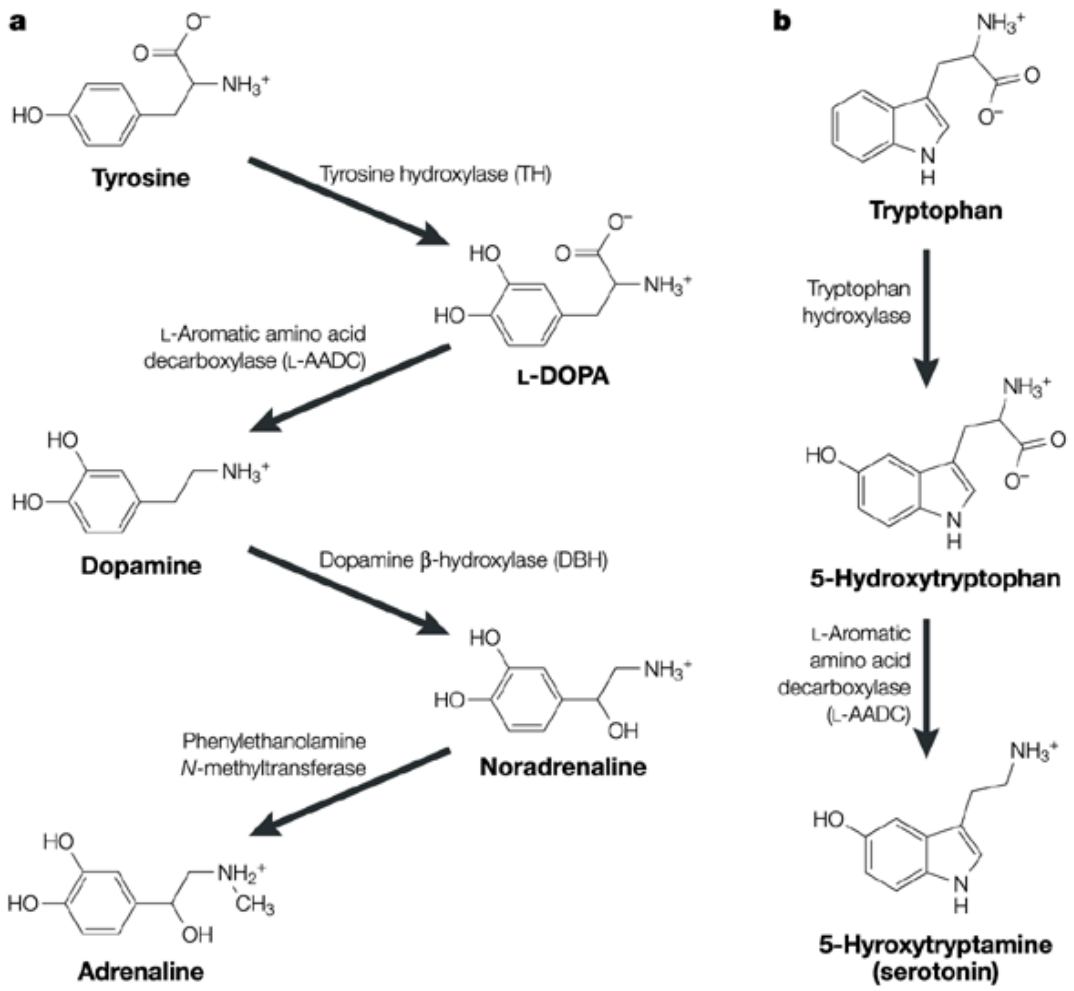


Fig.1.2. Metabolism of biogenic amines. Dopamine, noradrenaline and adrenaline are derived from the amino acid tyrosine (Left panel), while serotonin (5-HT) is generated from tryptophan (right). (From Goridis and Rohrer, 2002).

1.4.1.2 Serotonin (5-HT) and aggression.

5-HT is a biogenic amine that has received most of the attention as a modulator of aggression. Large number of studies have suggested that low levels of 5-HT are associated with aggression both in animal models and in humans (reviewed by Olivier, 2004). In most cases, the activity of 5-HT was estimated from measurements of 5-

HIAA (5-hydroxy indole acetic acid), the product of 5-HT metabolism. In mice, levels of both 5-HT and 5-HIAA were lower in aggressive males compared with non-aggressive (Giacalone *et al.,* 1968), and similar results have been found in hamsters and rats (reviewed by Miczek and Fish, 2006). Tests of CSF levels of 5-HIAA in human individuals with history of aggressive behaviour show reduced levels (Brown *et al.*, 1979). Inhibition of 5-HT synthesis (Vergnes *et al.,* 1986), or ablation of serotonergic neurons (Molina *et al.*, 1987) promotes aggression in rodents and primates.

However, the causative role of 5-HT in aggression is difficult to demonstrate: most of the evidence of 5-HT levels comes from measurements *following* the behaviour. To address the problem of temporal separation, *in vivo* micro-dialysis studies have been developed. These studies showed reduced 5-HT in aggressive rat males, but only after the fight has already started (e.g. van Erp and Miczek, 2000). Interestingly, 5-HT levels declined also in rats that were expecting a fight (following a regular daily interaction), although the fight never occurred (Ferrari *et al.,* 2003), indicating anticipating aggression itself elicits 5-HT reduction.

The role of 5-HT in aggression was also addressed by studying the 5-HT receptors. Over 14 receptors have been identified, most of which are GTP-binding proteins (G proteins), but the 5-HT_1 receptors have received most of the attention (Olivier, 2004). Mice whose 5-HT_{1B} receptor was knocked-out by homologous recombination, showed increased aggression (Saudou *et al.,* 1994). In contrast, knocking out the 5-HT_{1A} receptor decreases aggression (Zhuang *et al.,* 1999). The role of 5-HT was also studied pharmacologically using agonists and antagonists of these receptors. Agonists of the 1A receptors inhibit aggression in various species, and the fact that this effect can be attenuated by 1A antagonists, indicates that the agonists target the 1A receptor specifically. Agonists of the 5-HT_{1B} were also shown to reduce aggression (reviewed by Miczek and Fish, 2006), and without any apparent changes in locomotion that are often seen in 1A agonists treated subjects.

1A receptors function as autoreceptors: they are located in serotonergic neurons on the presynaptic terminals and respond to serotonin by decreasing its release, constituting a negative feedback loop. A recent study for example, revealed that in aggressive mice, levels of 5-HT are reduced, but the sensitivity of the 1A autoreceptors

is increased (Caramaschi *et al.*, 2007). Overall, these experiments support the serotonin deficiency hypothesis of aggression, although some studies suggest the opposite, including the fact that lack of MOA, which metabolises serotonin, is widely accepted as promoting aggression (see above).

1.4.1.2 Dopamine (*DA*) *and aggression*

Evidence for the role of DA in aggression goes back nearly 40 years, where destroying dopaminergic structures in the brain by the neurotoxin 6-OHDA reduced aggression (Reis and Fuxe, 1968). Postmortem measurements in aggressive mice and rats revealed increased level of DA (reviewed in Miczek and Fish, 2006). These early studies were corroborated later by *in-vivo* micro-dialysis measurements that showed increases in extracellular levels of DA in aggressive resident rats, but also increased in the submissive intruder males (van Erp and Miczek, 2000). Anticipation of a fight, which is achieved by 10 consecutive days of entrainment by introducing intruders at the same time, also led to a significant increase of DA (Ferrari *et al.*, 2003).

The role of the dopaminergic system in aggression is difficult to decipher, because it is involved in many other behaviours such as exploring novel environments, feeding, maternal or sexual behaviour (reviewed in Miczek and Fish, 2006). Because many of these behaviours are associated with pleasure, the DA system is often referred to as the 'rewarding pathway'. This rewarding aspect of aggression was demonstrated by a recent study (Couppis and Kennedy, 2008) in which the resident-intruder paradigm was modified so the resident mouse learned to press a trigger to get further access to the intruder which was bitten earlier. Resident males were apparently actively seeking further aggressive encounters to gain the rewarding sensation, and this behaviour was significantly reduced in males injected with the DA receptor (D1 and D2) antagonists SCH-23390 and sulpiride. Importantly, the treatment did not alter other functions like open-field behaviour (Couppis and Kennedy, 2008).

1.4.1.3 Biogenic amines and aggression in invertebrates

A number of studies have indicated the importance of biogenic amines in invertebrate aggression. In both crayfish and lobsters for example, injection of serotonin into subordinate males (Yeh *et al.,* 1996; Huber *et al.,* 1997b; Kravitz, 2000) results in a renewed willingness of these animals to engage in further agonistic encounters. A study in crabs compared the level of the neurohormone serotonin, octopamine and dopamine in animals before and after fighting (Sneddon *et al.,* 2000) Winners had higher concentrations of octopamine, dopamine, and serotonin than losers, and this difference reflected the difference in basal levels before the fights. The results suggested that the levels of the resting values of these amines could therefore be used as a predictor for which crab would be the more aggressive and win the fight. Similarly, studies in the Tarantula spider (*Aphonopelma hentzi*) by Punzo and Punzo (2001) showed that the level of octopamine and serotonin were significantly higher in the dominant compared to the subordinate spiders (but not dopamine, noradrenaline and adrenaline), although these levels decreased significantly 30 min after fighting in both opponents.

The role of biogenic amines in aggression has also been tested in insects. In crickets serotonin depletion had no influence on aggression or the renewed willingness of the loser to fight, while depletion of octopamine and dopamine (using specific pharmacological inhibitors) led to reduction in the duration and frequency of aggressive encounters (Stevenson *et al.,* 2000) Interestingly, testing the levels of these modulators during escape behaviour indicated that this trait is enhanced by serotonin depletion, but depressed by dopamine or octopamine depletion (similar to crustaceans).

1.4.1.4 Other molecular modulators of aggression.

The increased aggression in human males compared with females, suggests a link between the male hormone testosterone and aggression (reviewed by Birger *et al.,* 2003). Dabbs and Morris (1990) found high testosterone levels in a sample of 4,462 men, who were associated with anti-social behaviour, substance abuse and aggression. Similarly, a study comparing testosterone in male prisoners found a significant difference in levels of testosterone between inmates jailed for violent crimes and those whose crime did not involve aggression (e.g. drug abuse). The effect of testosterone on

aggression starts early in life: a study monitored preschool children found a significant correlation between aggression and testosterone levels in boys (Sanchez-Martin *et al.*, 2000).

Another important molecule implicated in aggression is nitric oxide (NO), which is an endogenous signalling molecule involved in various physiological functions. NO is a gaseous neurotransmitter which is generated through receptor activation and conversion of L-arginine to L-cirtrulline. NO is important in the conversion of GTP to cGMP, which is a key second messenger involved in numerous physiological and behavioural responses (Chiavegatto *et al.*, 2006). Mice lacking the gene nNOS (encoding NO synthase) showed a significant increase in aggression in the resident-intruder tests (Nelson *et al.*, 1995). Consistently, wild-type mice that were treated with a nNOS blocker (7-NI) showed increased aggression, which importantly, was not accompanied with changes in other locomotor activities, implying a specific effect on aggression (Demas *et al.*, 1997). It is possible, that the effect of NO on aggression is mediated through interaction with serotonin (5-HT). In nNOS knockout mice, an increase in 5-HT metabolism was observed, and this could have been the driver of increased aggression in these animals (Chiavegatto *et al.*, 2001). 5-HT agonists that bind to serotonin receptors decrease aggression in wild-type mice, but the effect is much more dramatic in nNOS knockout mice, again, indicating requirement of NOS in the serotonergic system (Chiavegatto *et al.*, 2001).

Vasopressin (VP) is a hormone that has been also implicated in aggression. Injecting hamsters with a VP blocker resulted in inhibition of aggression (Ferris and Potegal, 1988) prolonging the latency for resident male to attack an intruder and decreasing the number of bites. In contrast, injecting VP into the same brain region (anterior hypothalamus) leads to the opposite effects (Ferris, 1996). Increased aggression was also observed following injection of VP into other brain regions such as the ventrolateral hypothalamus hamsters, or the amygdala or lateral septum in rats (reviewed by Ferris, 2006). High levels of VP in the cerebrospinal fluid were associated with aggression in both rats and humans (Coccaro *et al.*, 1998) The role of VP on aggression was also demonstrated by knocking-out the vasopressin 1b receptor (V1bR),

leading to reduced aggression in the knockout mouse (Wersinger *et al.*, 2002). Ferris *et al.* (1997) suggested that VP interacts with the serotonergic system, each with opposite effects on aggression. The co-existence of both neurotransmitter systems in the anterior hypothalamus (a region particularly important for aggression) was observed by using double-staining immunofluorescence, and functionally tested by injecting VP into Prozac treated hamsters. Prozac (fluoxetine) inhibits reuptake of 5-HT, which then accumulates in the synapses, inhibiting aggression. Indeed, the data showed that the aggression-enhancing effect of VP was counteracted by 5-HT accumulation (induced by Prozac) indicating an interaction between the two systems (Ferris *et al.*, 1997).

1.5 Genes associated with aggression in *Drosophila*

Drosophila has been intensively used as a model organism to study the genetic basis of behaviours (Sokolowski, 2001). Some early observations of aggressive behaviour in Drosophila, which will be described in detail in Chapter 3, have led to more intensive research to reveal the genes involved in fly aggression (most of these studies have been published after the launch of this project, in 2003, and will be described here and in Chapter 4).

Although mutagenesis screens are commonly used in Drosophila, to date no such screen has been attempted to isolate aggression genes. Instead, several candidate genes, for which null mutants were already isolated, have been tested for their role in aggression. The first two loci that were associated with aggression were *ebony* (e) and *black* (*b*), in which levels of β -alanine (and dopamine) are respectively increased and decreased. (Jacobs, 1978). The increased aggression of the *ebony* mutants compared to *black* was later reproduced (Baier *et al.*, 2002). In this study (Baier *et al.*, 2002), a significant reduction in aggression in octopamine null mutants was also observed (the mutant lacks a functional tyramine β-hydroxylase enzyme that converts tyramine to octopamine). This finding, which has been recently reproduced (Hoyer *et al.*, 2008) is consistent with findings in crickets (Stevenson *et al.*, 2000, see above) where depletion of octopamine reduced aggression, but contrasts with observations in crustaceans where increased levels of octopamine lead to low aggression (Huber *et al.*, 1997a).

High aggression levels were also observed in *fruitless* (*fru*) *mutants*, in which males are engaged in head-to-head interactions, which were interpreted as aggressive (Lee and Hall, 2000). *fru* encodes a transcription factor which is spliced in a sex-specific manner, and plays a critical role in the sex-specific development of the nervous system, and consequently in adult courtship behaviour (reviewed in Douglas and Levine, 2006). Because both fly sexes exhibit aggression, which includes sex specific elements (Nilsen *et al.*, 2004), the role of the sex-specific isoform FRU^M was tested (Vrontou *et al.*, 2006). The flies that were used in that study were previously engineered to express constitutively the fru^M and fru^F alleles irrespective of the fly sex (Demir and Dickson, 2005). Indeed, inappropriate *fru* sex-specific expression reversed the sex-specific elements of aggression. In addition, the dominance hierarchy which is normally established in males but not in females (Nilsen *et al.*, 2004) was abolished in fru^F males (showing the female pattern). However, fru^M females also failed to produce dominance relationship, apparently because this transgene caused females to court rather than to fight each other (Vrontou *et al.*, 2006).

Serotonin (5-HT) is a major modulator of aggression in a broad range of species (see above). Dierick and Greenspan (2007) have studied the role of 5-HT in fly aggression by a series of pharmacological and genetic experiments. While no change in transcript levels in 5-HT-related genes was observed in microarray data or qPCR experiments, overexpressing tryptophan hydroxylase (*dTrh*) that converts typtophan into 5-HTP using the GAL4-UAS system (see Chapter 2 for overview of the method) resulted in an increase in 5HT and significant elevation of aggression (Dierick and Greenspan, 2007). Silencing the serotonergic neurons by driving the expression of the light chain of tetanus toxin induced low level of aggression and renders the flies irresponsive to administration of 5-HT (in contrast to control flies that show elevated aggression).

The same study also found that the *Drosophila* neuropeptide Y (NPY) homologue (known as NPF in *Drosophila*) is involved in repression of aggression similar to its mammalian counterpart (Karl *et al.*, 2004). Silencing *npf* neurons using the GAL4-UAS system as above was associated with increased aggression. However,

in contrast to the mammalian system where the effect of NPY is mediated by the serotonergic system, in *Drosophila* the two systems appear to independently modulate aggression (Dierick and Greenspan, 2007).

A large number of candidate genes have been associated with aggression in several microarray studies that aimed to profile the global changes in expression related to aggression (Dierick and Greenspan, 2006; Edwards *et al.*, 2006; Wang *et al.*, 2008). These studies will be reviewed in detail in Chapter 4.

1.6. The rationale and aims of the current study

Drosophila is a powerful model organism for genetic analysis. *Drosophila* resources, which have been developed over the years, including an enormous mutant collection, transgenic strains, deficiencies and other chromosomal aberration strains, the GAL4-UAS binary system, and the fly genomes, all make the fly the most compelling model system for behavioural analyses. *Drosophila* has been used in genetic analysis of a broad range of areas, including human health. As much as 77% of known human disease genes are conserved in the fly genome (Reiter *et al.*, 2001), providing an opportunity for understanding the function of these genes. Perhaps one of the best examples of the utility of using *Drosophila* has been in the field of circadian biology. The *Drosophila period* (*per*) gene was the very first circadian clock gene to be identified in 1971 (Konopka and Benzer), and 26 years later the mammalian orthologue was cloned in the mouse (Tei *et al.*, 1997), where it serves a similar function. A mutation in one of the human *per* paralogues (*hPer2*, probably the fly orthologue), in the same region as the original fly per^{s} mutation, leads to a sleep disorder and a faster running circadian clock (Toh *et al.*, 2001), just as it does in the fly.

The rationale for the current study is built on early observations of aggression in *Drosophila* (this will be further reviewed in Chapter 3) and is aimed to bring the powerful *Drosophila* genetic `toolbox` into the study of aggression. The completion of the fly genome sequencing project (Adams *et al.*, 2000) and the emergence of other genomic *Drosophila* resources paved the way for studying behaviour at the genomic level. The goal of the current study was to analyse the fly transcriptome using commercial microarray chips (Affymetrix) to identify genes that show differential

expression induced by, or associated with, aggressive behavior [Note: Throughout this thesis, I use the term *aggressive flies* as a shorthand for a pair of flies that show high score in the paradigm described in chapter 3].

This approach provided an opportunity to examine the regulation of aggression at the systems level, discovering functions and pathways (gene ontologies) that are associated with this behaviour, as well as identifying regulatory elements targeted by aggression-specific transcription factors. Several genes, which have been identified as differentially expressed, were further studied by more traditional molecular approaches using mutant and transgenic strains that allowed the manipulation of expression and testing for its functional effect on aggression. Since the launch of this project (2003), several other studies have taken a similar, but not identical approach (Dierick and Greenspan, 2006; Edwards *et al.*, 2006; Wang *et al.*, 2008). The methodological difference between these studies, and their results provided additional insight into the molecular basis of aggression and will be discussed in Chapter 4.

Chapter 3 explores the behaviour, particularly the temporal structure of aggression, and sets the stage for the following genetic and molecular experiments. The fundamental part of this study, the microarray experiments, is described in Chapter 4. This set of experiments provided a genome-wide perspective on aggression as well as generating a list of differentially expressed genes. The gene *dopamine N-acetyltransferase* (*Dat*), which was shown to be upregulated in aggression, was selected for further investigation. An alternative splicing event associated with the expression of this gene was studied using the GAL4-UAS system and is described in Chapter 5. Two other genes that were shown to be down-regulated in the microarray experiments in aggressive flies, *CG6480* and *Slh* were studied by RNA interference and the results are described in Chapter 6.

Chapter 2

MATERIALS AND METHODS

This chapter describes general procedures and reagents. Additional information will be provided in the Materials and Methods sections in each of the following Results chapters.

2.1 Fly Stock maintenance

Fly stocks were reared on sugar/agar medium (4.63 g of sucrose, 4.63 g of live yeast, 0.71 g of agar and 0.2 g of nipagen dissolved in 100 ml of ethanol/ 1 L of water) in glass vials (10 cm x 2.2 cm). The flies were kept at 18 ºC or 25 ºC in temperature-controlled rooms or incubators, and subjected to a 12:12-hour light/dark cycle. The strains of flies that were used are listed in the methods section of each chapter.

2.2 Plasmid DNA isolation

Small-scale plasmid preps (~20 µg) were made using the Qiagen DNA Spin Miniprep Kit. Larger scale plasmid preps (100-500 µg) were made using the Qiagen DNA Maxiprep kit.

2.3 Bacterial Strains and Transformation

The bacterial strains used for cloning were sub-cloning Efficiency DH5α chemically competent *E. coli* from Invitrogen. Plasmid DNA was mixed with the DH5α *E. coli* chemically competent cells and then left on ice for 20 minutes. The cells were then heat-shocked by placing them in a 42 ºC water bath for 45 s, then immediately transferring them back onto ice for 2 min. The cells were then transferred to a tube containing 950 µl of Luria-Bertani (LUB) broth (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) and shaken for 1 h at 37 ºC. After incubation, 200 µl were plated on LUB agar plates containing the appropriate antibiotic. In addition, the remaining 800 µl of cultures were centrifuged at 5000 rpm for 1 min, most of the supernatant was removed and the cells were re-suspended in a remaining supernatant (around 200 µl) and plated onto a second

plate. The plates were incubated overnight at 37 ºC. Single transformant colonies were isolated and grown overnight in 2.5 ml of LB and antibiotics at 37 ºC. These cultures were then used for isolating plasmids for further work.

2.4 Fly DNA isolation

Genomic DNA was isolated from single flies (Gloor *et al.*, 1993). Each fly sample was placed in a 1.5 ml Eppendorf tube and frozen at -20 ºC. To each tube containing a fly were added 50 µl of SB (squishing buffer: 10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl) and 200 µg/ml proteinase K. Each fly was then homogenised thoroughly using a clean pestle and ground until the solid material appeared well homogenised. The result was incubated at 37 ºC for 30 min, followed by inactivation of the proteinase K by heating to 95 ºC for 1-2 min. The samples were then stored at -20 ºC.

2.5 Ligation

All ligations were performed using the enzyme T4 DNA ligase from New England BioLabs, following the conditions recommended by the supplier.

2.6 Dephosphorylation of linearised plasmids

Dephosphorylation was performed when cloning a specific insert into a linearised plasmid, in order to increase ligation efficiency by preventing plasmid recirculation. The shrimp Alkaline phosphatase (SAP) enzyme was used. The dephosphorylation reactions were conducted by following the instructions accompanying the SAP enzyme supplied by New England BioLabs.

2.7 Restriction enzyme digestion of DNA

In order to digest DNA with a restriction endonuclease, the required amount of DNA was incubated with the enzyme (or enzymes) of choice with the appropriate buffer. The reaction was then incubated for a maximum of 2 h, usually at 37 ºC or at the optimum temperature for the enzyme. A small sample of the products was then run out on an agarose gel to check DNA restriction.

2.8 Recovery of DNA from an agarose gel

For isolation of a specific fragment from an agarose gel after it had been separated by electrophoresis, the QIAquick gel extraction kit was used, following the protocol described in the manual supplied with the kit.

2.9 PCR

All PCR reactions were performed on a MJR DNA Engine Dyad (MJ Research). The PCR reactions were set up as required for the experiment and according to the primers' annealing temperature. Primers were designed using the Primer3 software (Rozen and Skaletsky, 2000) using the default parameters. The primer sequences, and the specific PCR programs are describes in the following chapters. Primer oligonucleotides were synthesised by Invitrogen.

2.10 Sequencing

DNA sequencing reactions were performed by the PNACL DNA sequencing service at the University of Leicester, and by Lark Technologies Inc (Essex).

2.11 Total RNA extraction

Unless specified otherwise, RNA was extracted from fly heads. In order to isolate the heads, the fly samples were kept in 15 ml sterile falcon tubes, frozen in liquid nitrogen and vortexed. Heads were separated from the bodies using double sieves over a dry ice tray. RNA was extracted by using the TRIzol reagent from Invitrogen Life Technologies following the manufacturers instructions.

2.12 DNase treatment

DNA-free™ from Ambion was used to remove genomic DNA from RNA samples. The samples were purified and DNase enzyme and buffer were removed after treatment, following the manufacturer's recommended procedure.

2.13 cDNA synthesis and RT PCR.

Reverse transcription from total RNA was performed by using the SuperScript II reverse transcriptase from Invitrogene. 1-1.5 µg of RNA was incubated with the following components in a nuclease–free tube; 1 µl Oligo(dT)12-18 (500 µg/ml) or 50-250 ng random primers or 2 picomole gene-specific primer (GSP), 1 µl dNTP mix (10 mM each), 12 µl DEPC treated water. This reaction mix was incubated at

65 °C for 5 min and then chilled on ice for 1 min, followed by a brief centrifugation for 30 s. To this RT mix was then added: 4 µl 5X First-Strand Buffer, 2 µl 0.1 M DTT, 1 µl RNaseOUT™ recombinant ribonuclease inhibitor (40 units/µl). The mixture was incubated at 42 °C for 2 min. If random primers were used, the mixture was incubated at 25 °C for 2 min (manufacturer's recommendation). Then 1 µl of SuperScript™ II RT (200 units) was added to the mixture and the reaction was incubated at 42 °C for 50 min, and followed by heat inactivation at 70 °C for 15 min. Finally, 1 µl (2 units) of *E. coli* RNase H was added to remove RNA complementary to the cDNA, and incubated at 37 °C for 20 min.

2.14 Inverse PCR

In order to find the exact location of the micro-injected transgene in the transgenic flies, a single-fly DNA isolation protocol was used (Gloor *et al.*, 1993; Engels *et al.*, 1990; Ochman *et al.*, 1988). Each fly sample was placed in a 1.5 ml Eppendorf tube and frozen at -20 ºC. To each tube containing a fly were added 50 µl of squishing buffer (SB) (10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl) and 200 µg/ml Proteinase K. Then each fly was homogenised thoroughly using a clean pestle and grinded until the solid material appeared well-homogenised. This was incubated at 37 ºC for 30 min, followed by inactivation of the Proteinase K by adding 1 µl 0.1 M PMSF to each tube and heating to 65 ºC for 10-15 min. 10 µl DNA was then digested with the restriction enzymes MspI and Sau3A, following the optimum conditions for the enzymes, to a total reaction volume of 25 µl. 5 µl digested DNA were added to 45 µl of ligation mix (1 µl T4 DNA ligase, New England BioLabs 20,000u 5 µl ligase buffer, 45 µl dH2O) and incubated at 4 ºC overnight. The inverse PCR cycling program was: 95 ºC for 5 min, 95 ºC for 30 s, 60 ºC for 1 min, 72 ºC for 4 min for 35 cycles and last step of 72 ºC for 10 min.

2.15 Transformation of *D. melanogaster*

DNA preparation

Flies from the w^{1118} strain carry a deletion of the white gene (Hazelrigg *et al.*, 1984) were used for the transformation. The transgene was constructed using the pUAST plasmid as a vector. This vector contains the mini-white gene as a selectable marker, conferring red eye colour in transformant flies. In addition a helper

plasmid, the plasmid pUCksΠΔ2-3 (Mullins *et al.*, 1989), was used. This helper plasmid contains a source of transposase that is needed for the initial integration of the transgene into the fly's genome. The `transgene plasmid` (the gene of interest cloned into the pUAST vector) and helper plasmid were ETOH precipitated and then re-suspended in 1x injection buffer solution (1:1 mM Na_2HPO_4, 5 mM KCl solution 2 : 0 0.1 mM NaH_2PO_4, 5 mM KCl, gradually adding solution 1 to 2 until a pH of 6.8 is reached, filtered, sterilised and stored at -20 ºC). The `transgene plasmid` and the helper plasmid were mixed at a ratio of 400 ng/µ and 200 ng/µ respectively and injected into dechorionated fly eggs.

Egg collection and dechorionation

Flies approximately five days old were kept in large open-ended plastic tubes on top of a small Petri dish containing egg-laying medium (sugar/agar medium). To ensure injection prior to the time of pole cell formation, eggs were collected every 30 min. Dechorionation of the eggs was carried by using a mounted needle, moving the eggs over a piece of double-sided adhesive tape. Each batch of eggs was dechorionated within a 10- to 15-mininterval. The tape was attached to a glass slide, and the dechorionated embryos were moved to the edge of the tape with the posterior end (where the pole cells develop) protruded over the edge, ensuring easy access for injection. The injected embryos (with the slide) were then kept in a desiccator containing silica gel crystals for 10 minutes, then covered with mineral oil (Voltalef grade 10s) and transferred to an inverted microscope at x100 magnification for injection.

Embryos injection

Pulling of the microinjection needles was carried by a flaming Brown needle puller set to the following programme: 1) H 830 P 30 V 50 T 120. 2) H 960 P100 V 220 T 55. The needles were cut at the tip to allow the injection DNA mixer to pass through, and then back-loaded by passing the needle through a Bunsen flame. The injection was carried out directly into the posterior tip of the embryos before the development of the pole cells. After injecting all the embryos, the strip of adhesive tape holding the embryos was removed from the glass slide and placed inside a small glass jar quarter-filled with sugar nutrient, which was sealed with cotton wool. Once emerged, the male G0 flies were crossed to five virgin w^{1118} females

and the female G0 flies were crossed to three young males. The crosses were placed into sugar nutrient vials and searched for red-eye 'transformant' G1 flies. The transformants were crossed to the double balancer stock w^{1118}; *CyO/Sco; MKRS/TM6B* to determine on which chromosome the transgene had inserted (for more details please see methods Chapter 5)

2.16 Microarrays

RNA from flies classified according to their aggression level was extracted. The RNA samples were then used for hybridisation with commercial microarrays (Affymetrix) that represent the whole fly genome. The microarrays were run by the UK Drosophila Affymetrix Array Facility in Glasgow, using the Drosophila Affymetrix expression genechip V.1. (for more details please see Chapter 4).

2.17 The UAS-GAL4 system.

The bipartite UAS-GAL4 expression system is widely used in *Drosophila* for the overexpression of transgenes (Brand and Perrimon, 1993) and utilises the yeast transcription factor *GAL4* and its target sequence *UAS,* to activate gene transcription (Laughon and Gesteland, 1984). *GAL4* is placed under the control of a *Drosophila* promoter and by introducing a second transgene in which *YFG* (*your favourite gene*) is fused downstream of UAS, any gene can be overexpressed/misexpressed in a pattern dictated by the corresponding promoter (Fig 2.1).

The plasmid pUAST

The vector pUAST consists of five GAL4 binding sites (UAS), followed by the *hsp70 TATA* box and transcriptional start, a polylinker with unique restriction sites for EcoRI, BglII, NotI, XhoI, KpnI and XbaI, the SV40 small t-intron and a polyadenylation site. The pUAST vector contains in addition the *white* gene and an ampicillin-resistant fragment. In the current study the transgenic flies were generated by insertion of constructs made by subcloning the gene of interest into the pUAST vector.

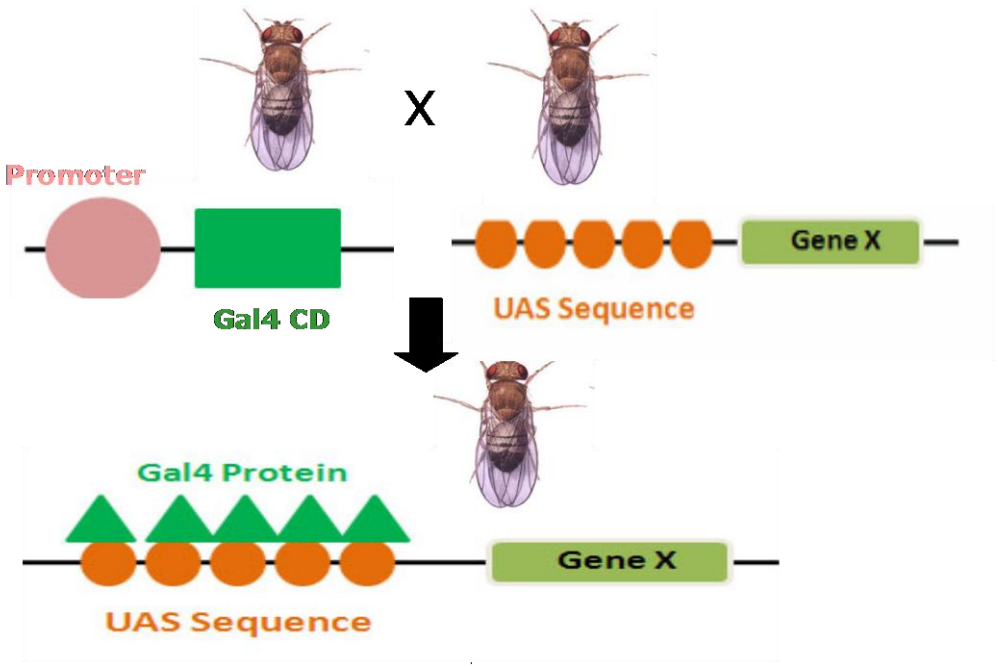


Fig 2.1: The UAS-GAL4 system. In order to activate transcription, the flies carrying the *UAS- gene 'X'* (right) are crossed to flies with a promoter::Gal4 fusion (left) that will express GAL4 in particular tissues; resulting progeny will over/mis-express gene 'X'. Modified from *http://www.hoxfulmonsters.com*.

2.18 Statistical analysis

The aggression data were not normally distributed, thus non-parametric statistics were used. For comparison of two groups, the Wilcoxon Rank Sum test (identical to Mann–Whitney test) was used. When more than two groups (e.g. genotypes) were compared, the Kruskal-Wallis ANOVA was first applied and then followed by a multiple comparison test after Kruskal-Wallis (Siegel and Castellan 1988, pp. 213-214). A similar approach was used in other aggression studies (e.g. Dierick and Greenspan, 2007). The tests were carried out using the R statistical software (R Development Core Team, 2007). Additional statistical tests and details are described in the various Results chapters.

Chapter 3

AGGRESSIVE BEHAVIOUR IN *DROSOPHILA*

3.1 Introduction

It has been known for some time that aggressive interactions occur between males in several *Drosophila* species and that they mainly involve the defence of a territory (Spieth, 1968). For example, males of *D.pseudoobscura*, *D. immigrans* and *D. melanogaster* use rotting fruit as breeding sites, and defend areas used by females for oviposition (Partridge *et al.*, 1987).

Male territorial aggression in *D. melanogaster* was first observed by Jacobs (1960) who noted that in laboratory cages, males defended patches of food against other males by charging and tussling with them. However the earliest study directly addressing aggressive behaviour in *D. melanogaster* was carried out by Dow and von Schilcher (1975) who observed six fly males in a cage defending a territory of a limited resource of food and successfully establishing a hierarchy that was significantly correlated with their mating success. Dow and von Schilcher also described various aggressive behaviours: "wing threat" that consisted of wings spread, raised and twisted (Fig.3.1), and "boxing" which included intensive slashing and tapping with the front legs, occasionally while both males stand on their hind-legs (Dow and von Schilcher, 1975).

Fig. 3.1. Aggressive actions in Drosophila. Left: lunging, middle: tussling, right: wing threat. Images from Dankert *et al.* 2009.

Evolutionary aspects of territoriality in *D. melanogaster*

The evolutionary aspects of aggression in *D. melanogaster*, particularly in relation to territoriality, have been addressed by Hoffmann in a series of studies (reviewed in Hoffmann, 1994). These studies, carried out under laboratory conditions, showed that territorial males (residents) were more successful in more common non-escalating fights, while intruders were successful in displacing the residents in infrequent escalating fights. In the escalated encounters, heavier males tended to be the winners, although weight differences were not a consequence of territory ownership. These studies (Hoffmann and Cacoyianni 1990) also showed that territorial behaviour is a conditional strategy that depends on various factors. Males ceased to be territorial when (i) territories (food cups) were larger (for a fixed male density), (ii) density of males was increased, and (iii) females were not present (or were not attracted to the territory, because of low quality of the food). This was consistent with optimality reasoning, in which males were expected to be territorial as long as the benefits of mating advantage prevailed over the costs involving defending the territory. This conditional strategy may be the evolutionary mechanism that maintains genetic variation in territorial behaviour, and also led to the notion that the tendency to be territorial (and aggressive) is a quantitative (continuous) trait (Hoffmann, 1994). The extent of genetic variation in territoriality was addressed in artificial selection experiments (Hoffmann, 1988). After 20 generations of selection, a substantial divergence was observed in the selected lines indicating that genetic variation for the male territoriality was ubiquitous in natural populations and that this behaviour can evolve rapidly in populations. Interestingly, change in body size was not associated with the variation in territoriality in these experiments.

Importantly, there is also a substantial non-genetic contribution to the phenotypic variation in territorial behaviour (Hoffmann, 1990) For example, in cages with males of various age, most territories were rarely occupied by one-day old males and they rarely were observed trying to displace older resident males. Also, isolated males show an increased tendency to defend territories compared with males that were grown in the presence of other males (Hoffmann and Cacoyianni, 1990)

The structure of aggressive behaviour in *Drosophila*

Nearly 30 years had passed since the study of Dow and von Schilcher (1975) before a detailed study of the fine structure of the behaviour was attempted (Chen *et al.*, 2002). The rationale behind this kind of study was to generate a fine description of the behavioural elements (i.e. the motor-output) that can be used to identify the underlying neural and molecular circuits. Chen *et al.* generated an ethogram which included nine offensive elements (approach, low-level fencing, wing threat, high level fencing, chasing, lunging, holding, boxing and tussling) and four defensive actions (walk away, defensive wing threat, run away/being chased, fly away); for a detailed definition of these action see Chen *et al.* (2002) Table 1. These behavioural elements were recorded in 73 fights and a transition matrix describing the occurrences of each action following each of the rest of the elements was constructed. The matrix allowed the generation of a model describing the probability of moving from one action to another during the fight (Fig.3.2). Some of the common actions (and their transitions) such as approaching and chasing are also exhibited during courtship behaviour, but the wing-threat and higher-intensity elements such as tussling make the distinction between aggression and mating obvious. Recently, a software algorithm was developed, that allows the automatic detection of these elements by the computer, enabling an objective and high-throughput analysis of fly interactions (Dankert *et al.*, 2009). The system has permitted the verification of the basic aggressive pattern (Fig.3.2) and the difference between agonistic and mating behaviour.

Following the report that *Drosophila* females also show aggressive behaviour (Ueda and Kidokoro, 2002), a detailed analysis of their aggression pattern was carried out (Nilsen *et al.*, 2004). This study revealed that some of the aggressive actions (e.g. wing threat) are common to males and females, but some elements are sex-specific. For example, boxing and tussling are unique to males, whereas head butt and thrust with a wing threat characterize female aggression. Another difference between the genders was the lack of any dominance hierarchy in female fights.

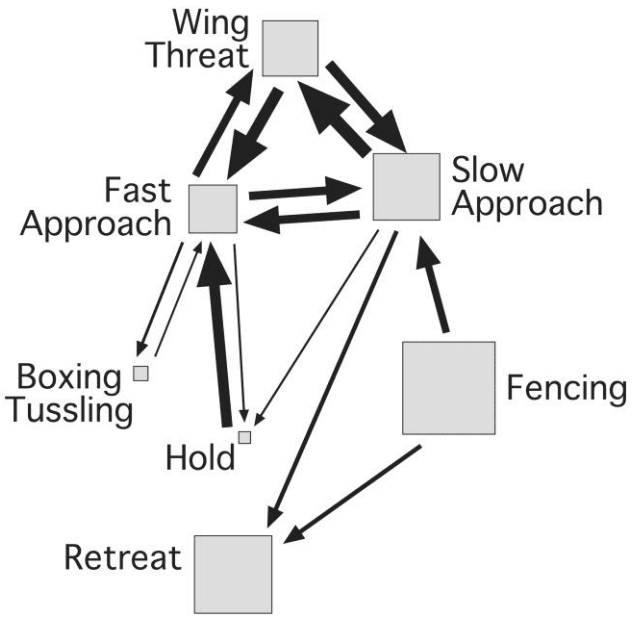


Fig. 3.2. Graphical description of structure of aggressive behaviour in *D. melanogaster.* This ethogram depicts the probabilities from a transition matrix of 9031 actions during 2074 interaction (73 pairs). The frequency of each action is represented by the size of the gray square, and the frequency of each transition is given by the size of the arrows. From Chen *et al.* 2002.

Unlike males, in which dominance can be clearly established (for example, the number of wins of a dominant male continuously increases), in an ongoing encounter between two females, there can be many reversals in the wining pattern (Nilsen *et al.*, 2004). These fine gender differences have been used to identify the role of sex-specific isoforms of the gene *fruitless* (Vrontou *et al.*, 2006, described below).

The dynamics of aggression, gradually moving from low- to high intensity aggression (Fig. 3.2) is consistent with theoretical predictions, allowing the flies to gain more information about their opponent before embarking on a full scale confrontation (Maynard-Smith and Price, 1973 See Chapter 1). One may expect that this assessment process is linked with learning and memory circuits, a link which was recently tested (Yurkovic *et al.*, 2006). The results of that study showed that male fights can be as long as 5 hr, but the frequency of interactions drops quickly with time. Males show experience-dependent changes in their fighting pattern: the frequency of lunges for example, gradually increased in winners, but declined in losers. Importantly, when males were separated for 30 min after a fight, and then placed again with a new male, their behaviour against the new opponent reflected their previous status: winners tended to lunge, losers tended to retreat. Additional experiments even suggested that males

recognized individuals that they fought previously and adjust their behaviour accordingly; the number of interactions was significantly smaller when males were paired with a familiar opponent compared to pairing with unfamiliar males. Because male are frequently tapping their opponents (fencing), it was suggested that individual recognition is mediated by cuticular hydrocarbon profiles that may vary among flies (Yurkovic *et al.*, 2006).

The current study was aimed at isolating fly males that exhibit either high or low aggression for further molecular characterisation. This chapter describes the behavioural screen that has been used to assess aggression in male flies and uncovers several properties of the behaviour, particularly the time structure, that were not previously addressed.

3.2 Materials and Methods

Fly strains

Preliminary experiments with a wild-type Canton-S strain showed a low level of aggression and frequent inter-male courtship behaviour, which confounded the analysis. Therefore, to increase the aggression component of behaviour during encounters, the *ebony* (e^{11}) mutant which is known to be aggressive was used (Jacobs, 1978; Kyriacou *et al.*, 1978). The *ebony* flies have elevated levels of the enzyme beta-alanyl-dopamine synthase. Since the homozygote mutant is visually impaired, heterozygotes flies were used. To reduce genetic variation, attached X virgin females (XˆXY) were crossed to e^{11} homozygous males to produce F1 males, which carried the paternal X chromosome[1]. These males were isolated on ice soon after eclosion. Flies were maintained on sugar/agar food (4.63g sucrose, 4.63g dried brewers yeast, 0.71g agar and 0.2g Nipagin in 100ml of H20) in 1/3 pint milk bottles or glass vials (10cm x 22cm). Fly stocks were maintained at either 18°C or 25°C in temperature-controlled rooms, in 12:12 light: dark cycle.

[1] This cross produces four genotypes: XˆX,X (lethal), YY (lethal), XˆXY (female) and XY (maternal Y, paternal X).

The behavioural paradigm

Male flies were collected on the day of eclosion and individually isolated in a separate vial containing sugar/agar nutrient, and were transferred to fresh food every other day. The flies were kept at 24ºC and subjected to a 12:12LD cycle. The behavioural experiments were carried out after four days of isolation. On the third day of isolation during late afternoon, the flies were transferred to glass tubes (diameter 0.7 cm, length 10 cm) with agarose to keep tubes moist. On the fourth day, between 10:10-12:00 AM (the light phase started at 10:00 AM), flies were introduced to the observation chamber (Fig. 3.3) by inserting the glass tubes into the chamber (3.7 cm in diameter and 4 cm deep). The chambers were moulded from transparent Perspex and fitted in a small Petri dish. A food cup (2 cm in diameter) containing standard medium and a drop of fresh baker's yeast, was placed at the centre of the chamber. The experiments were performed at 24 ºC under standard room illumination. A pair of male flies was introduced simultaneously to each chamber and allowed to acclimatise for 5 min. Six chambers were observed in each session that lasted 30 min, each chamber being observed for 10 s each minute (timed by a digital metronome). This time sampling recording faithfully captured the relative aggression levels compared with continuous monitoring (see Results).

The level of aggression was scored as follows: each aggressive interaction that involved body contact (kicking, pushing, boxing), was counted as one score. The maximum score in a single 10 s observation interval was 3, which was also used in cases when the flies fought continuously through the whole interval (i.e. tussling). At the end of the experiment each pair of flies was immediately frozen on dry ice and then stored at -80 ºC.

Data analysis

The aggression scores were not normally distributed (Shapiro-Wilk normality test, $W = 0.9754$, $p < 0.0001$), and therefore non-parametric statistics were used (Siegel and Castellan 1988), using R (R Development Core Team, 2007).

To analyse the distribution of latencies that preceded aggression, survival analysis was carried out that tests the pattern of waiting times to the occurrence of an event (Budaev, 1997). Briefly, survival analysis generates curve-fits for distribution of waiting times, such as survival time of patients. The analysis was carried using the *survival* library of the R package. The curves were generated using the non-parametric Kaplan-Meier estimator, and the difference between various survival curves was tested using the log-rank test.

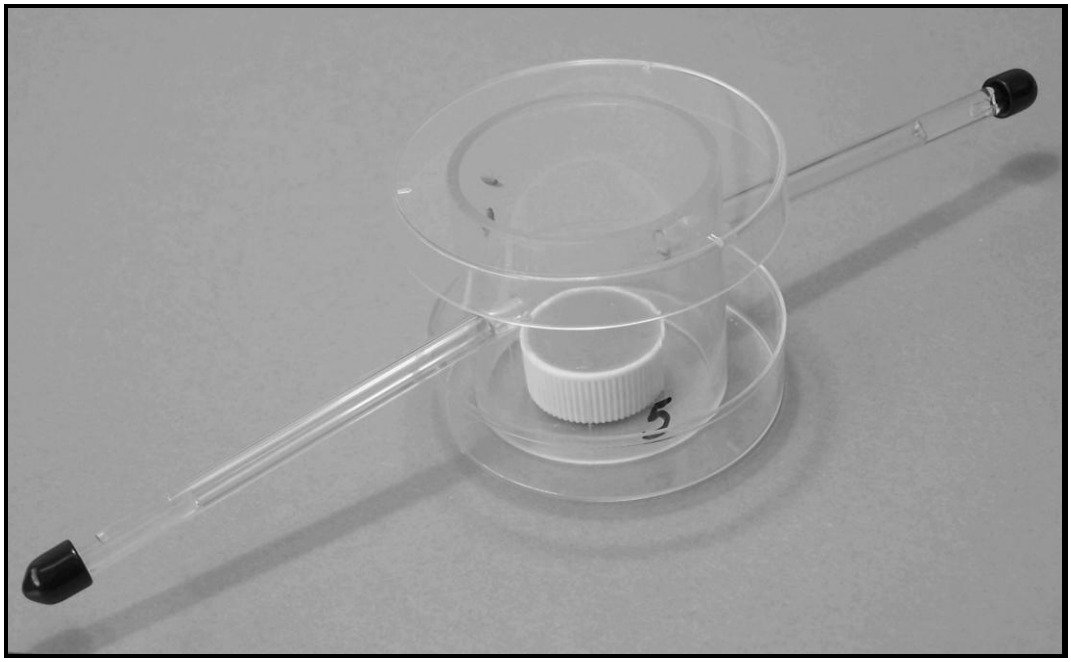


Fig 3.3: The observation chamber. Prior to the experiment, individual males are kept in glass tubes, which are then inserted to the chamber via holes in the cylinder. A small food plate is placed at the centre. The flies are then free to explore the chamber (see attached DVD for film of aggressive encounters).

3.3 Results

To test the validity of the time-sampling recording, 12 pairs of males were observed continuously, in separate observation sessions, and their aggression was recorded. The aggression of the 12 fights was ranked based on the total aggression. Next, each minute of observation was divided into six 10 s bins (1-10 s, 11-20 s, etc.)

and the aggression score in each bin was tabulated. These data were used to generate simulated aggression scores that would have been recorded in by time-sampling observation: for each pair of males, a bin was randomly sampled, and the 12 pairs were ranked again based on the score of the sampled bins. In a given simulation for example, the score of pair 1 was estimated by using the bin 11-20 s, the score of pair 2 estimated by bin 51-60 s, etc. This sampling was repeated 100 times, to generate 100 simulated rankings. The close resemblance among the simulated ranks, and between the ranks of simulated and the original data indicated that loss of information by the time-sampling recording is not substantial, and that this method can reliably capture the relative aggression of different pair of males (Fig. 3.4).

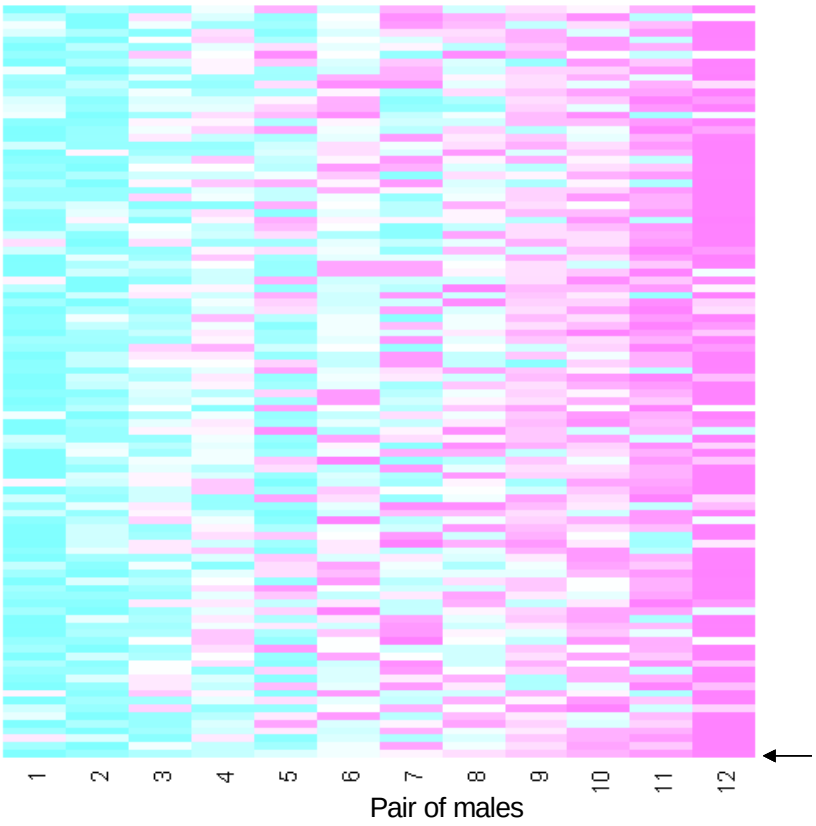


Fig.3.4 Comparison of time-sampling vs. continuous observation of aggression. This heatmap depicts the ranks of simulated data sampled from continuous observation of 12 fights (bottom line, arrow). Each row represents a random sampling of 10 sec bins from the continuous data (n=100). Ranks are coded by colours (purple: high aggression, light blue: low aggression). The 12 fights were sorted based on ranks of the continuous data, with most aggressive flies shown to the right (See text for more details).

During the first 10 days of adulthood, the level of aggression significantly correlated with age (Kendall's rank correlation $\tau = 0.38$, $z = 5.799$, $p = 6.676e\text{-}09$; Spearman's rank correlation $\rho = 0.52$, $S = 159746.2$, $p = 4.06e\text{-}10$; N=126), increasing from a median score of 2 on the first day to median of 12 on the 6th day (Fig. 3.5).

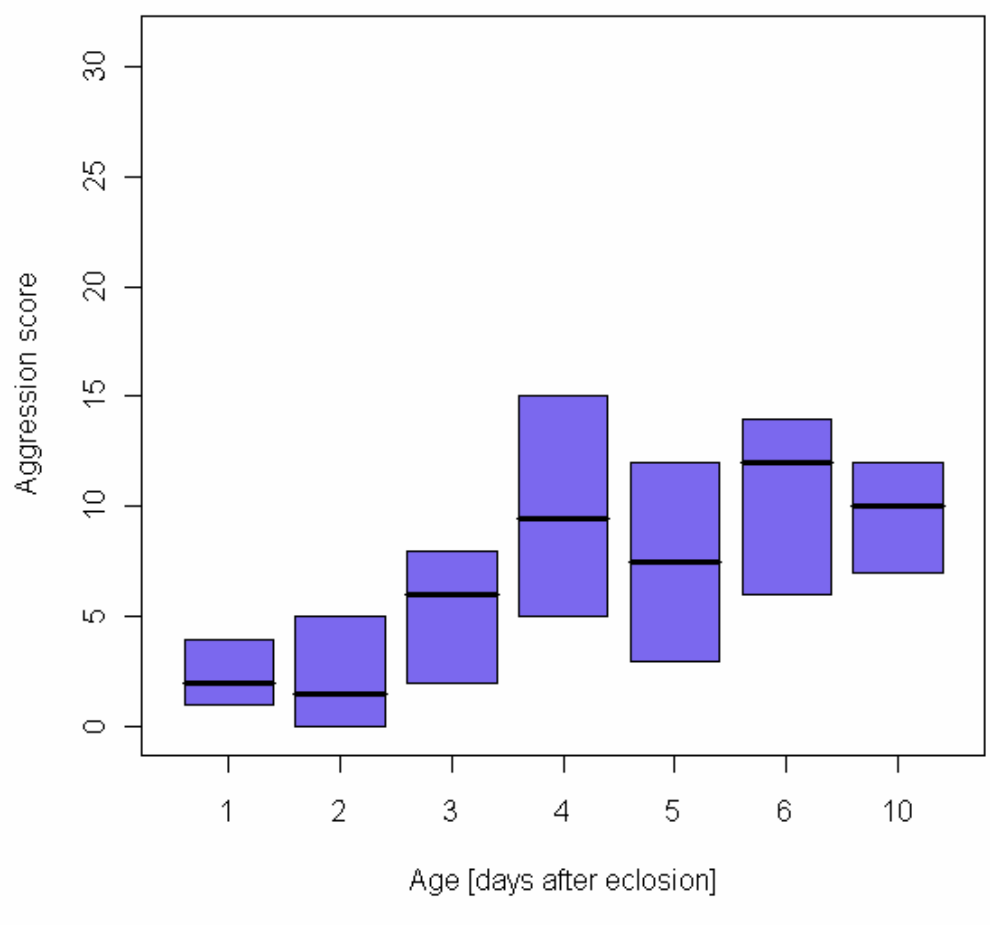


Fig.3.5 Change of aggression with age. A box plot showing the median level of aggression (line within box) and the 25 and 75 percentiles (bottom and top of box). In each age group, 18 pairs of males were tested.

When flies were introduced to the observation chamber they usually spent the first few minutes exploring the chamber. After the initial exploration they would then come to the food cup, where most interactions occurred. The flies demonstrated all types of aggressive interaction previously reported in the literature (see Introduction), including kicking, pushing, boxing, wing threat and tussling. Often, one of the males would leave the food cup and then later come back, trying to attack the male on the food (see attached DVD).

Fig. 3.6 presents the distribution of the aggression levels in 910 fights. The distribution is right-skewed. The median of aggressive scores was 12 and the first and the third quartiles were 7 and 18 respectively. Although these experiments were not aimed at monitoring the specific behavioural elements in the fights, it was evident that highly aggressive flies also showed extreme aggressive elements (e.g. tussling), reflecting the escalation of the fight. It is also noteworthy that flies exhibiting low levels of aggression (or none) were not necessarily non-active; these flies typically spend more time exploring the chamber instead of trying to monopolize the food cup. Fig. 3.7 depicts the time structure of the behaviour in nine different fights (aggression core: 18). Although no clear structure is evident, a fine analysis of various temporal parameters described below indicated consistent changes that were correlated with the intensity of aggression.

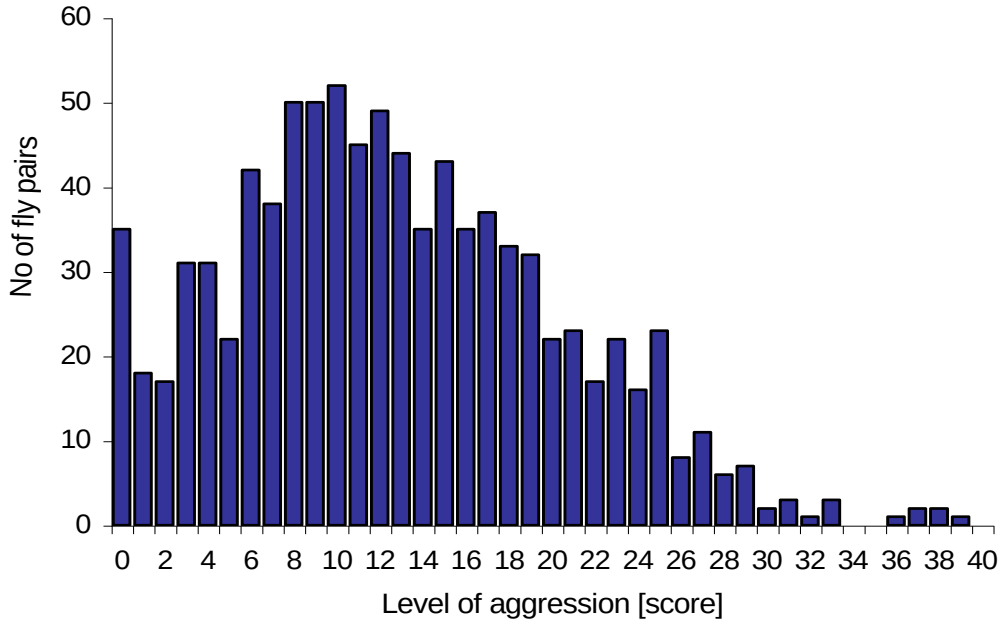


FIG.3. 6. Distribution of aggression levels. The aggression of 910 fighting pairs of males was scored (see text), and 240 flies (120 pairs) from each of the right and the left tails of the distribution were classified as aggressive and non-aggressive respectively. RNA extracted from heads of these flies was used for the microarray profiling (chapter 4).

The interval from the start of the observation to the first aggressive interaction ('latency to attack') was analysed using survival analysis (Fig. 3.8). Latencies in fights with different levels of aggression (7, 12, 18 and 25, each based on 30 fights) were compared using the log-rank test and showed a significant difference (χ^2 = 16.1, df=3, p = 0.001). In general, an intense level of aggression was associated with shorter latencies to attack, and this is most obvious when comparing intense (score 25) and low-aggression (score 7); in intense fights, 90% of the pairs were engaged in fighting after 1 min (Fig. 3.8 green curve), while in weak fights (score 7, black curve), the same proportion of pairs were engaged in fighting only after 10 min. The pairs with intermediate level of aggression (score 12 and 18) showed intermediate latencies (and showed overlapping survivor curves).

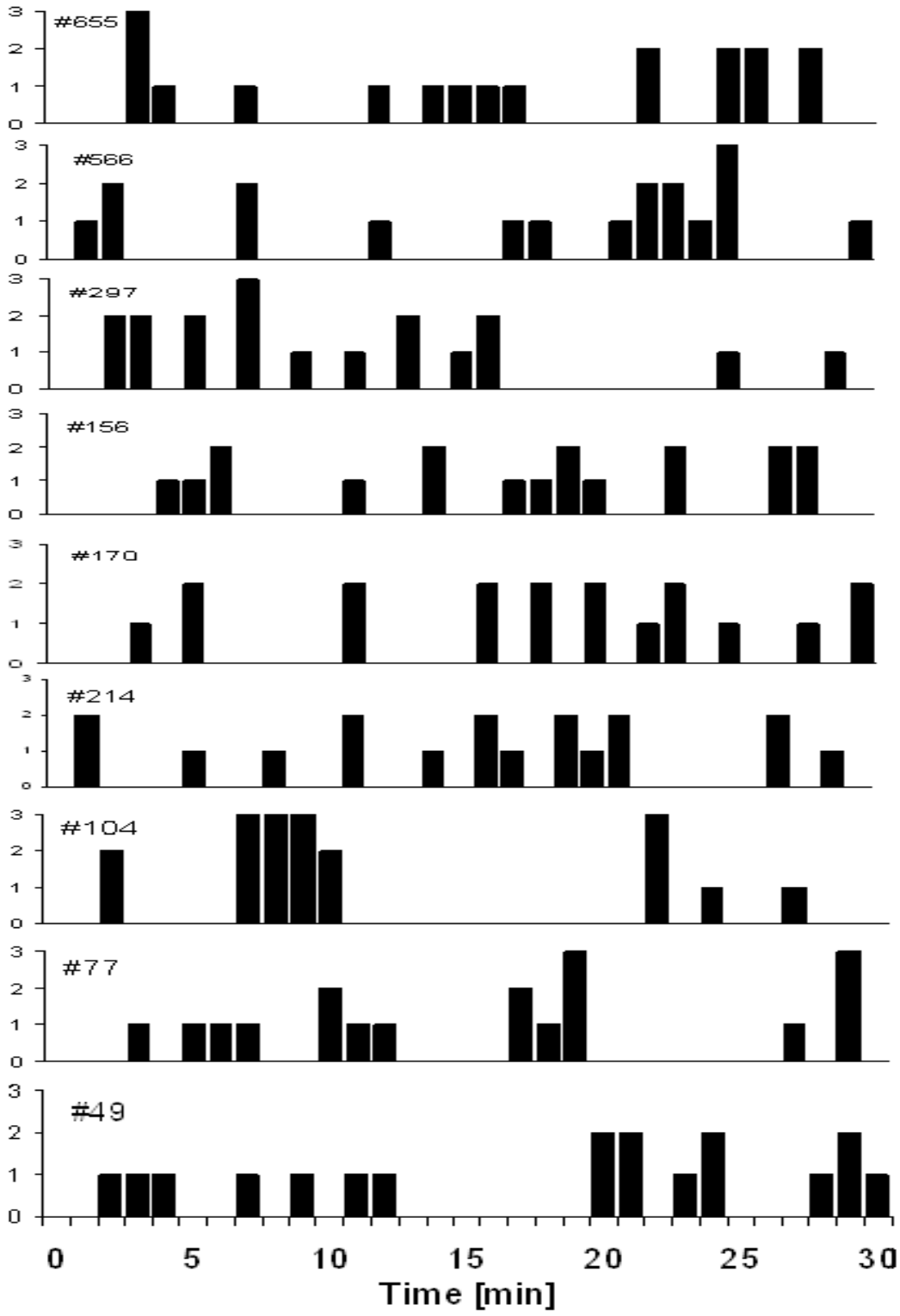


Fig. 3.7. Bouts of aggressive interactions during 30 min observations. Nine examples are shown (the pair identity number is also given). The Y-axis represents aggression score (all fights had a score of 18). Flies are observed 10s per minute (see text).

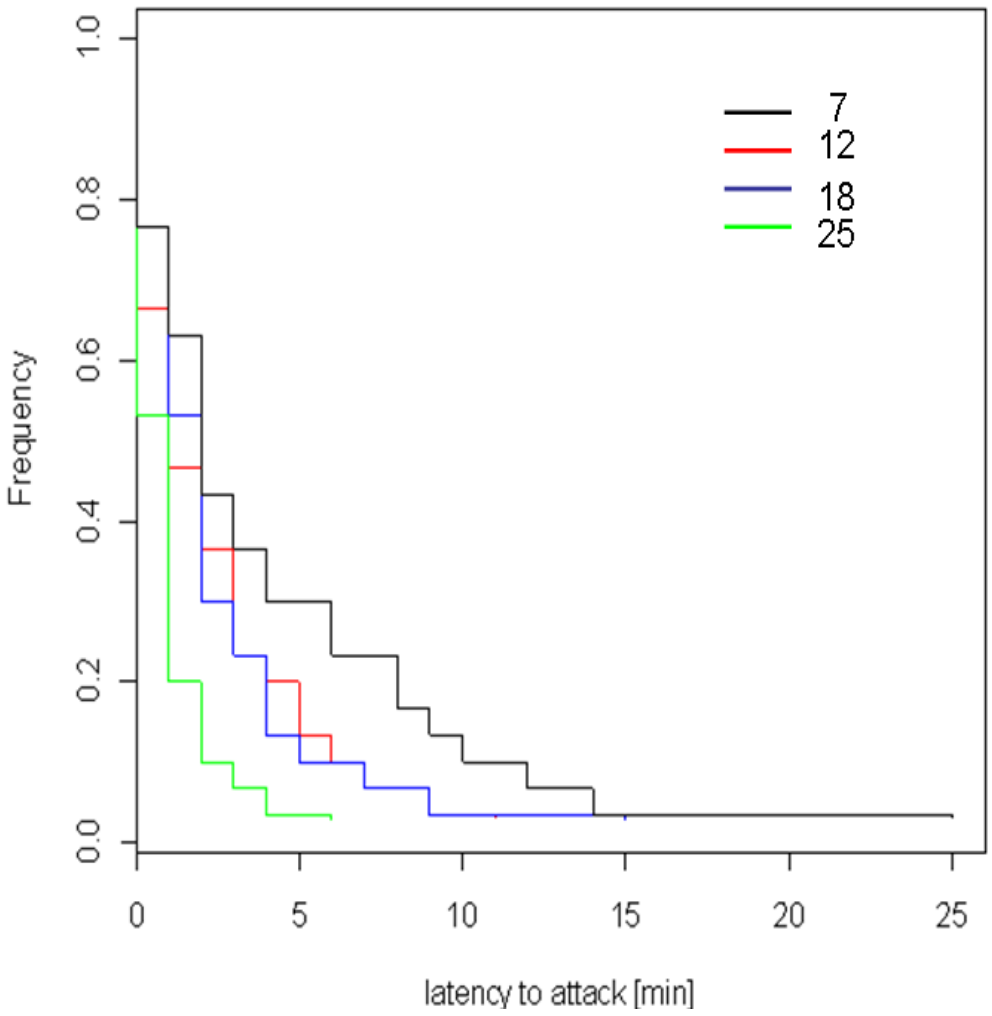
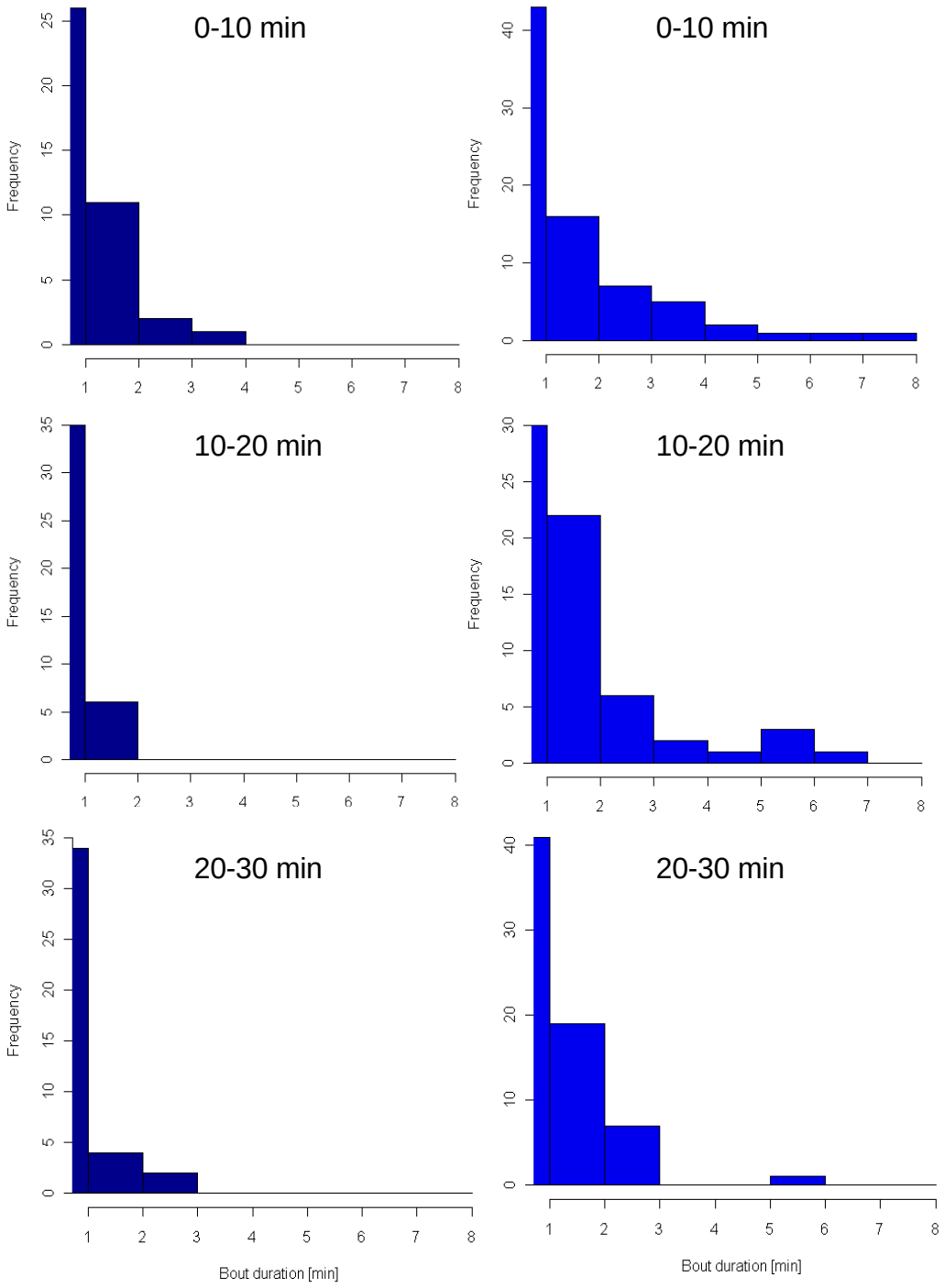


Fig. 3.8. Survival analysis of latencies to attack. Kaplan-Meier curves showing the frequency of male pairs prior to fighting at time t. Latencies were analysed in fights with different aggression level: Low aggression (score 7, black); intermediate (score 12, red), high (18, blue) and intense (25, green). Each group consists of 30 fights. A log-rank test indicated a significant difference between the curves (χ^2 = 16.1, df=3, p = 0.001).

The bout duration of aggressive interactions was estimated by counting successive observation bins (1min) where aggression was present (although observation was not continuous, and each pair was 'visited' for only 10 sec per min). Fig. 3.9 shows the distribution of these 'clusters' of interactions during the three sections of 30 min observation (i.e. 0-10, 10-20, 20-30 min), in low aggression (score 7) and intense fights (score 25). In the low-aggression fights, a Kruskal-Wallis test revealed a significant difference between the distributions during 30 min (χ^2=6.57, df = 2, p = 0.037), which was due to slightly (but significant) longer bout durations during the first 10 min, compared with the following 10 min (Wilcoxon rank-sum test, W = 996, p-value = 0.027, note higher frequency of 2 min bouts in Fig. 3.9). In contrast, during intense fighting, bout duration tend to be longer as expected, and does not change significantly during the 30 min observation ($\chi^{2=}$3.26, df = 2, p = 0.19).


0-10 min
0-10 min
10-20 min
10-20 min
20-30 min
20-30 min
Frequency
Bout duration [min]

3.4 Discussion

This chapter describes the behavioural screen aimed at identifying *Drosophila* males that exhibit either strong or weak aggressive behaviour. Unlike other behaviours, aggression, and social phenotypes in general, are 'difficult' phenotypes to measure (Jones, 2007 p.31). In fact, aggression *per se* is not a phenotype, but a set of behaviours, which can serve as a measurable phenotype. The difficulty however might be that as a social phenotype, it depends on other individuals that are present, and their behaviour. Nevertheless, this study, as well as other recent studies on aggression in *Drosophila* (Dierick and Greenspan, 2006; Edwards *et al.*, 2006; e.g. Wang *et al.*, 2008), suggests that reliable phenotyping of aggression is feasible, and opens the way for high-throughput screening for molecular correlates of aggression.

The dynamics of aggression is predicted by theory (Maynard Smith, 1982). In dominance contests, absolute aggression appears to be a poor predictor of the outcome, compared with the behaviour that individual exhibits relative to its opponent. As the asymmetry between the opponents (e.g. size) increases, contests become shorter and are less aggressive. In contrast, symmetry between individuals leads to escalation of the contests and longer duration of the contests. The sensory cues that fly males use to gauge their opponents (e.g. visual, olfactory) are as yet unknown and in the current study males were randomly paired. It is rather likely that most cases involved asymmetrical contests and therefore resulted in low-or intermediate aggression, which was manifested in the right-skewed distribution of the observed levels of aggression (Fig. 3.6). This may also explain the distribution of aggressive bout duration (Fig. 3.9): In low-aggression encounters, which may represent asymmetric pairing, dominance is established quickly and aggression decays after 10 min, with the subordinate male leaving the food patch. In high-aggression encounters (score 25), bout duration is obviously longer without a significant change during the 30 min observation. It also should be noted that various stochastic factors may contribute to the asymmetry of the fight: for example, the male who first arrive at the food (by chance), may establish ‘ownership’ that affects the starting conditions of the fight. The extent to which these stochastic factors are important is difficult to estimate, and to control.

While bout duration is likely to depend on the real-time information that the opponents pickup up from each other, the latency to first attack may more closely represent the propensity to fight. This parameter has been extensively used in aggression studies in mice (e.g. Wersinger *et al.*, 2002; Ferris *et al.*, 1997), but has never been monitored in flies. In mice, a popular paradigm is the resident-intruder design where a 'tester' mouse is introduced into the cage of mouse (resident) whose aggression is to be measured. Under these conditions, the resident mouse invariably will be the first to attack the intruder, and latency to attack can be used as proxy to measure aggression in a single mouse. The situation in the current study was slightly different: here latency was inversely correlated with escalation of the fights (Fig.3.8), which may imply that this parameter is dependent on both opponents. This finding is interesting because if the propensity of one fly to attack is very high, a short latency to attack will establish an asymmetric match, and under these conditions the fight usually is not expected to escalate (see above). Perhaps, the correlation between short latencies and fight escalation is caused by individuals that are less efficient in gathering information about their opponents (e.g. reduced sensitivity to sensory cues from other conspecifics). Then, symmetry ceases to be an important factor and fights are likely to escalate.

Practically, the fact that escalating fights are relatively rare is a challenge for molecular research that is aiming to compare gene expression associated with aggression (e.g. Chapter 4), since longer and more elaborate behavioural screens are required in order to isolate individuals that exhibit high aggression. The finding that latency to attack is a reliable predictor of aggression in flies is very useful, as this may significantly shorten future screens. Instead of observing long intervals of interaction between males, one can envisage designs where only latency to attack is measured perhaps automatically as in (Dankert *et al.*, 2009), significantly shortening the phenotyping process. In this study it appears for example, that 10 min interval might have been sufficient to detect 90% of the latencies, thus distinguishing between low intensity and fierce fights (Fig. 3.8).

Chapter 4

GLOBAL ANALYSIS OF GENE EXPRESSION INDUCED BY AGGRESSION IN DROSOPHILA

4.1 Introduction

Some complex phenotypes, such as certain diseases or behaviours depend on the interactions between multiple genes, rather than changes in a single causal gene. Traditionally, the role of genes involved in a given complex trait was studied one at a time, using approaches like Northern or Western blots, which make the identification of the underlining network a slow process (Primrose and Twyman, 2003). The development of microarray technology during the mid 90's paved the way for *global profiling* where the expression of all genes in the genome is measured simultaneously in a single experiment. This new approach has accelerated the process of identifying new gene interactions, allowed a holistic view of the molecular mechanism, and initiated the study of a trait at the genome level.

Although techniques for monitoring global *protein* expression have been developed (e.g. 2D PAGE, protein microarray), most studies have focused on measuring RNA expression, with the underlining assumption that transcript level closely reflects protein abundance. This might not be always the case, because of post-transcriptional and post-translational regulation. Yet, DNA microarrays (for transcript profiling) currently have the potential to monitor a higher numbers of genes, and the technique is practically easier and cheaper than proteomic analysis, making it a popular method for global profiling.

Besides gene expression, DNA microarrays have also successfully been used in other applications, including genotyping, sequencing, DNA copy number analysis, and DNA-protein interactions (Hoheisel, 2006). Due to its high flexibility, microarray

technology becomes an indispensable tool not just for gene expression analysis but also for applications involving protein and cell analysis.

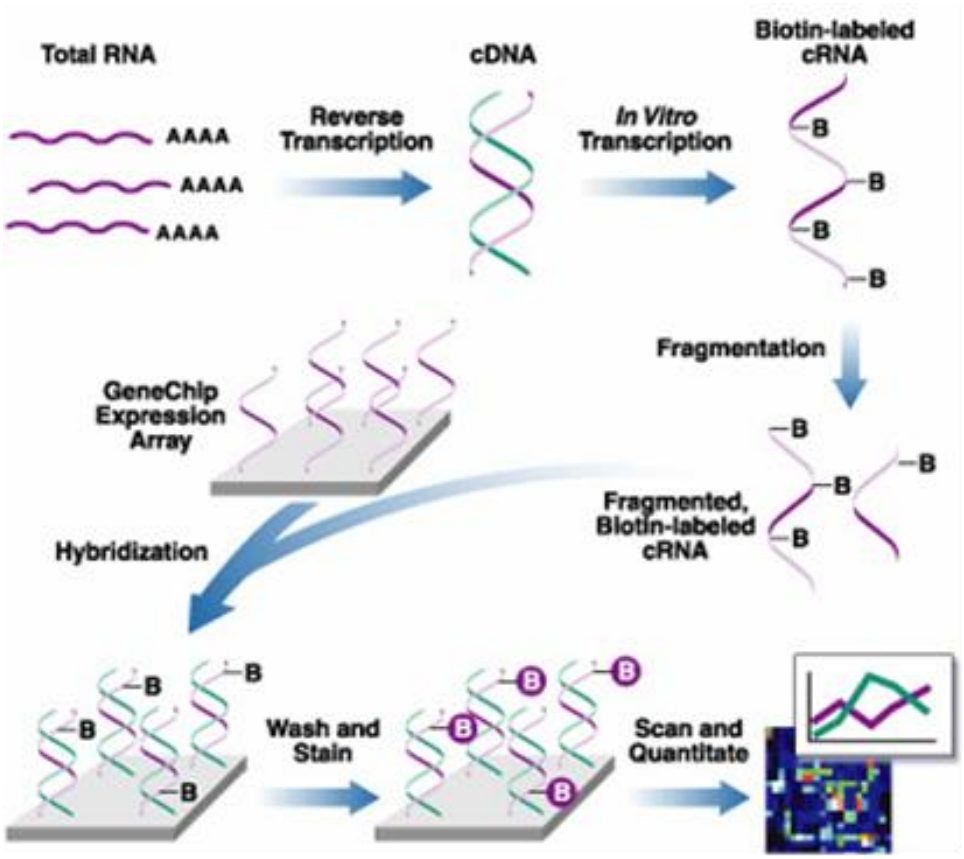


Fig. 4.1 Schematic overview of microarray experiment. Labeled cDNA or cRNA targets derived from the mRNA of an experimental sample are hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each DNA location, it is possible to infer the abundance of each mRNA species represented. From http://www.dkfz.de/gpcf/24.html.

DNA microarrays: the technology

A DNA microarray consists of thousands of microscopic spots of DNA attached to a solid surface. Each spot contains millions of copies of a specific DNA sequence. The DNA is attached to a solid surface (glass slide, silicon chip) by a

covalent bond to a chemical matrix (Fig. 4.1) or microscopic beads (Illumina) instead of the large solid support. The spotted DNAs are used as probes to hybridize to a fluorescently labelled DNA sample, the "target". Probe-target hybridization is usually detected and quantified by a laser fluorescent scanner. The intensity of the fluorescent spot corresponds to whether the gene was transcribed and to what degree. Computer algorithms are then used to analyze the thousands of genes expressed in the sample (Knudsen, 2004).

The two main formats of DNA microarrays are cDNA microarrays and oligonucleotide microarrays. cDNA microarrays are produced by isolating the mRNA from the sample, that is then reverse transcribed into cDNA. Each spot represents a single gene and consists of cDNA isolated from the organism (typically a few hundreds bp). In contrast, in oligonucleotide microarrays, DNA sequences are directly synthesised onto the slide and each gene is represented by a set of short sequences (25-30 bp).

While cDNA microarrays (typically 'home-made' by individual laboratories) have the advantage of being suitable for profiling of non-model organisms for which no sequence data are available, commercial oligonucleotide microarrays gradually became the method of choice with the completion of genome sequencing of various organisms such as yeast, fly, mouse and human. These microarrays usually perform better because the probes can be carefully designed and selected to allow uniform and stringent hybridisation parameters and to minimize cross-hybridisation between genes.

Microarray applications

The first reported use of microarrays for gene expression profiling was in 1995 when a differential expression measurements of 45 *Arabidopsis thaliana* genes were made, representing a small fraction of the total number (25,498) of the *Arabidopsis* genome (Schena *et al.*, 1995). The first whole genome microarray analysis was carry out a few years later on the complete genome sequence of the yeast *Saccharomyces cerevisiae* (Lashkari *et al.*, 1997), showing that under different environmental conditions (e.g. heat shock), large number of genes were differentially

expressed. Since then the number of microarray studies has exponentiated, and they have been used to investigate an enormous number of phenotypes. For example, a microarray study on human acute leukemia generated a test that can classify cancer, allowing a distinction between myeloid leukemia and lymphoblastic leukemia, which require a different type of treatment (Golub *et al.*, 1999). Studies in mice, led to new insights into gene expression in embryonic development, analysis of brain regions, and during apoptosis (Ko *et al.*, 2000; Pan *et al.*, 1999; Zirlinger *et al.*, 2001).

Microarrays have also been used to study gene expression in invertebrates. For example, gene expression profiles in the brain of young and old honeybees (*Apis mellifera*) were compared, and an elevated expression of high numbers of genes involved in spatial learning and memory was observed during the age-related transition of adult honeybees from hive worker (nurses) to foragers (Whitfield *et al.*, 2003).

D. melanogaster has also been a pioneering model organism for using DNA microarrays, particularly in developmental studies. One notable example investigated the expression of 4028 genes in wild-type flies throughout development, including fertilization, embryonic, larval and pupal periods, and adulthood (Arbeitman *et al.*, 2002). Various clusters of co-regulated genes were associated with specific organs, or with particular biological processes. These results suggested a strong association between the modulation of transcriptional activity and morphogenesis and reveal how microarray data from appropriately designed experiments can shed light on tissue-specific gene regulatory hierarchies.

In the current study, I have used Affymetrix microarrays to study global gene expression associated with aggression in *Drosophila*. The comparison of the head transcriptome of flies that distinctly differ in their aggression level revealed 347 genes that show significant differential expression. These genes have been further analysed for enriched gene ontologies and shared regulatory elements. Towards the completion of this study, a few other expression studies of aggression in *Drosophila* were published (Edwards *et al.*, 2006; Dierick and Greenspan, 2006; Wang *et al.*,

2008). The results of these studies, which used different methodologies will be reviewed and compared in the Discussion.

4.2 Materials and Methods

Flies

The flies that were analysed in this study were described previously (Chapter 3). Briefly, the aggression of 910 pairs of males was observed and scored using the paradigm described in Chapter 3. The tails of the distribution of the aggression level (Fig 3.6) was used to identify pairs of flies that were extremely aggressive or non-aggressive. Flies were placed in dry ice following the 30 min observation, and then kept in -80ºC. Heads from 240 aggressive flies were harvested, and the same number of heads was collected from flies showing the lowest level of aggression. Each group was divided into three separate samples, based on the aggression score of the flies, in a way that would generate three independent replicates (80 fly heads in a sample, 6 samples in total Fig. 4.2).

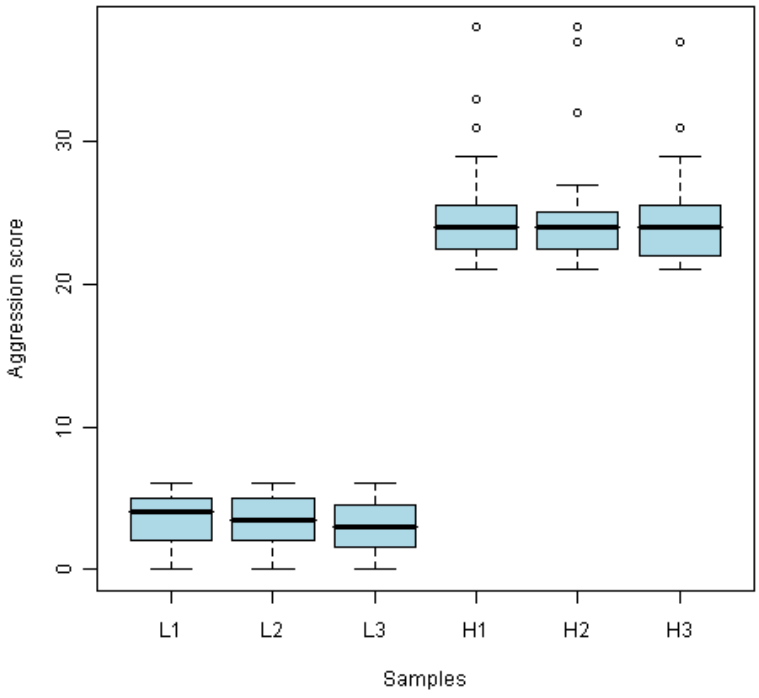


Fig4.2. Aggression scores of flies used in the microarray analysis. A box plot showing the distribution of aggression level in each of the six samples analyzed in this study.

RNA preparation and hybridization

From each group of flies, 10-20 ug total RNA was extracted and was used for hybridisation to three chips for each category (i.e. three replications of aggressive and three replications of less-aggressive pairs of flies). The chip hybridisation was carried following the manufacturer instructions by the UK Drosophila Affymetrix Array Facility at the University of Glasgow.

Affymetrix Microarrays

The Affymetrix Drosophila Genome Array 1.0 GeneChips were used. Each chip carries 14,010 probe sets, representing the complete fly's genome. The design of this microarray is based on the Perfect Match/Mismatch probe strategy (Fig.4.3). For each probe designed to be perfectly complementary to a target sequence (Perfect Match, PM), a partner probe is generated that is identical except for a single base mismatch in its centre (Mismatch probe, MM). These probe pairs, allows the quantification and subtraction of signals caused by non-specific cross-hybridisation. The difference in hybridization signals between the partners serves as indicators of specific target abundance. A t-test is used to compare the expression of all PM vs. MM probes in a set. Affymetrix uses a classification system where genes whose PM is significantly different from MM are flagged as present (P), while genes with $p > 0.05$ (indicating high noise, or cross hybridisation) are flagged absent (A); genes with $p = 0.05$ are considered marginal (M).

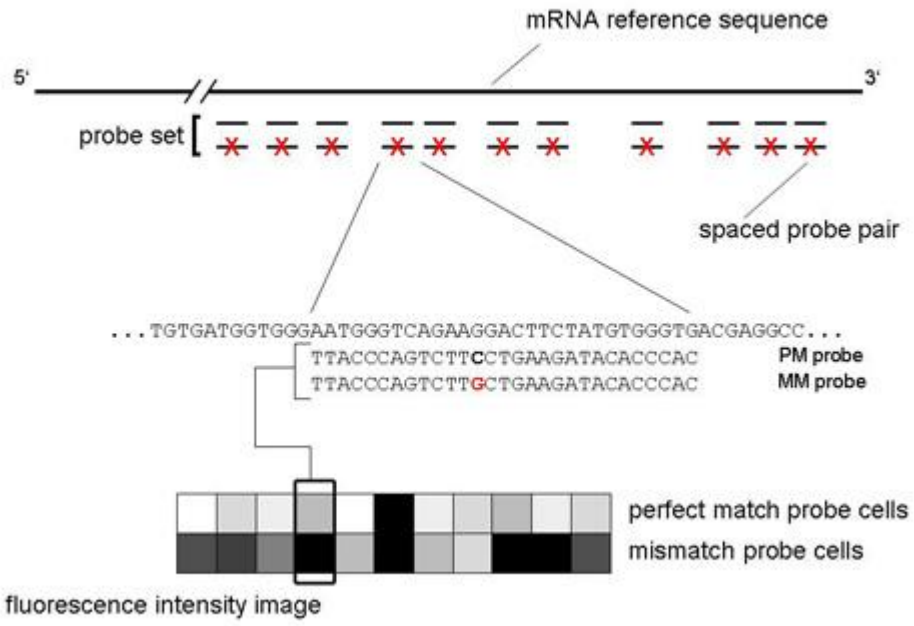


Fig. 4.3: The Affymetrix GeneChip design. Each gene is represented by 11-20 pairs of probes. Each pair composed of two probes, one which is a perfect match (PM) to the target mRNA, and another mismatch probe (MM), which differ by a single nucleotide; the chip carries 5,000-20,000 probe sets. Hybridization of fluorescent mRNA to these probes pairs on the chip is detected by laser scanning of the chip surface. (image modified from Lipshutz *et al.*, 1999))

Data analysis

Data pre-processing

The signal intensities of the microarray were processed and analysed using the GeneSpring v.6 software (Silicon Genetics). After data transformation (to convert any negative value to 0.001) and filtering out genes that were not flagged present (P) in all six chips, normalization was performed. To adjust for systematic non-biological variations in intensity between chips (for example, due to inconsistent washing, inconsistent sample preparation, or other microarray production) per-chip median normalization was carried:

$$\frac{\text{signal strength of gene A in chip X}}{\text{median signal of all genes taken in chip X}}$$

In addition, I carried out gene median normalization, (as recommended by the GeneSpring manual)

$$\frac{\text{signal strength of gene A in chip X}}{(\text{median of every measurement taken for gene A from all chips}}$$

These normalizations generated similar distributions for all chips and centred the distribution of signal intensity of each chip on the same value (Fig. 4.4).

The assumption behind this type of normalization is that most of the genes do not show significant change throughout the experiment due to true biological activity, which would cause the median of one chip to be higher than another. Indeed, behavioural changes are not expected to involve global transcriptomic changes (as would for example, induced by heat-shock).

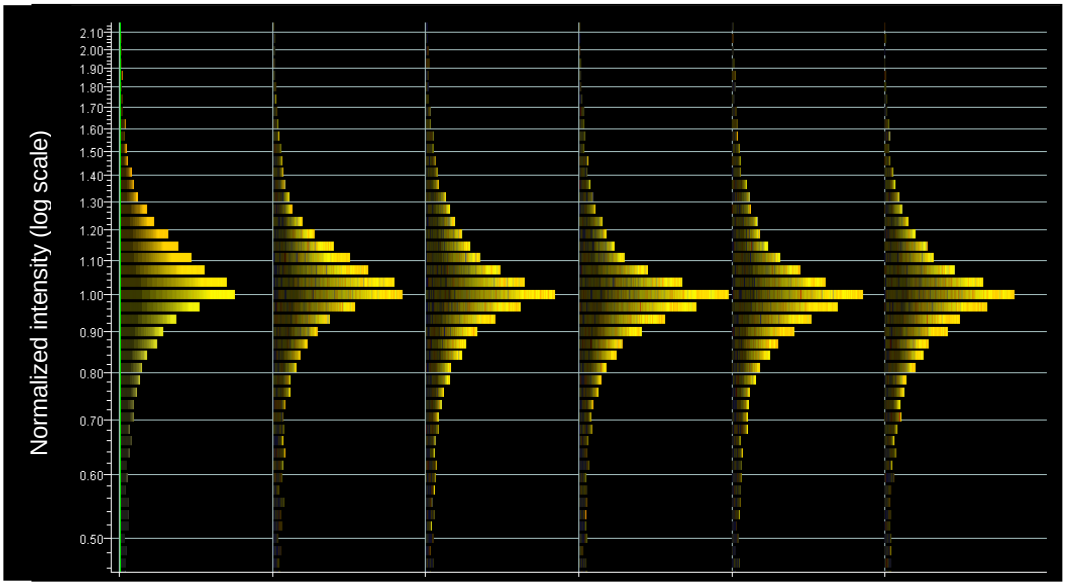


Fig.4.4. Distribution of signal intensities. The normalized intensities (log scale) are shown following a per-chip, per-gene median normalization.

Identifying differentially expressed genes

The signals of all probe pairs of a gene are summed to produce an average intensity for that gene (Average difference) using the following formula:

$$AvgDiff = \frac{\sum_N (PM - MM)}{N}$$

Where N is the number of probe pairs used for the calculation (probe pairs which deviate by more than 3 standard deviations from the mean were excluded). Note that genes with negative or very small AvgDiff will be flagged as Absent (see Introduction and Data pre-processing) and will be excluded from the analysis at the earlier stage.

Signal intensities were transformed to logarithms and Welch's approximate t-test for two groups (variance between groups is not equal) were used to compare the levels of each gene in the aggressive chips vs. the non-aggressive chips. To correct for multiple testing, the R package *locfdr* was used (Efron *et al.*, 2009). The package calculates the local false discovery ratio (FDR), which may be considered as the probability for a gene to be a false positive.

Gene ontologies

The gene ontology (GO) project is a collaborative effort to produce a unified standard annotation (Ashburner *et al.*, 2000; Blake and Harris, 2008), generating a vocabulary that classifies genes according to three classes: cellular component (e.g. nucleus), molecular function (e.g. transporter activity) and biological process (e.g. signal transduction). A given gene can be associated with GO from different classes and also can have multiple GO from the same class (e.g. a few cellular component). The organization of GO is semi-hierarchical, with specific GO (child) nested in a more general one (parent) for example, nuclear chromosome (GO:0000228) is nested in chromosome (GO:0005694); however, a `child` can have more than one `parent`.

The classification of the differentially expressed genes based on their Gene Ontology (GO) was carried using the EASE software (Hosack *et al.*, 2003) implemented in the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 (Dennis *et al.*, 2003). The EASE score (a modified Fisher- Exact test) was used to test the significance of the GO of each of the differentially expressed genes, by comparing the observed frequency against its representation in the entire genome.

For example, in the *Drosophila* genome (~14,000 genes) 158 genes are involved in the immune response. In a given gene list of 347 genes, 11 immune response genes are present. One is interested in testing if 11 out of 347 are more than chance compared to the Drosophila background of 158 out of 14,000.

Fisher Exact test may be used to compare the proportions (e.g. immune genes in the list vs. the whole genome), resulting in a p-value = 0.003, suggesting that this gene list is specifically enriched in immune genes than by chance. The EASE Score is calculated in the same way, but the number of genes used is adjusted[1] 11-1=10,

[1] This correction addresses the problem of categories with only one member, which otherwise, if present in the list, would appear to be over-represented.

resulting in a p-value (EASE Score) of 0.008. The EASE score is therefore more conservative.

Finding transcription factor binding sites

Genes whose transcription is induced by the same transcription factor are expected to show a similar expression profile. Conversely, identifying genes with the same expression profile may share a binding site that is targeted by the same transcription factor. Here, I used the GeneSpring software to identify common upstream motifs in those genes that show significant differential expression. Up to 1000 bp upstream of each gene were analysed for enrichment of motifs of 5-8 bp long. The analysis allowed for up to 3 point discrepancies (mismatches). It also allowed for up to 4 gaps (Ns) in the exact middle of the motif, allowing one to look for sequences such as *ACGnnnCGT*, which is biologically relevant due to loops and non-binding areas (for example, ***ACG***ac***CGT, ACG***ttgg***CGT, ACG***acta***CGT*** are all counted as the same motif).

The observed frequency of each sequence was compared with its frequency in the entire genome. The calculated probability was further corrected for the total number of tests that were carried out (this number varies depending on the length of the given oligomer and number of N's).

For example, the sequence *TATCGATA* was observed upstream in 42 out of the 300 upregulated genes. Only exact matches were counted. This was compared to the frequency (0.0272) over the length searched of that sequence in the entire fly genome. If the distribution of bases were random one would expect to see that sequence upstream of 0.0272 of the genes. The probability that this particular sequence is that common due to chance is 1.38e-17. However since 5,242,880 tests were done, the software reports the adjusted probability of 7.22e-11.

Validating differentially expressed genes by qPCR

A qPCR was used to validate the results of the microarray experiments, using the absolute method (Nolan *et al.*, 2006). This method is based on running serially diluted standards along with the samples. SYBR green, which is incorporated into the

dsDNA, was used to monitor the amplification of the PCR product. The reaction was run on a DNA Engine Opticon 1 system (MJ research).

The samples for the standard curve were produced by PCR. The product was run on the gel, purified, and quantified by UV-spectrophotometry (Eppendorf). Serial dilutions of the purified DNA were prepared and were stored in siliconised tubes (Sigma).

The standard curve was made by using six PCR-amplified standard samples (concentration of 300,000 to 30 copies, in 10 fold dilutions), run in triplicate. The threshold cycle (Ct) that marked the beginning of the exponential amplification phase was detected for each of the standards and used to produce the log-linear standard curve. The slope of the standard curve was used to calculate the efficiency of the PCR. Only reactions that show 90-100% efficiency were used. The standard curve was used to calculate the level of the target in the experimental samples. To control for variation in the amount of cDNA in the different samples, the expression of the target was divided by the amount of the house-keeping gene *RP49* that was also quantified in each sample (note: *RP49* was not found to be differentially expressed in the microarray experiments). Melting curves were also generated at the end of each reaction and were analysed to verify the specificity of the reaction (indicated as a single peak in the melting curve).

Primers were designed to amplify ~ 200 bp with approximately 60º C annealing temperature (Appendix 4), and were selected from flanking exon regions to prevent any bias by genomic DNA contamination. For each set of primers, the PCR reaction was optimised.

The same RNA samples that were used for the microarray experiments were used for the real-time PCR. The RNA was treated with DNAseI (Ambion) to ensure that there was no DNA contamination. cDNA synthesis was carried out using Superscript II (Invitrogen) and 2ug RNA. For the real-time PCR 100 ng cDNA were used as a template in each reaction. The Real-Time PCR reaction included: Mix Syber green 10X 0.5ul, dnTP 12.5Mm O.5ul, Mg Cl2 25Mm, Primers 10Mm 0.75ul ,

AmpliTaqGold 0.125ul, H2O 14.875ul, Gold buffer 2.5 ul. template 5ul. (Total reaction volume 25ul). A typical Real-Time PCR program was: 92°C for 5 min; 40 cycles of [92°C for 30s; 62°C for 30 s; 72°C for 8 s] 72°C, 2 min. Reactions were typically carried out in a 96-well plate, quantifying three genes (of which one was the house-keeping gene). Six standard dilutions run in triplicate were used together with six experimental samples (3 high, 3 low), each run in duplicate (total of 12 samples). The average level of the reference gene (*RP49*) of each of two technical replicates was used to normalise the level of the target genes of the corresponding cDNA sample, and these ratios were log-transformed. The statistical difference between the high and the low aggression samples was tested by ANOVA using the *nlme* library of the R statistical package (R Development Core Team, 2007).

4.3 Results

Identifying differentially expressed genes

Out of 5463 genes that were used for the analysis (i.e. genes that were flagged Present in all the chips) 347 showed a significant difference in their expression level (t-test $p < 0.05$), of which 45 were significant at level of $p < 0.01$. 306 of these genes were upregulated in the aggressive flies[2] (Fig. 4.5), and the rest (41) were downregulated. The fold change of all genes was small (<2). The top 30 differentially expressed genes are shown in Table 4.1. The full list is given in Appendix 1. To correct for multiple testing, the R package *locfdr* has been used (Efron *et al.*, 2009). The package computed the local false discovery rate (FDR) based on the distribution of the t-statistics. When fixing the FDR to 0.2 (the software's default value), the number of significant genes was 68 (this list included the genes *Dat*, *CG6480*, and *Slh* which were selected for further analysis and described in chapters 5 and 6).

The expression level of several genes has been tested also by qPCR. A significant difference was observed in the level of the gene *Slh* ($F_{1,5}=54.5$, $p < 0.001$). The expression of *Dat* was tested separately for each of the existing splice forms: The difference was significant in the case of *DatB* ($F_{1,5}=28.6$, $p = 0.0031$) but not for *DatA* ($F_{1,5}=0.21$ $p = 0.6636$). Three other genes that show a significant differentiation in the microarray, *CG6480*, *CG6762*, and *Syx13*, did not show a comparable difference in the qPCR assays. In addition, the gene *Dopamine 2-like receptor* (*Dop2R*) was also analysed, as this gene is involved in dopamine signalling, and showed a significant difference in the microarray experiment. However it was not included in the 5463 genes used for the final analysis because expression in one of the six chips was flagged marginal (M, see Methods). The qPCR showed that this gene was significantly upregulated in aggressive flies ($F_{1,5}= 7.4$ $p < 0.05$) when measured by qPCR.

[2] The reader is reminded that from hereafter gene expression output is the average of three samples, each averaged across a pool of 120 pairs of flies.

Table 4.1 List of top 30 differentially expressed genes. For each gene, the direction of regulation is shown relative to aggressive flies. Genes in bold are discussed in the following chapters.

Gene	P-value	Regulation
Int6	0.00108	Up
CG10363	0.00116	Up
Dat	**0.0016**	**Up**
CG8311	0.00161	Up
CG4729	0.00173	Up
CG17820	0.00205	Up
CG17108	0.00211	Up
RpL1	0.00224	Up
Obp56e	0.00237	Up
PyK	0.0024	Up
CG6762	0.00252	Up
CG6673	0.00258	Up
CG2025	0.00272	Up
Ef1&agr;48D	0.00291	Up
Tfb4	0.00357	Up
CG8067	0.00404	Up
CG6113	0.00434	Up
CG14969	0.00483	Up
CG7966	0.00497	Up
CG8426	0.00543	Up
Slh	**0.00558**	**Down**
CG6480	**0.00561**	**Down**
CG15400	0.0057	Up
eEF1&dgr;	0.00571	Up
CG17146	0.00575	Up
janA	0.00586	Up
EG:171D11.6	0.00603	Up
mbf1	0.00605	Up
CG10026	0.0062	Up
CG10409	0.00698	Up

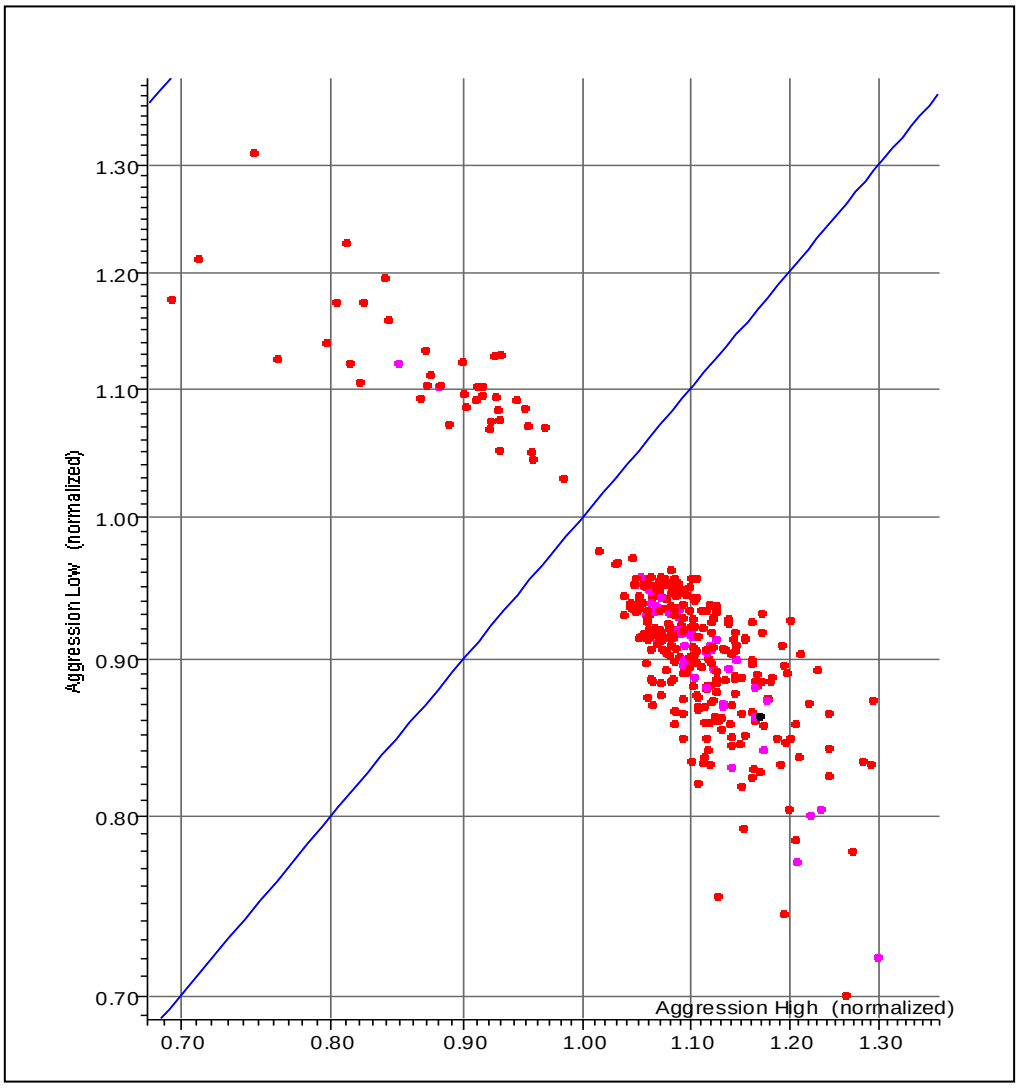


Fig. 4.5 Microarray expression levels. Signal intensities for 347 differentially expressed genes in aggressive (X-axis) and low-aggression (Y-axis) flies. The diagonal represent fold change = 1 (no change) and points below the diagonal represent genes upregulated in aggressive flies. Genes labelled in pink were significant at level of $p < 0.001$. The gene *Dopamine-N-acetyl transferase (Dat)* which came towards the top of the differentially expressed genes is depicted in black.

Functional classification of aggressive genes

The DAVID Functional Annotation Clustering algorithm grouped the 347 significant genes into 47 gene-ontology biological process categories (Fig. 4.6).

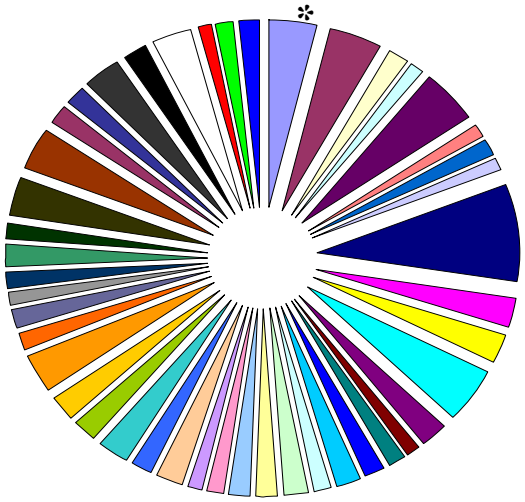


Fig. 4.6 Functional classification of aggressive genes. The DAVID 2008 tool was used to group annotations into clusters (the legend lists a representative member of each cluster). Complete lists of over-represented functional classes are reported in Appendix 2.

- GO:0007559~histolysis
- GO:0002778~antibacterial peptide production
- GO:0046903~secretion
- GO:0001558~regulation of cell growth
- GO:0017111~nucleoside-triphosphatase activity
- IPR001128:Cytochrome P450
- GO:0046034~ATP metabolic process
- IPR006625: pheromone/odorant binding protein PhBP
- GO:0006006~glucose metabolic process
- GO:0009260~ribonucleotide biosynthetic process
- GO:0015986~ATP synthesis coupled proton transport
- GO:0046356~acetyl-CoA catabolic process
- IPR002110:Ankyrin
- GO:0048489~synaptic vesicle transport
- GO:0031966~mitochondrial membrane
- GO:0009581~detection of external stimulus
- GO:0007568~aging
- GO:0007617~mating behavior
- GO:0048858~cell projection morphogenesis
- GO:0008415~acyltransferase activity
- GO:0035107~appendage morphogenesis
- GO:0031410~cytoplasmic vesicle
- GO:0000398~nuclear mRNA splicing, via spliceosome
- GO:0042626~ATPase activity
- GO:0016859~cis-trans isomerase activity
- GO:0006813~potassium ion transport
- GO:0006612~protein targeting to membrane
- GO:0009150~purine ribonucleotide metabolic
- GO:0012501~programmed cell death
- GO:0003746~translation elongation factor activity
- GO:0006096~glycolysis
- GO:0048278~vesicle docking
- GO:0006413~translational initiation
- GO:0006865~amino acid transport
- GO:0007517~muscle development
- GO:0003924~GTPase activity
- GO:0007281~germ cell development
- GO:0008298~intracellular mRNA localization
- GO:0050684~regulation of mRNA processing
- dme00940:Phenylpropanoid biosynthesis
- GO:0048812~neurite morphogenesis
- GO:0051321~meiotic cell cycle
- GO:0000278~mitotic cell cycle
- GO:0008236~serine-type peptidase activity
- Serine/threonine-protein kinase

The clustering algorithm groups the interrogated genes, based on their overlapping annotations, within several similar GOs. In Fig 4.6 these groups are named by using the GO of one of the group members. The largest group with 20 genes was associated with nucleoside-triphosphatase activity, followed by programmed cell death group (11 genes). Other categories included secretion and vesicle docking, glucose metabolism (two related categories with total of 9 genes), synaptic (and neurotransmitter) transmission, and RNA splicing. Interestingly, there were also groups associated with detection of external stimuli, pheromone (odorant) binding proteins and mating behaviour.

Analysis of the annotations of the 'aggression' genes revealed 147 categories (out of 210 tested) that were significantly over-represented in this list ($p < 0.05$), compared with their expected frequency in the genome (of which 21 genes were significant after applying Bonferroni correction). Table 4.2 lists the top 30 categories and the complete list can be found in Appendix 2. A few categories were related with ribosomal proteins, which may suggest increased translation activity (genes that were associated with this function were all upregulated in the aggressive flies). Other categories included immune response, ion transport, and sensory transduction.

Table 4.2 Top 30 over-represented biological function categories. Terms derived from Swiss Prot PIR keywords (SP_PIR_KEYWORDS), gene ontologies (e.g. GOTERM_CC_ALL) and the KEGG pathway database.

Category	Term	Count	%	P-Value
SP_PIR_KEYWORDS	ribonucleoprotein	20	5.76%	4.61E-13
SP_PIR_KEYWORDS	ribosomal protein	18	5.19%	3.27E-12
GOTERM_CC_ALL	GO:0005830~cytosolic ribosome	18	5.19%	8.12E-10
GOTERM_CC_ALL	GO:0005737~cytoplasm	105	30.26%	1.59E-09
GOTERM_CC_ALL	GO:0044445~cytosolic part	20	5.76%	1.65E-09
GOTERM_CC_ALL	GO:0005811~lipid particle	19	5.48%	3.06E-08
GOTERM_CC_ALL	GO:0044444~cytoplasmic part	88	25.36%	3.17E-08
GOTERM_MF_ALL	GO:0003735~structural constituent of ribosome	21	6.05%	1.11E-07
KEGG_PATHWAY	dme03010:Ribosome	17	4.90%	1.80E-07
SP_PIR_KEYWORDS	signal	23	6.63%	2.69E-07
GOTERM_CC_ALL	GO:0033279~ribosomal subunit	20	5.76%	2.79E-07
SP_PIR_KEYWORDS	alternative splicing	24	6.92%	8.64E-07
GOTERM_CC_ALL	GO:0005843~cytosolic small ribosomal subunit	10	2.88%	1.26E-06
GOTERM_CC_ALL	GO:0005840~ribosome	21	6.05%	2.07E-06
SP_PIR_KEYWORDS	endoplasmic reticulum	12	3.46%	2.20E-06
SP_PIR_KEYWORDS	transport	23	6.63%	2.68E-06
GOTERM_CC_ALL	GO:0015935~small ribosomal subunit	12	3.46%	4.68E-06
GOTERM_CC_ALL	GO:0005829~cytosol	30	8.65%	5.26E-06
SP_PIR_KEYWORDS	Secreted	14	4.03%	7.96E-06
SP_PIR_KEYWORDS	cytoplasm	17	4.90%	2.40E-05
SP_PIR_KEYWORDS	membrane	31	8.93%	5.41E-05
GOTERM_MF_ALL	GO:0005198~structural molecule activity	32	9.22%	1.20E-04
GOTERM_CC_ALL	GO:0030529~ribonucleoprotein complex	26	7.49%	1.55E-04
GOTERM_BP_ALL	GO:0006810~transport	67	19.31%	1.88E-04
GOTERM_BP_ALL	GO:0051234~establishment of localization	68	19.60%	1.98E-04
SP_PIR_KEYWORDS	hydrolase	34	9.80%	3.40E-04
GOTERM_BP_ALL	GO:0006952~defense response	21	6.05%	3.98E-04
GOTERM_BP_ALL	GO:0044249~cellular biosynthetic process	45	12.97%	5.20E-04
GOTERM_CC_ALL	GO:0005842~cytosolic large ribosomal subunit	8	2.31%	7.95E-04

Identifying regulatory elements in aggression genes

The analysis of the upstream regions of the 300 genes that were upregulated in aggressive flies revealed 21 oligomers that were significantly over-represented compared with their overall occurrences in the genome (Table 4.3). These sequences may represent promoter motifs that could be targeted by transcription factor(s) that would drive the upregulation of these genes. The most significant motif was the palindrome *TATCGATA* ($p < 0.00001$) that appeared in 42 out of 300 sequences. This sequence is known as a DRE element (DNA replication-related element) that activates promoters of various genes in *Drosophila* (Matsukage *et al.*, 1995). Close inspection of Table 4.3 suggests other sequences that share a similar motif *(ATCGAT)* with DRE.

Table 4.3 Over-represented motifs in the upstream region (1Kb) of up-regulated genes in aggression. The percentage (%) of occurrences in the whole genome is also shown. *Sequences sharing the motif *ATCGAT.*

Sequence	Frequency	P-Value	%
*TATCGATA	42/300	7.22E-11	2.78%
TCTGnTAA	267/300	4.01E-07	9.03%
ATTTnnAAA	143/300	2.97E-06	18.48%
TAGCGATA	125/300	0.000153	1.90%
*ATCGATAG	27/300	0.000233	1.90%
GTATnAAG	246/300	0.000317	9.01%
*ATCGATA	63/300	0.000321	9.04%
GGTCACAC	21/300	0.00059	0.89%
TACTnnnnAGC	224/300	0.00727	6.25%
CGTTnnACA	232/300	0.00792	6.25%
CGATAGCA	124/300	0.00955	1.30%
TATAnnnnTAAG	197/300	0.0127	4.05%
*TATCGAT	59/300	0.0147	9.03%
GGTCACA	37/300	0.0155	4.30%
*GATCGATA	130/300	0.0185	1.90%
GTCACACT	20/300	0.0232	1.30%
TCGAnAGC	40/300	0.0273	4.30%
*CTATCGAT	24/300	0.029	1.90%
TATTnnAGG	248/300	0.0298	9.01%
TATTnnTAA	100/300	0.0349	18.47%
*ACTAnCGAT	113/300	0.0374	1.90%

4.4 Discussion

Microarrays offer a powerful tool for analysing gene expression on a genome scale. The current study exploited the fact that the *Drosophila* genome has been fully sequenced and the availability of commercial microarrays, to study the molecular correlates of aggression. This study showed a significant change in expression, associated with aggression level in 347 genes (Fig. 4.5).

The large number of statistical tests ('multiple comparisons') involved in analysing microarray data is an inherent challenge to this approach. For example, at the level of p = 0.05, in a genome of 15,000 genes, 750 genes are expected to show a significant difference by chance alone. To pass the scrutiny of traditional correction methods such as Bonferroni , the p-value of differentially expressed genes needs to be extremely small. This problem is likely to intensify in microarrays of behavioural phenotypes where the fold changes of significant genes is moderate, resulting in p-values in the range of 0.01-0.05. This may explain why most of the published behavioural microarray studies have not implemented any formal statistical correction (Edwards *et al.*, 2006; Dierick and Greenspan, 2006; e.g. Wang *et al.*, 2008; Toma *et al.*, 2002; Carney, 2007). In the current study, an estimation of the local false discovery suggested that the top 68 genes fall below the threshold of 0.2 FDR. However, for comparison with other aggression studies (see below) and characterisation of biological functions and putative *cis*-regulatory elements, the non-corrected list (347) genes has been used.

Aggression and social phenotypes in general are 'difficult' phenotypes to measure (Jones, 2007, p.31). Indeed we see aggression *per se* not as a phenotype, but as a set of behaviours, which can serve as a measurable phenotype. The difficulty however, is that as a social phenotype it depends on other individual that are present, and their behaviour. The complexity associated with aggression makes it less amenable for genetic analysis (Jones and Mormède, 2007). The current study compared gene expression in *pairs* of flies that show either high or low levels of aggressive interactions. On one hand, this simplified the screening because individual labelling

and monitoring of flies was not necessary. On the other hand, because escalating fights are usually rare in *Drosophila* (Chapter 3), this significantly slowed the process of obtaining pairs of aggressive flies. Presumably, in most cases the aggressive tendency of the males in a pair does not always match, so dominance is established and subordinate males soon leave the territory, resulting in overall intermediate/low level of aggression (Fig 3.3). The focus of the current expression study in *pairs* of males from the extremes of the experimental scores was critical in capturing the expression differences between asymmetrical pairing (low-aggression) and symmetrical matches (leading to high aggression).

Nevertheless, this approach has been successful here, and in other studies (Demir and Dickson, 2005; Dierick and Greenspan, 2007; Hoyer *et al.*, 2008), in identifying molecular correlates of aggression.

While this work was underway, several other microarray studies of aggression in *Drosophila* have been published (Edwards *et al.*, 2006; Dierick and Greenspan, 2006; Wang *et al.*, 2008). Each of these studies has employed a different methodology to study aggression. The study of Dierick *et al.* (2006) used artificial selection to generate flies that are highly aggressive. Flies were observed in population cages where multiple 'territories' were present (similar to the small plates used in the current study). Territorial males, chasing other males away from the territories were collected in each generation to produce the next generation of selected line. After only eight generations of selection, the selected lines showed a significant increase in aggression compared with the control line (suggesting a genetic variation in loci affecting this phenotype). After 21 generations, the change in expression gene in aggressive compared to the control flies was analysed using microarrays and 130 genes showed significant differential expression. Artificial selection has also been used in another aggression study (Edwards *et al.*, 2006). Aggression was again quantified by the total number of aggressive encounters, and both high and low selected lines were produced by repeatedly selecting males with extreme phenotypes to produce the next generation. In this study, flies exhibited a robust response to selection indicating substantial genetic variation for this trait. After 25 cycles of selection, the flies were tested for changes in

gene expression, and over 1,500 genes (~10% of the genome) were found to be differentially expressed.

There are two main differences between the artificial selection experiments described above and the current study. First, the selection studies have compared genetically different lines and the differential expression may reflect the difference between different alleles that were fixed or had changed in frequency in the selection lines. In the current study, isogenic flies produced from two inbred lines were used and the differential expression reflects mainly environmental variation that might cause individual variation in transcript levels. It might be that some of the genes found in the selection studies (Dierick *et al.*, Edwards, *et al.*) are a result of polymorphic alleles that were randomly fixed in the selection lines, which might not have a role in aggression, or alleles in linkage disequilibrium with 'aggression' genes. In the current study, this potential problem does not exist and differential expression is more likely to be associated with the phenotype rather than any genetic variation among individuals. The use of microarrays to study expression of selection lines also has the potential problem that difference in the measured signals may reflect different efficiency of the hybridisation of the polymorphic alleles with the array's probes, and not differences in expression levels. Again this is unlikely to be a problem in the current study because the samples were derived from flies with the same genetic background.

The other difference between the selection studies and the current study is that here, flies were profiled immediately following aggressive interactions, while in the selection studies the specific flies that were sampled were not associated with aggression at the time when collected. Thus, the expression profiles revealed in Edwards *et al.* (2006) and Dierick and Greenspan (2006) represent the general level of expression of these genes, while the profile presented in this study reflects also the effect of the fight itself. In other words, in the selection studies expression profiles represent the tendency of a male for aggression while here it reflects expression changes induced by aggression itself (and possible differences prior to the fights). However, the interval used here (30 min) may have been too short for allowing large

changes in transcript level, which may explain the low levels of fold change (However, fold change was also very low in the published selection studies).

Another microarray study focused on the effect of social experience on aggression (Wang *et al.*, 2008). Group housing of flies suppresses aggressiveness (Hoffmann, 1990) (which is why males tested in the current study were isolated after eclosion, - Chapter 3) Wang *et al.* used microarrays to test differential expression between single- and group-housed flies. Flies from the two treatments were randomly sampled but were not directly scored for their aggressiveness. Nevertheless, their significant gene list included *Cyp6a20*, a gene that encodes a cytochrome P450 and was highlighted by the study of Dierick *et al.* as a major locus regulating aggression (This gene was not included in the gene list of Edwards *et al*, nor in the list of the current study). Members of the cytochrome P450 family are involved in broad range of functions including development reproduction and detoxification. In addition, *Cyp6a20* is also a circadian regulated gene (McDonald and Rosbash, 2001; Ueda *et al.*, 2002) that shows daily circadian oscillation in transcript abundance, although Dierick *et al.* excluded the possibility that the expression difference in the selected lines may reflect a different daily sampling time, or different circadian rhythmicity of the flies.

The different experimental methodologies between the studies (and data processing) may explain the small overlap between the gene lists (fig. 4.7). A similar situation has also been observed in the field of chronobiology, where five different *Drosophila* microarray studies targeting genes that show circadian oscillations initially seemed to share a disappointedly small number of genes (reviewed by Duffield, 2003). Later however, meta-analysis methods have been developed, which indicated a much larger concordance between these lists (Keegan *et al.*, 2007). Nevertheless, the intersection of the gene lists of the different aggression studies (including the current study) revealed two genes, *CG13794* and *Obp99b*, which seem highly likely to be related to aggression (Fig. 4.7). Excluding the very large list of Edwards *et al.* from this analysis, the probability to get two shared genes from three random lists from a genome of 14,000 genes (Fig. 4.7 left panel) is $p < 0.001$; this was calculated by a re-sampling simulation of 30,000 datasets carried out with the R package.

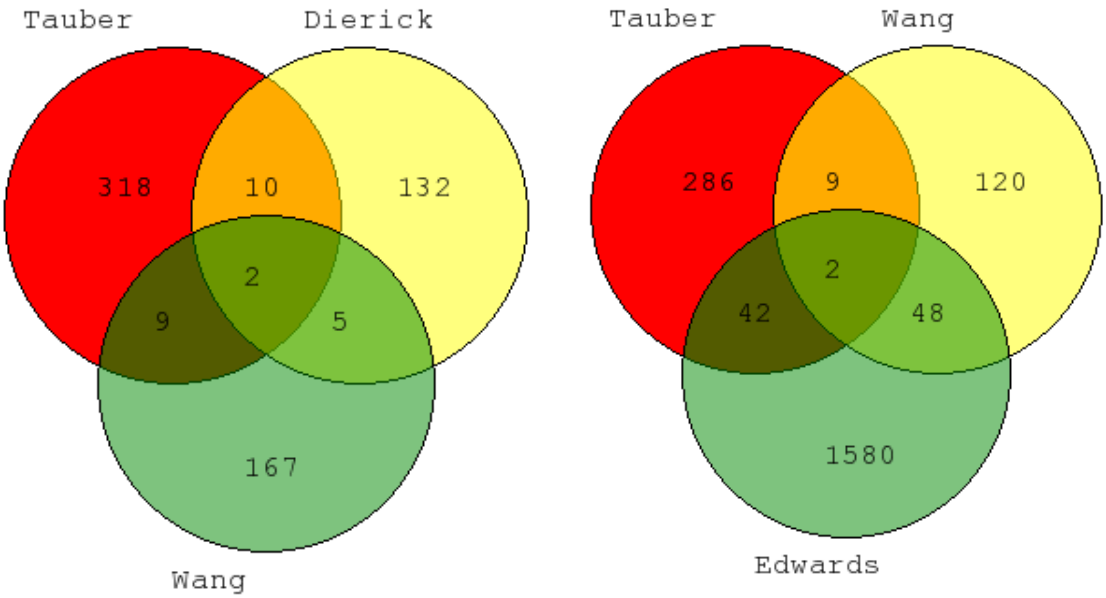


Fig. 4.7 Overlap between aggression microarray studies. These Venn diagrams showing the overlap between the significant gene list of the current study with (Dierick and Greenspan, 2006; Wang *et al.*, 2008) (left) and (Edwards *et al.*, 2006; Wang *et al.*, 2008) (right). Only 339 (out of 347) genes have been used in this analysis due to lack of annotation (the size of the other lists is also slightly reduced because of this reason). The same two genes, *CG13794* and *Obp99b* are shared by all lists in the two comparisons.

The function of the *CG13794* gene is unknown, but sequence analysis reveals that this 595 amino acid protein (mapped to the 2L chromosome arm) belongs to the Sodium: Neurotransmitter symporter superfamily (SNF, PSSM Id: 93180). These proteins are neurotransmitter transporters that use the co-transport of Na^+ to get energy which is used to transport neurotransmitters such as GABA, dopamine and serotonin, into the cell from the synaptic cleft, against their concentration gradient (Amara and Arriza, 1993). The fact that this gene is upregulated in aggressive flies may reflect

increased synaptic activity and amplified neural excitability during aggression. This is consistent with the GO analysis that showed a number of GOs over-represented in the aggression genes (Appendix 2), including GO:0015662 ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism ($p = 0.017$), and potassium transport ($p = 0.022$), ATPase activity coupled to transmembrane movement of ions (GO:0042625, $p = 0.045$), and calcium ion transport (GO:0006816, $p = 0.048$).

Obp99b (encoding an odorant binding protein) is the other gene that was listed in all four aggression microarray studies. This gene was upregulated in males maintained in isolation (Wang *et al.*, 2008) and consistently upregulated in aggressive males. It is reasonable to assume that this protein is part of the input pathway to the aggression system and is probably tuned to olfactory cues from conspecific males. Increased expression of this protein may lead to amplified responses to sensory signal from other males, and reduced threshold for aggressive responses. Interestingly, sequence analysis of natural alleles of odorant binding proteins (including *Obp99b*) reveals high levels of nucleotide variation (Wang *et al.*, 2007). Comparison of the variation in *Obp99b* alleles to orthologous alleles in the closely relates species *D. simulans*, indicated far more segregating polymorphisms in *D. melanogaster* than what would be expected under neutrality, suggesting that this variation is maintained under balancing selection (Wang *et al.*, 2007). It is possible that a trade-off between aggression (territorial) and non-aggressive (non-territorial) strategies exists, and this drives the balancing selection that keeps 'peaceful' and 'violent' alleles of *Obp99b* (low and high level of expression of this gene respectively) segregating in the population. *Obp99b* (also known as *tsx*) is regulated by the sex-determination genes *Sex-lethal* (*Sxl*), *transformer* (*tra*) and *tra2*, leading to expression of this gene only in males (Fujii and Amrein, 2002). Over-expression of *Obp99b* in females reduces the receptivity of females to male's courtship, indicating a role for this gene in social behaviour. This idea was further supported by a recent microarray study that tested global expression in males following courtship of females and identified *Obp99b* as one of the top differentially expressed genes (Carney, 2007). Galindo and Smith (2001) used promoter-LacZ fusion and found expression of *Obp99b* mainly in subsets of sensillae in the olfactory organs, the maxillary pulps, and third antennal segments.

Previous studies have highlighted the role of biogenic amines in aggression (see Introduction). The current study uncovered the biogenic amine gene, *Dat* (encoding dopamine N-acetyl transferase) as one of the top hits (Appendix 1). This gene was also observed in the Wang *et al.* list as upregulated in males maintained in solitary conditions, which tend to be more aggressive. The analysis of flies carrying a null mutation of *Dat* and transformant flies miss-expressing this gene will be described in the following chapter). The comparison of 'aggression genes' found in the current study with that of Wang *et al.* (2008) is particularly informative, as both studies tested the expression in 'solitary' males. Because Wang *et al.* did not test the expression immediately following the aggressive interactions (but the current study did), this may indicates that the significant upregulation of *Dat*, which was observed in both studies, is an inherent property of aggressive flies, and not necessarily induced by the fight itself. This logic may be extended to the other nine genes (e.g. *Obp99b*) that are shared by both lists (Appendix 3). In the current study, variation in *Dat* levels would have to be environmentally mediated, and this stochastic variation in *Dat* was then manifested in variation in aggression.

Aggression is a specific phenotype and not merely a general increase in activity: Dierick *et al.* showed that the activity level of selected lines was not different from that of control flies. Notably, flies mutant for the *Dat* locus, show similar levels of activity to wild-type flies (Shaw *et al.*, 2000). Yet, aggression itself involves metabolic costs, and one concern at the beginning of this study was that because aggressive males fight more, the expression of metabolism genes would mask the effect of aggression genes. The results however suggest that this is not the case, as metabolism-related genes did not dominate the differentially expressed list (Appendix 1) nor the GO classes (Appendix 2).

Although statistically significant, the fold-change of differentially expressed genes was rather small (Fig. 4.5, mainly around 1.5). This seems to be a general property of microarray studies of behaviour as similar magnitude of fold change was observed in other studies (Edwards *et al.*, 2006; Dierick and Greenspan, 2006; Wang *et al.*, 2008). This may reflect that behaviour is driven by many genes with small effect,

and/or in small subset of neurons in the brain. The small fold-change is perhaps the main limit of this technology in studying behaviour since this reduces the power of the statistical tests: a small fold-change implies a lower significance level, which in turn leads to higher false-positive rate estimation. This is probably the reason this type of correction was not applied in neither of the other microarray studies (Edwards *et al.*, 2006; Dierick and Greenspan, 2006; Wang *et al.*, 2008). The small fold-change also constitutes a problem for detecting differences by qPCR (Nolan *et al.*, 2006). Yet, the overlap between the different microarray studies demonstrates the validity of this approach. A development of meta-analysis methods in the future will hopefully allow a better estimation of the false-positive rates in these aggression gene lists.

Chapter 5

THE EFFECT OF DOPAMINE N -ACETYLTRANSFERASE (*Dat*) ON AGGRESSION

5.1 Introduction

In the microarray experiment (Chapter 4), the gene encoding dopamine N-acetyltransferase (*Dat*) was among those that showed the most significantly altered expression. This enzyme is important in the catabolism of the neurotransmitter dopamine (Fig 5.1), and was upregulated in the aggressive flies. This is particularly interesting as dopamine has been implicated in aggressive behaviour in several arthropod and vertebrate species (see Introduction). The *Drosophila Dat* gene (18,177bp) is located on the right arm of the second chromosome (60B12-60C1), and encodes a protein that has a Gcn5-related arylakyl N-acetyltransfarase activity domain (GNAT). GNAT is a superfamily of enzymes that use acetyl coenzyme A (AcCoA) to transfer an acetyl group to a substrate, a reaction implicated in various functions, from mammalian circadian rhythm to bacterial antibiotic resistance (Neuwald and Landsman, 1997). Within the GNAT superfamily, *Dat* is related to the arylalkylamine N acetyltransferases (aaNAT) group of enzymes. In vertebrates, the aaNAT enzymes are mainly found in the pineal gland, where they catalyze the rate-limiting step of the rhythmic production of melatonin from its precursor serotonin (Klein, 2006) (Fig. 5.2). In insects, aaNAT carry out several physiological functions, including inactivation of monoamine neurotransmitters, hardening of the cuticle, and (as in vertebrates) melatonin biosynthesis (Hintermann *et al.*, 1996).

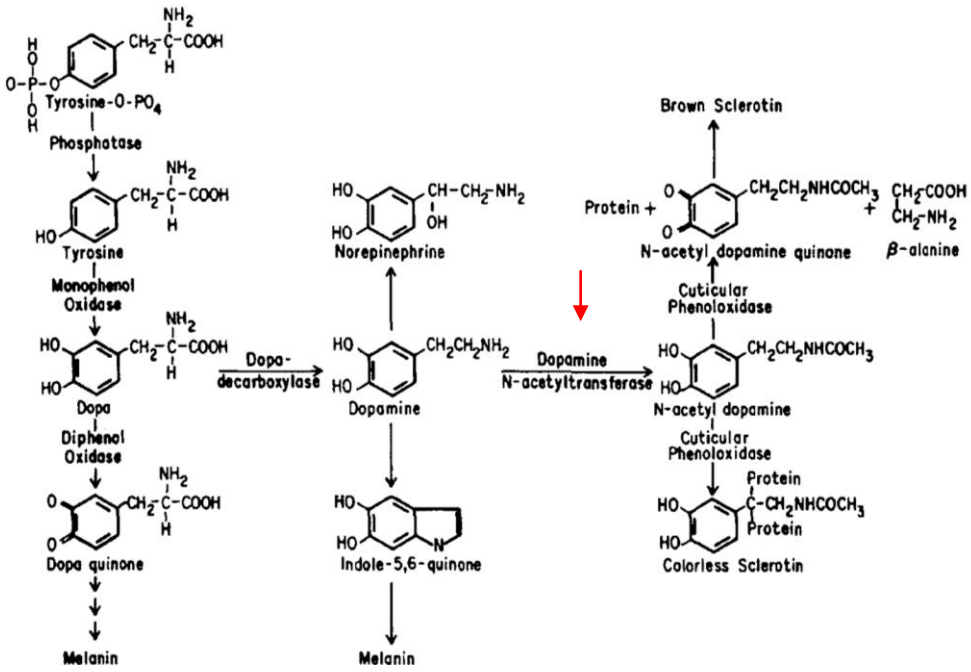


Fig 5.1: Metabolism of dopamine in *Drosophila*. Dopamine N-acetytransferase (Dat, see arrow) is catalyzing the reaction that converts Dopamine into N-acetyl dopamine, hence reducing the levels of dopamine. This is part of the pathway of sclerotization (shown on the right) and melanisation (bottom) (From Wright, 1987).

In *Drosophila*, *Dat* is thought to encode a new type of aryalkylamine N-acetyltransfarase (aaNAT), since it shows no obvious homology to already known acetyltransferases, except in two conserved regions that are found in several bacterial and yeast species (Hintermann *et al.*, 1996). It is not clear whether *Dat* is essential for the process of sclerotization (Hintermann *et al.*, 1996; but see Brodbeck *et al.*, 1998). However, like the vertebrates aaNAT, Dat[1] is involved in the catabolism of monoamine neurotransmitters such as dopamine and octopamine. For example, Dat inactivates dopamine by transferring acetyl group from CoA into dopamine to make N-acetyl dopamine (Fig 5.1).

[1] Note: Dat refers to the protein (as opposed to *Dat* referring to the gene). The symbol DAT is reserved for another protein, the dopamine transporter.

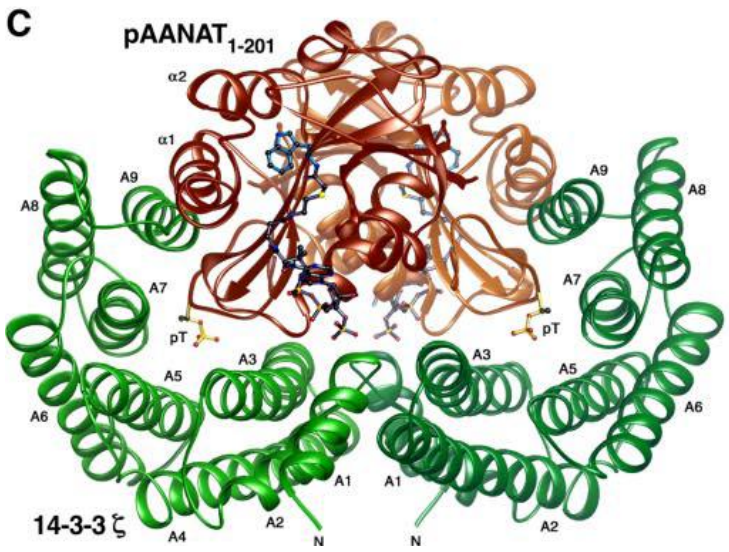


Fig 5.2 The vertebrate AANAT protein (brown) with the 14-3-3 zeta complex (green). This complex modifies AANAT's activity and affinity for its substrates (depicted in blue) by stabilizing a region involved in substrate binding. Although the sequence of AANAT shows poor homology with the *Drosophila* Dat, it may show some similarities in the 3D structure due to functional constraints (The 3D structure of Dat has not been yet determined experimentally). (Figure from Obsil *et al.*, 2001).

The *Dat* gene encodes two transcripts generated by alternative splicing: *Dat A* and *Dat B*[2] (Fig. 5.3) (Brodbeck *et al.*, 1998). The two transcripts encode two isoforms that differ by 35aa at the N–terminal.

[2] The two transcripts have been annotated in the opposite way by Flybase. The current work refers to the Flybase descriptions of the transcripts. Another difference is that the fly genome project finds an additional upstream untranslated exon for the *DatB* transcript *1b*.

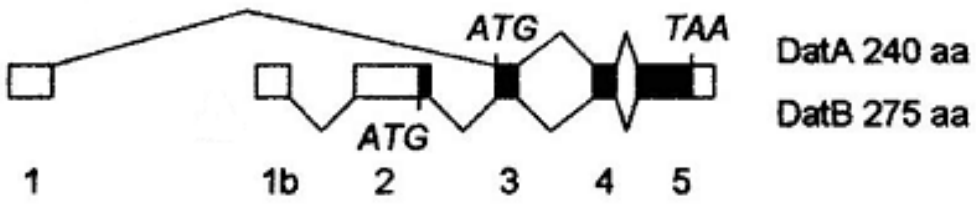


Fig 5.3. Genomic organization of *Dat.* The first exon contains sequences specific for isoform *DatA.* Boxes indicate exons, and introns are indicated by thin lines (black boxes indicate translated region). The second exon and an upstream additional untranslated exon (1b) are specific for the isoform *DatB.* The last three exons are shared by both transcripts.

Previous studies have provided preliminary evidence that the two transcripts may be under tissue-specific control (Hintermann *et al.*, 1996; Brodbeck *et al.*, 1998). The more abundant isoform, *DatA* first appears to be expressed during late embryonic stages in the brain, ventral nerve cord, and the midgut. In the adult, it can be detected in the brain, and the midgut. The less abundant isoform *DatB* appears only during late pupal stages and in the adult brain. Any differential brain expression of the two isoforms is poorly documented, so while the two isoforms may show different expression patterns during development, they nevertheless appear to share a preference for the same substrates, dopamine and with less affinity to octopamine. Both transcripts and proteins do not show circadian cycles in their expression levels (Brodbeck *et al.*, 1998).

A spontaneous mutation in the *Dat* locus (Dat^{lo}) has been identified (Brodbeck *et al.*, 1998) and shows a reduced enzyme activity. The mutation is caused by insertion of two transposable elements, *MDG412* and *Blastopia*, spanning 12 kb and located about ~1.5 kb downstream of exon 1 (fig 5.4). In Dat^{lo} flies, *DatB* isoform levels remain unaffected while the levels of *DatA* are significantly reduced (Brodbeck *et al.*, 1998).

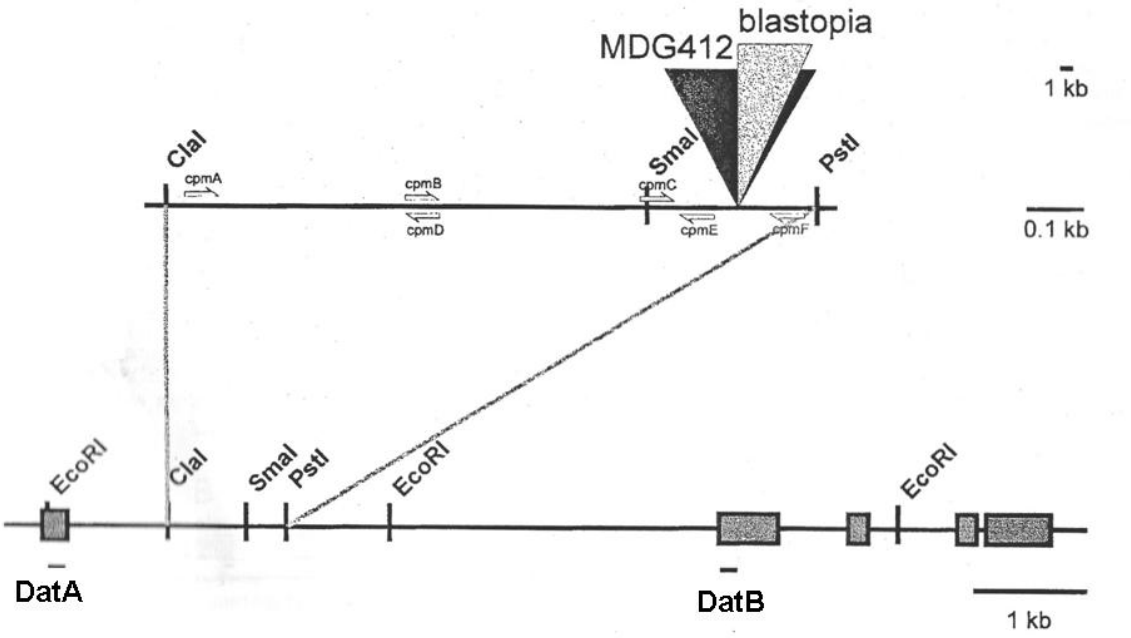


Fig 5.4. Arrangement of the *Dat* locus in the mutant strain (*Dat*^{lo}). Insertion of two transposable elements in the first intron results in 90% reduction of the *DatA* isoform (Figure from Brodbeck *et al.*, 1998).

While the precise location of the neurons in the fly's brain expressing *Dat* is not known, a preliminary study of mutant flies by immunohistochemistry indicated reduced staining in the optic lobes. However, since the antibody used does not distinguish between the two protein isoforms the specific expression pattern of each isoform is awaiting further study. Similarly, a recent study (Wang *et al.*, 2006) attempted to map dopaminergic (DA) neurons using an antibody to tyrosine hydroxylase (TH), an enzyme that converts tyrosine to DOPA (named monophenol oxidase in Fig. 5.1). This antibody targeted DA neurons that may express both *Dat* splice forms (Fig. 5.5).

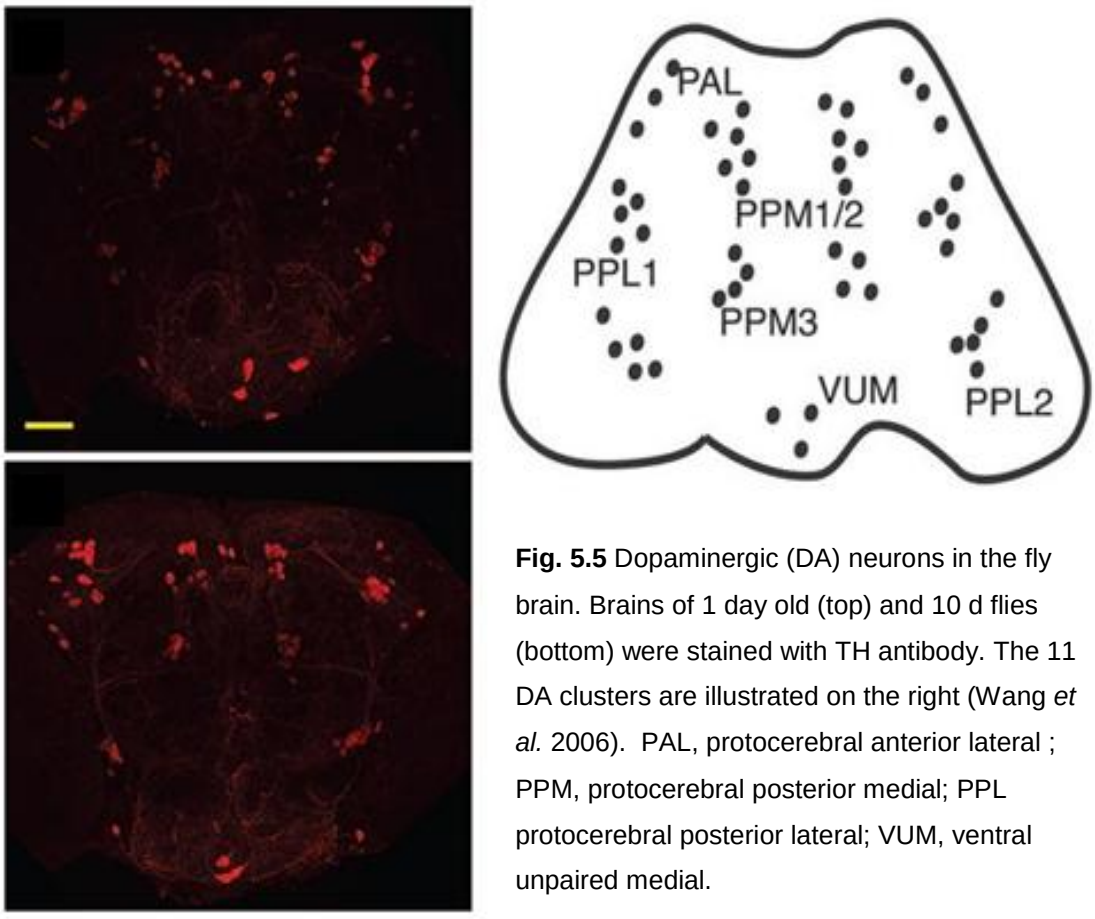


Fig. 5.5 Dopaminergic (DA) neurons in the fly brain. Brains of 1 day old (top) and 10 d flies (bottom) were stained with TH antibody. The 11 DA clusters are illustrated on the right (Wang *et al.* 2006). PAL, protocerebral anterior lateral ; PPM, protocerebral posterior medial; PPL protocerebral posterior lateral; VUM, ventral unpaired medial.

The aim of the present study was to test the role of *Dat* in the regulation of aggression by studying *Dat* mutant behaviour, and by constructing transgenic flies that express one or other of the splice forms using the GAL4-UAS system (see Chapter 2). In addition, transformant flies carrying a UAS-dsRNAi construct were used to knock down *Dat* expression. A number of UAS transgenic flies were used with different GAL4 drivers, allowing missexpressing of *Dat* constructs in various patterns followed by testing their functional effects on aggression.

5.2 Materials and Methods

Fly Strains

The fly strains that were used were w^{1118}, Canton-S (wild-type), bw^{1}, $Dat^{lo}bw$, *w;CyO/Sco;MKRS/TM6B* (double balancer). Overexpression of *Dat* in transgenic flies was driven by GAL4 strain carrying *elav-GAL4* insertion (Bloomington fly stock, No. 8760), and *Dcd-GAL4* insertion strain (Bloomington fly stock, No. 7009).

Dat^{lo} flies are carrying the *brown* (*bw*) marker (i.e. *bw*, Dat^{lo}). For experiments testing the aggression of these flies, males of bw^{1} and $Dat^{lo}bw$ were each crossed to females *w; CyO*. Males and females carrying the balancer chromosome (*CyO*) were crossed to each other, and F2 males homozygous for the original 2nd chromosome (i.e. without *CyO)* were selected for the experiments. This generated two type of males, with and without Dat^{lo}, whose genetic background was similar (shuffled with the genome of the *w; CyO* strain).

In addition, transgenic strains harbouring a UAS construct that induces *Dat* knockdown by RNA interference (RNAi)[3] were obtained from the Vienna Drosophila RNAi Centre. The two strains used were *Dati2* (transformant ID: 47906, inserted in chromosome 2) and *Dati3* (transformant ID: 47907, inserted in chromosome 3). Both carried the same construct (ID 10935) producing a hairpin length of 311 bp. These flies were first crossed to line w^{1118} to verify that the flies are homozygous for the transgene by assessing the eye marker carried on the transformation construct.

Cloning of *Dat*

To harvest the heads, collected flies were kept in 15 ml sterile falcon tubes, frozen by liquid nitrogen, and vortexed. The heads were collected by using double sieves over dry ice tray, separating the heads from the bodies. RNA was extracted by using the TRIzol Reagent from Invitrogen Life Technologies. DNA was removed from the samples by treatment with DNase (DNA-free™ Ambion). cDNA synthesis was

[3] See Chapter 6 for detailed explanation of the RNAi technique to knockdown genes.

carried using total RNA with SuperScript II reverse transcriptase in the following mix: 1- 1.5 µg of RNA, 1 µl (2 pmole) gene-specific primer (primer DatRT, see primers list, Appendix 4), 1 µl dNTP Mix (10 mM each), 12 µl DEPC treated water . The reaction mix was incubated at 65°C for 5 min and then chilled on ice for 1 min and a brief centrifugation for 30 sec. To this mix was then added: 4 µl 5X First-Strand Buffer, 2 µl 0.1 M DTT, 1 µl RNaseOUT™ (40 units/µl recombinant ribonuclease inhibitor), The Mix was then incubated at 42°C for 2 min 1 µl (200 units) of SuperScript™ II RT was added to the mix and the reaction was incubated at 42°C for 50 min. The reaction was terminated by heating at 70°C for 15 min. Finally, 1 µl (2 units) of *E. coli* RNase H was added and incubated at 37°C for 20 min, to remove RNA complementary to the cDNA.

A fragment spanning the complete coding DNA of *Dat* was amplified by PCR from cDNA template. To avoid mutations, the Expand high fidelity PCR system (Roche) was used, which is composed of a thermostable Taq DNA polymerase, and Tgo DNA polymerase (a thermostable DNA with proofreading activity). The PCR reactions were performed using specific primers for each of the isoforms, designed from the 5'- and the 3'-UTR regions; *DatA* amplified with primers Dat3 and primer DatA5-N, and *DatB* with primers Dat3 and DatB5 (see Appendix 4 for sequences). The PCR reactions were set up according to the Expand high fidelity PCR system manufacturer protocol (Roche). Following the PCR, the samples were loaded on 1% agarose gel to isolate and purify the specific DNA fragment by electrophoresis. For the isolation of the DNA fragment from the gel QIAquick Gel Extraction Kit (QIAGEN) was used following the protocol provided with the kit. The purified DNA was used as a template in a second, nested-PCR, following the same conditions described above using the high fidelity PCR system. The primers for this nested PCR were Dat-tailA5`-N and Dat tail3`-N for isoform *DatA*, and Dat-tailB5 with Dat-tail3-N for amplification of isoform *DatB* (see Appendix 4). These nested primers were directed to the start and the stop codons, and included 'tails' encoding a restriction sites (see Appendix 4) to allow ligation of the insert to the pUAST transformation vector.

Next, the PCR products were loaded on 1% agarose gel to purify and isolate the specific DNA fragment by electrophoresis. The QIAquick Gel Extraction Kit was used following the protocol provided with the kit.

The amplified DNA fragments were subcloned into the plasmid pGEM using the pGEM-T easy Vector kit Promega, following the protocol provided. After ligation, the plasmid was introduced into DH5α *E. coli* competent cells by chemical transformation (see chapter 2). Single transformant colonies were isolated and grown in 2.5 ml of LB at 37ºC overnight. Transformant colonies carrying the plasmid pGEMDatA (with the *Dat A* sequence) and the plasmid pGEMDatB (with the *Dat B* sequence) were isolated. Small scale plasmid preps (~20 μg) were made using Qiagen DNA Spin Miniprep Kit, and samples were sequenced using *M13* reverse and *M13* forward as primers (Lark Technologies DNA sequencing services).

Plasmid pGEMDatA and pGEMDatB were digested with a restriction endonuclases to recover the inserts as follows: plasmid pGEMDatA was double digested with the Enzymes NotI and BglII and plasmid pGEMDatB was double digested with NotI and EcoRI. These reactions were incubated at 37ºC for 1.5 h. After restriction the samples were loaded on 1% agarose gel and the expected DNA fragments (1253 bp for isoform *DatA*, and 1417 bp for isoform *DatB*) were recovered from the gel using the QIAquick Gel Extraction Kit, and subcloned by ligation into plasmid pUAST (see plasmid maps in Appendix 5). The plasmid pUAST was digested with the above restriction enzymes respectively, and dephosphorylated by Shrimp Alkaline Phosphatase (SAP, Promega), following the protocol provided.

For the transformation into *E. coli* cells, 2 μl of ligation mix was added to DH5α *E.coli* chemically competent cells (35 μl, see chapter 2). Single transformant colonies were isolated and grown in 2.5 ml of LB at 37ºC overnight. These cultures were then used for isolation of the resulting *UAS-Dat* constructs: pUSDatA (with the *Dat A* sequence) and pUSDatB (with *Dat B*). Small scale plasmid preps (~20 μg) were made using Qiagen DNA Spin Miniprep Kit, and samples were verified by sequencing using primers pUASt-2 and PUAST-R (Appendix 4).

Transformation of *D. melanogaster*

The plasmid DNA was amplified by a maxi prep procedure (QIAGEN), and 1µg of DNA was used to produce transgenic flies by P-element transformation (Spradling and Rubin, 1982). The construct pUSDatB was transformed into w^{1118} fly embryos (see Chapter 2). The other construct pUSDatA was transformed using the same protocol by a commercial service (BestGene Inc).

Dat transformants

Five *DatB* transformant strains (independent insertions), and 10 *DatA* transformants strains were obtained. To verify that the transformant lines are carrying the transgene, a PCR reaction was setup using the genomic DNA extracted from the transformant flies with primer pUASt-2 annealing to the pUAST and primer Dat tail3`-N targeting the *Dat* sequence (Appendix 4). Out of these transformant strains, three were selected for further work and were further verified by additional set of primers targeting the UAS sequence (Fig. 5.6).

Mapping the inserts

Males of each of the transformant strains were crossed to a female double balancer stock *w; Cyo/Sco; MKRS/TM6B.* If the insert mapped to the *X* chromosome, all females in the F1 progeny would be red-eyed. To map the inserts on the autosomes, F1 red-eyed males (with the transgene) that carried both 2nd and 3rd chromosome balancers (*CyO* and *MKRS)* were crossed to w^{1118} females. This allowed mapping the insert to the 2nd chromosome (all red-eyed progeny will not carry *CyO)* or the 3rd.

Statistics

In most cases, the aggression scores did not follow a normal distribution and therefore non-parametric statistics was used. In experiments comparing more than two genotypes, Kruskal-Wallis one-way ANOVA by ranks was first used. Once a significant difference between the groups was indicated, the analysis followed by a multiple comparison test after Kruskal-Wallis (Siegel and Castellan 1988, pp. 213-214) using the library *pgirmess* of the R statistical package (R Development Core Team. 2007).

5.3 Results

Each of the splice forms was successfully transformed into flies and two transformant lines *A4* and *A9* carrying the *UAS-DatA* transgene (inserted in chromosome 2 and 3 respectively) were selected for further study, allowing the overexpression of the *DatA* isoform. Transformant lines *B2* and *B1* carrying the transgene for *UAS-DatB* (inserted in chromosome 2 and chromosome 3, respectively) were used for testing the overexpression of the *DatB* isoform. The presence of the transgenes was verified by PCR (Fig. 5.6)

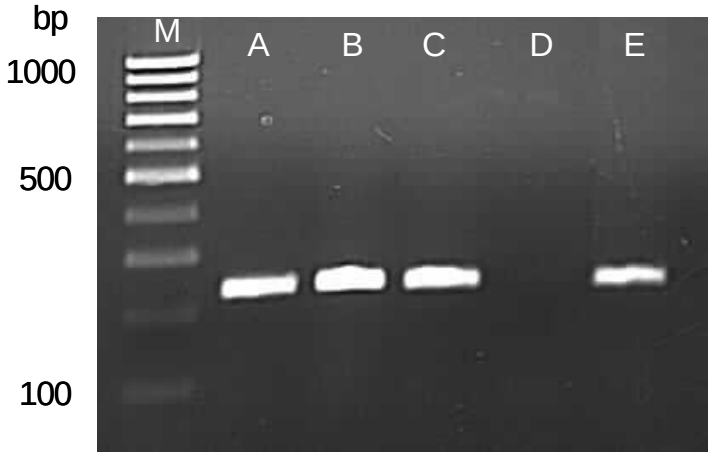


Fig. 5.6. The *UAS-Dat* transgenes verified by PCR. Two primers targeting the UAS sequence were used for PCR generating a 228 bp fragment. **A**. *UAS-DatA4.* **B**. *UAS-DatA9.* **C**. *UAS-DatB1.* **D**. Canton-S (negative control). **E**. pUASt plasmid DNA (positive control). **M**. FullRanger 100 bp DNA ladder.

qPCR for *Dat* isoforms.

These experiments used the same RNA samples that were used in the microarrays experiments. The real-time PCR quantification for each of the splice-forms revealed a significant 2.7 fold increase in the levels of the *DatB* isoform in the aggressive flies Fig. 5.7). The levels of *DatA* were not significantly different between aggressive and non-aggressive flies.

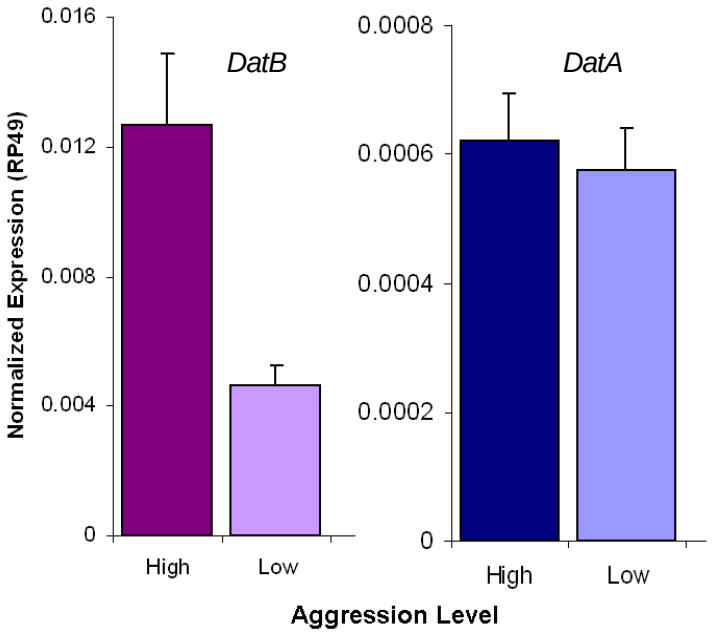


Fig. 5.7 Expression level of *Dat* transcripts measured by real-time PCR. The difference in expression of *DatB* (but not *DatA*) was significant between aggressive (High) and less-aggressive (Low) flies ($F_{1,5}$=28.6, p = 0.0031. No significant differences were observed for *Dat A* $F_{1,5}$=0.21, p =0.66).

Aggression in the mutant *Dat* ^{lo}

The mutant *Dat*^{lo} showed significantly higher levels of aggression compared with the control flies (Wilcoxon sum rank test, W=387 N1=N2= 22, p<0.001, Fig. 5.8). The mutant flies have low activity of the enzyme caused by insertion, which reduces the levels of *DatA*, but express normal levels of *DatB* (Brodbeck *et al.*, 1998). The fact that *Dat*^{lo} flies, which have lower *DatA* levels show a highly aggressive phenotype and that an increase in *DatB* is associated with aggression in normal flies (Fig. 5.7) suggests that the amount of aggression may be reflected in the balance between *DatA* and *DatB* levels.

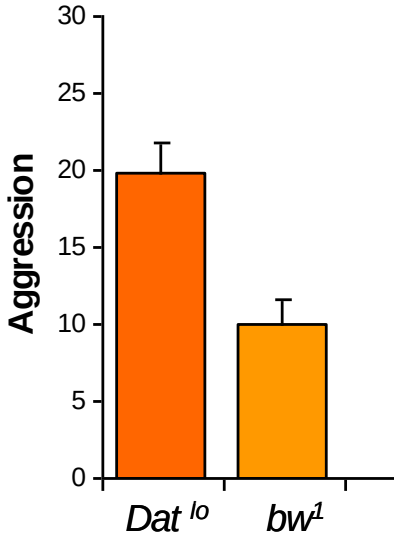


Fig 5.8. Aggressive behaviour of *Dat*^{lo} mutant flies compared with control (*bw*^{1}). Wilcoxon sum rank test, W=387 N1=N2= 22, p<0.001.

Over-expressing *DatA* and *DatB*

The aggression level of the transgenic flies carrying either the *UAS-DatA* or *UAS-DatB* with the *elav-GAL4* driver, and their respective controls, is shown in Figure 5.9. An overall comparison using the non-parametric Kruskal-Wallis one-way ANOVA by ranks test indicated a significant difference in aggression between the genotypes ($\chi^2 = 13.27$, df = 4, $p < 0.02$).

To analyse the aggression of each genotype, a multiple comparison test after Kruskal-Wallis was used for pair-wise group comparison (Siegel and Castellan 1988, pp. 213-214). Flies over-expressing *DatA* under *elav-GAL4* control showed a significant decrease in the levels of aggression ($p < 0.01$) compared with both control groups (flies carrying *UAS-DatA,* or *elav-GAL4*). Over-expressing *DatB* with the same driver (*elav-GAL4/UAS-DatB*) had no effect (Fig 5.9).

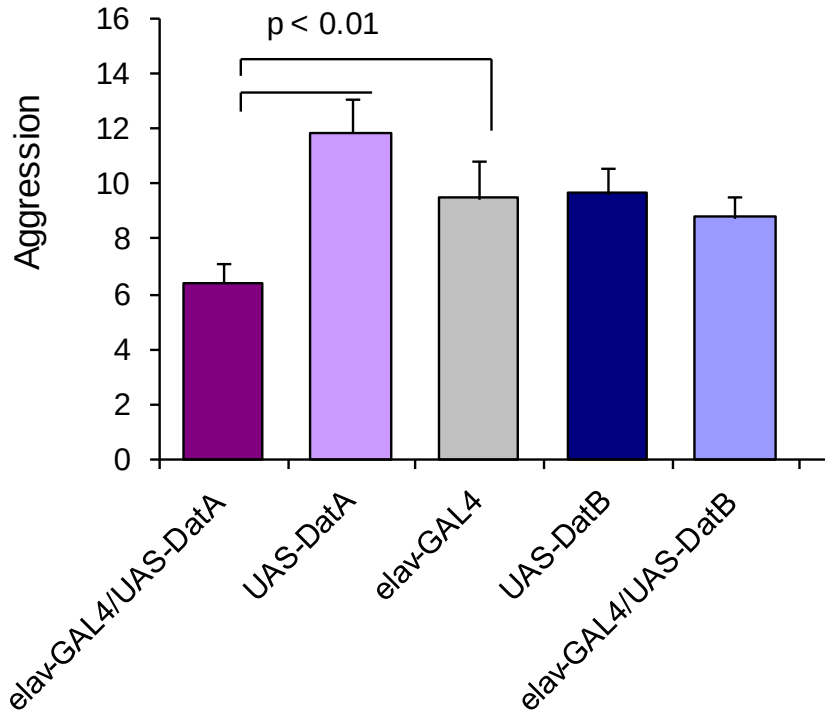


Fig 5.9. Overexpression of *Dat* isoforms using *elav-GAL4* driver. Only over-expression of *DatA* results in a significant difference in aggression compared to its corresponding *UAS* and *elav-GAL4* controls.

An even stronger effect was observed when the *Ddc-GAL4* driver (*Ddc*: *Dopa decarboxylase*, encoding a protein that is involved in biosynthesis of catecholamines, including dopamine) was used to over-express *DatA* and *DatB* (Fig 5.10). An overall comparison using the non-parametric Kruskal-Wallis one-way ANOVA by ranks test indicated a significant difference in aggression between the genotypes ($\chi^2 = 26.34$, df = 5, $p < 0.001$). A multiple comparison test after Kruskal-Wallis indicated that aggression in flies over-expressing *DatA* was reduced compared to flies expressing a single transgene, either the *UAS-DatA* or the *Ddc-GAL4* ($p < 0.01$), while the aggression of

flies over-expressing *DatB* was not significantly different from flies carrying only the *UAS* transgene.

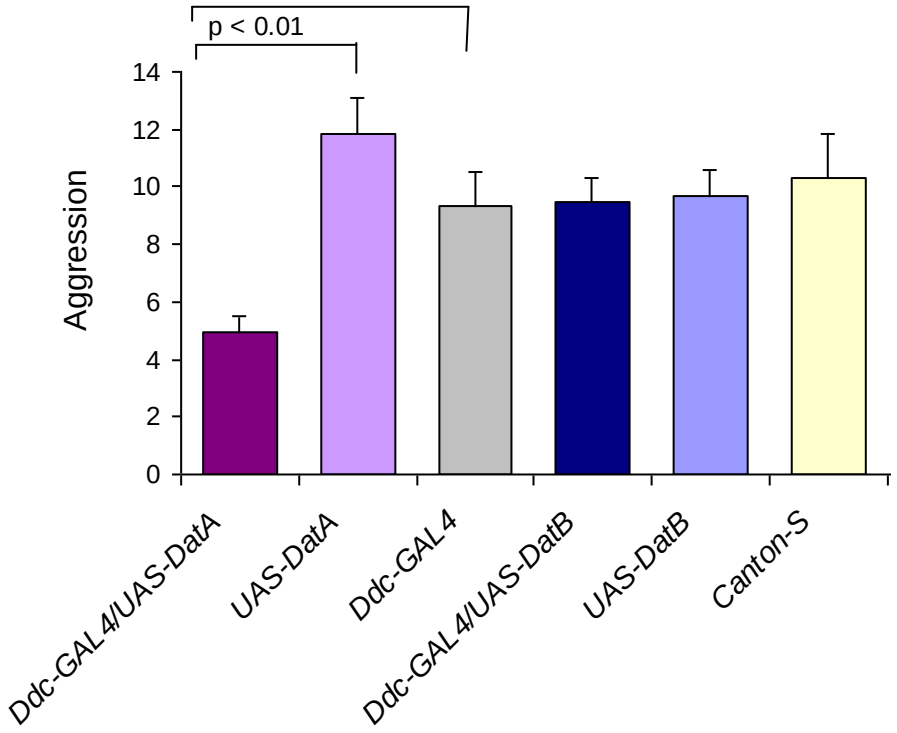


Fig 5.10. The aggression response of flies using the *Ddc-GAL4* driver. Overexpression of *DatA,* but not *DatB,* results in a significant reduction in aggression compared with the relevant controls.

Driving *UAS-DatB* with *elav-GAL4* in a Dat^{lo} homozygous or heterozygous background (Fig 5.11) revealed a significant increase in aggression compared to heterozygous Dat^{lo} carrying either *elav-GAL4* or *UAS-DatB* (Kruskal-Wallis, $\chi^2 = 59.3$, df = 5, $p < 0.0001$, followed by a multiple comparison test set at $p < 0.01$). Driving UAS-*DatA* with *elav-GAL4* on a Dat^{lo} homozygous or heterozygous background showed no difference in aggression when one or both copies of Dat^{lo} were eliminated, compared to heterozygous Dat^{lo} carrying either *elav-GAL4* driver or *UAS-DatA* (Fig 5.11).

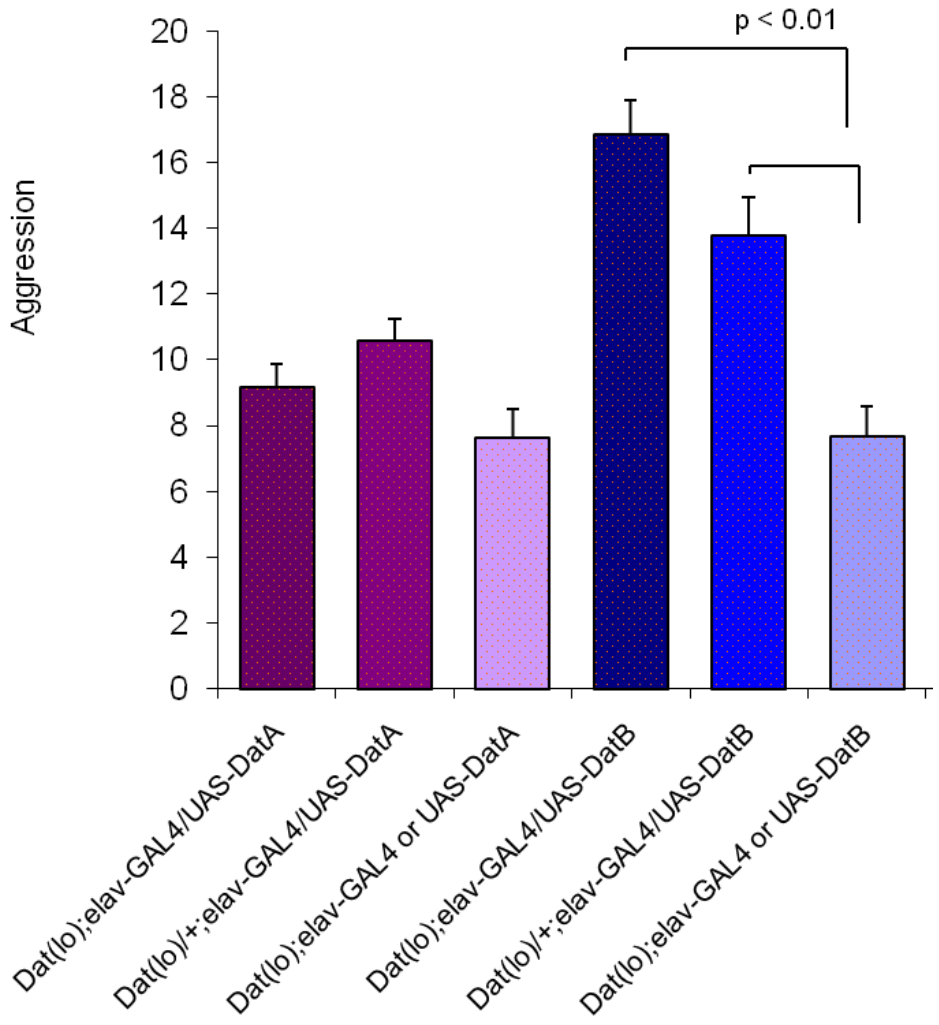


Fig 5.11. Overexpression of *Dat* splice forms in mutant backgrounds. *DatB* with *elav-GAL4* driver over Dat^{lo} homozygous or heterozygous background increases aggression. Overexpression of *DatA* showed no significant differences compared to its corresponding control.

Knocking down the expression of both *Dat* isoforms simultaneously by RNAi using the *UAS-Dati3* strain driven with *elav-GAL4* resulted in a significant

increase in aggressive behaviour (Fig. 5.12): overall comparison of the UAS-RNAi lines and their controls using Kruskal-Wallis ANOVA (χ^2 =19.76, df = 4, $p < 0.001$). Post-hoc multiple comparison test revealed that only one of the UAS lines *(Dati3)* showed an increased aggression compared to the *UAS* control ($p < 0.01$), but not compared with the *elav-GAL4* control. Even so, the absolute levels of aggression on this genetic background are rather low. The other UAS-RNAi strain, *Dati2,* did not show a significant change in aggression. The results of all the mis-expression experiments are summarised in Table 1.

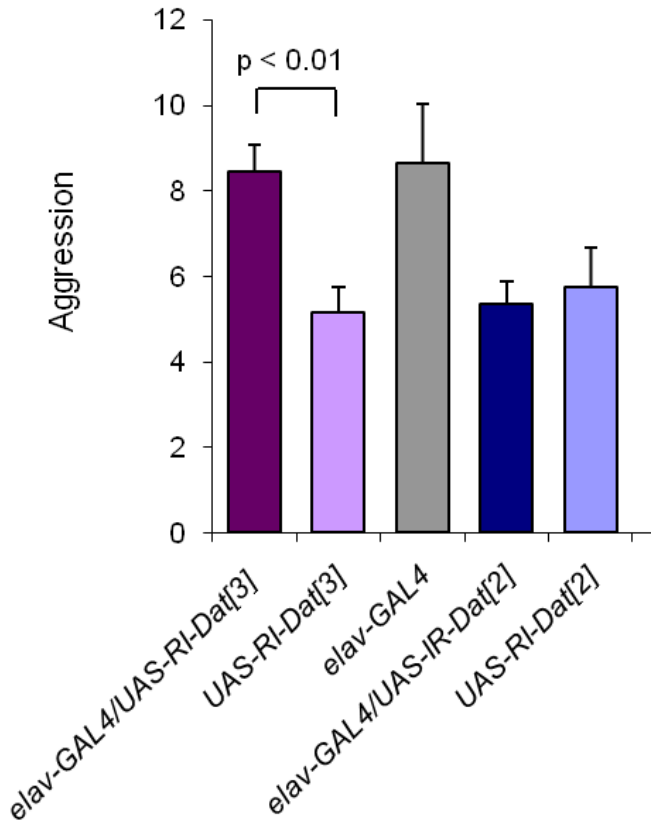


Fig 5.12 UAS-RNAi against both *Dat* isoforms increases aggression in *elav-GAL4* flies carrying the UAS transgene on chromosome 3 compared with the *UAS* control (but not with the *GAL4* driver). There is no apparent change in flies with the transgene inserted in chromosome 2.

Table 5.1. Summary of miss-expression experiments with *Dat* isoforms.

Description	**Genotype**	***DatA***	***DatB***	**Aggression**
Mutant Dat^{lo}	*;Dat^{lo}/Dat^{lo};*	--	++	Up
Dat^{lo} heterozygote	*;Dat^{lo}/+;*	-/+	++	nc[4]
Overexpressing *DatA*	*w ;; elav-GAL4/UAS-DatA*	Up	++	Down
Overexpressing *DatA*	*w ;; Ddc-GAL4/UAS-DatA*	Up	++	Down
Overexpressing *DatB*	*w ; ; elav-GAL4/UAS-DatB*	++	Up	nc
Overexpressing *DatB*	*w ; ; Ddc-GAL4/ UAS-DatB*	++	Up	nc
Overexpressing *DatA* over mutant (Dat^{lo}) background	*w ; Dat^{lo} ; elav-GAL4/UAS-DatA*	++?	++	nc
Overexpressing *DatB* over mutant (Dat^{lo}) background	*w ; Dat^{lo} ; elav-GAL4/UAS-DatB*	--	Up	Up
RNAi against *Dat*	*w;; elav-GAL4/UAS-RNAiDat*	Down	Down	Up?
Selected Aggressive flies		++	Up	Up

[4] nc: No change in aggression (compared to control flies).

5.4 Discussion

My earlier experiments using global microarray profiling indicated that *Dat* transcript levels are elevated in aggressive flies (Chapter 4). Here, by using mutant and transgenic flies I was able to verify that manipulating *Dat* levels alters aggression in *Drosophila* males. *Dat* converts dopamine to N-acetyl dopamine, suggesting that aggression-related change in *Dat* transcripts is associated with changes in dopamine level.

The results of this chapter indicate that high levels of dopamine induce aggression in *Drosophila* in the same manner as it does in other organisms (see below), since Dat^{lo} mutants, supposedly with elevated level of dopamine, exhibit increased aggression (Fig. 5.8). Knocking down *Dat* by RNAi (Fig. 5.12) also resulted in increased levels of aggression, but only in one line, and just with one of the controls so perhaps this result should be treated with some caution. The role of dopamine in *Drosophila* has been tested before by feeding flies either L-DOPA (dopamine precursor) or 3-iodo-tyrosine (dopamine inhibitor), but the results were not conclusive (Baier *et al.*, 2002).

Nevertheless, the microarray data indicated an increase of *Dat* levels in aggressive flies, which would have resulted in lower levels of dopamine[5] (Chapter 4). Importantly, the same result was observed in another recent microarray study (Wang *et al.*, 2008) where higher levels of *Dat* were observed in males that were kept individually and were more aggressive than flies kept in groups. However, the microarray probes (Affymetrix) cannot distinguish between the two alternative *Dat* splice forms. To get a better understanding of the function of this gene in aggression, I have quantified the level of each isoform in aggressive and non-aggressive flies by qPCR (Fig. 5.7). These experiments suggested that the effect of *Dat* on aggression is isoform-specific, with *DatB* (but not *DatA)* showing higher levels in the aggressive

[5] The enzyme also acetylates octopamine, but activity is much reduced (Brodbeck *et al.* 1998)

flies. Furthermore, in Dat^{lo} mutants, only the transcription of the *DatA* isoform is disrupted, but transcript levels of *DatB* are normal. These mutants also show higher levels of aggression (Fig 5.8), suggesting that the balance between these two *Dat* splice forms regulates aggression. Thus, the increased aggression in the selected H flies could be the result of the reduced ratio of *DatA*/*DatB* (0.0006/0.012 = 0.05) compared with the L flies (0.0006/0.004 = 0.15) (*Dat* levels taken from Fig. 5.7).

However, in apparent contradiction, pan-neural (by *elav*) or *Ddc* driven overexpression of *DatB* in a normal background (Fig.5.9 and 5.10, Table 1) does not increase aggression. Yet once the copy number of endogenous *DatA* was reduced using homo- and heterozygous background of Dat^{lo} the *elav-DatB* overexpression did give higher levels of aggression again supporting the 'balance' model. Furthermore, over-expression of *DatA* significantly *decreases* aggression, in line with the observation that reduced *DatA* levels in the Dat^{lo} mutant give higher levels of aggression. Overexpressing *DatA* while simultaneously reducing its levels with Dat^{lo}, rescued the mutant phenotype. Thus a simple balance model in which the increasing ratio of *DatA* to *DatB* attenuates aggression (and *vice versa*) fits the data. The microarray result with *Dat* has therefore been validated in a rather novel manner with the various *Dat* manipulations. The two isoforms seem to act in opposite ways to regulate aggression.

There are a couple of observations that might argue against such a simple model. One such 'fly in the ointment' is that increasing levels of *DatB* in the H lines gave higher aggression levels, whereas a similar manipulation by over-expression of *DatB*, on an otherwise wild-type background, did not. Yet this apparent discrepancy may be easily resolved by considering that the *e/+* heterozygotes from the aggression selection strain, may have higher dopamine levels to start with, as homozygous *ebony* mutants are well known to have excess dopamine (Hodgetts and Konopka 1973). Indeed one of the reasons these flies were chosen was because they were more aggressive than the CS strain we used initially (Chapter 3). Thus the reduced ratio of *DatA/DatB* in the H line, would be expected to give enhanced aggressive behaviour because this line is already 'sensitised' with higher dopamine levels Secondly,

reducing *DatA* dosage in heterozygotes does not give an increase in aggression. However as the mutant gives only a partial reduction in *DatA* levels (Brodbeck et al, 1998), this would clearly suggest that heterozygotes have enough *DatA* to maintain normal levels of aggression. Finally, knockdown of both transcripts gave equivocal results, in that one RNAi line gave an effect, whereas the other did not. Again in a balance model, reducing both transcripts might not be expected to give a dramatic phenotype. Indeed this seems to be the case.

The results of this study provide an example of regulation of behaviour by alternative splicing, specifically by altering the ratio of the splice isoforms. Another example of behaviour regulated by splicing is the male courtship behaviour in *Drosophila* that is controlled by sex-specific splicing of *fruitless* (*fru*), (Demir and Dickson, 2005). Several splice isoforms are produced from the *fru* locus, including number of isoforms which are male specific (fru^M). Demir *et al.* showed that male courtship behaviour and sexual orientation requires fru^M and that expressing these isoforms in females resulted in inhibition of female reproductive behaviour, and caused females to behave as males. Though both cases indicate to how alternative splicing can effect behaviour, in the case of *fru*, splicing is a developmental process, whilst *Dat* splicing reflects an ongoing regulation of behaviour by the changeable ratio between the two isoforms, and while the *fru* isoforms are important in shaping sex-specific neural structures, *Dat* isoforms influence synaptic transmission (via dopamine), thus, continuously affecting behaviour. Another example of regulating behaviour by splicing is the *period* (*per*) gene, where low temperature and short day-length promote the splicing of a downstream intron located in the 3'UTR region, leading to change in the daily activity profile of flies (Majercak *et al.*, 2004; Majercak *et al.*, 1999; Collins *et al.*, 2004). The splicing leads to overall increase in transcript level and earlier "evening" activity, which was suggested to be a seasonal adaptation of the circadian clock.

Interestingly, dopamine plays important role in regulation of sleep and wakefulness in both mammals and *Drosophila* (Wisor *et al.*, 2001; Shaw *et al.*, 2000; Cirelli *et al.*, 2005). The levels of *Dat* were found to be higher in awake flies (relative

to rest), and Dat^{lo} mutants showed a greater rest rebound after sleep deprivation (an effect that was even stronger in flies carrying a deficiency for the *Dat* locus, in which both *DatA* and *DatB* are reduced in these flies). These experiments may suggest a link between aggression and awareness or arousal, and may explain the reduced threshold for aggression often seen in sleep-deprived humans (Kahn-Greene *et al.*, 2006). Importantly, Dat^{lo} mutants show a similar level of activity, and circadian rhythms as wild-type flies (Shaw *et al.*, 2000) indicating that the increased aggression in Dat^{lo} mutants observed in my experiments was not merely reflecting increased locomotor activity.

To understand the regulation of aggression by the *Dat* splice isoforms, further experiments are needed. To date, the promoters for *DatA* and *DatB* are unknown. Cloning of various upstream and intronic fragments may allow construction of GAL4 lines that would drive the expression of each of the splice forms, but such a promoter-bashing strategy as yet to be applied. Using such *Dat-GAL4* lines with UAS-GFP will allow mapping the expression of the two splice forms in the brain. In addition, these GAL4 lines will allow the specific ablation of dopamine neurons if used in combination with UAS constructs expressing cell death genes such as *hid* or *reaper* (UAS-hid, UAS-rpr), and test the effect on behaviour. Monitoring the two isoforms at the protein level is important but might proof unfeasible as they only differ in 35 residues. Another approach (that was used in the *fru* studies, Demir and Dickson, 2005) would be to fuse a florescent tag to each of the unique exons of *DatA* and *DatB* using homologous recombination to allow the simultaneous detection of the isoforms in the fly brain by confocal microscopy. In another set of experiments, RNAi constructs may be prepared for knockdown of each of the splice forms. The accurate expression of these UAS constructs would be achieved by using the specific GAL4 drivers mentioned above. In addition, probes targeting the unique exons of *DatA* and *DatB* may be prepared and used in *in-situ* hybridisation to brains of aggressive and non-aggressive flies.

In conclusion, the novel ‘selection’ approach that was taken in this study has revealed a number of genes that were implicated in aggressive behaviour. One of

these, *Dat*, was validated by a number of neurogenetic manipulations, which suggested that the two splice forms act as a molecular switch, between low and high levels of aggression. These findings reveal that even in these days of 'omic' and network analyses, that there is no real substitute for treating each individual gene on its merits. Neither global transcriptomic profiling nor any kind of network analysis would have uncovered the antagonistic functions of the two *Dat* transcripts. Future studies of the *Dat* locus will need to address the molecular basis of how the two isoforms interact to generate the aggressive phenotype.

Chapter 6

THE ROLE OF *SLH* AND *CG6480* IN AGGRESSION: AN RNA INTERFERENCE STUDY

6.1 Introduction

Manipulating the activity of genes allows exploration of the biological role of their protein products. One way to target gene expression is to use double-stranded RNA interference (RNAi). RNA interference is the process whereby the introduction of double-stranded RNA of a specific gene into a cell inhibits gene expression (Hammond *et al.*, 2001). This approach uses a natural process in which translation of some of a cell's messenger RNA is prevented, due to the presence of matching double-stranded RNA sequences. This RNAi pathway, which is significant for some forms of innate immune response, is believed to protect the cell against viruses, and also plays a role in regulating development and genome maintenance (Hannon, 2002).

In the RNAi pathway, specific proteins target the messenger RNA (mRNA), and break it down into smaller segments that can no longer be translated into protein. The RNAi pathway is initiated by the Dicer enzyme. Dicer is a ribonuclease, belonging to the RNase III family, which cleaves double-stranded RNA. It catalyses the first step in the RNA interference pathway and initiates the formation of large ribonucleoprotein complex, the RNA–induced silencing complex (RISC). RISC, whose catalytic component Argonaute is an endonuclease, is capable of degrading messenger RNA as part of the post-transcriptional RNA silencing pathway (Jaronczyk *et al.*, 2005).

Dicer cleaves long dsRNA molecules into short fragments of 20–25 base pairs called small interfering RNA strands (siRNA). These fragments guide RISC to find complementary mRNA sequences (Fig 6.1). One of the two strands in the siRNA, known as the guide strand, is incorporated into the RNA–induced silencing complex (RISC) and pairs with complementary sequences. When the guide strand

specifically pairs with an mRNA molecule, it induces the mRNA cleavage by Argonaute, the catalytic component of the RISC complex, and gene silencing is achieved.

The RNAi mechanism can silence the expression of specific genes .Its selective nature makes it a valuable research tool, since synthetic dsRNA, introduced into cells, can induce suppression of specific genes of interest.

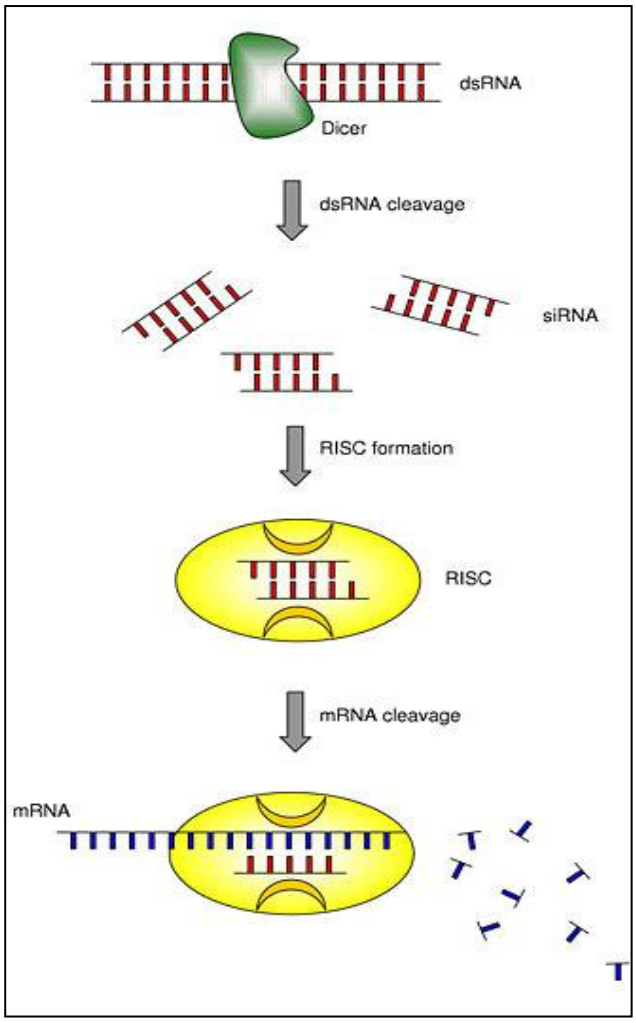


Fig 6.1: Mechanism of RNA interference (RNAi). The Dicer enzyme cuts double-stranded RNA, forming small interfering RNA fragments (siRNA). These RNA fragments are incorporated into the RNA-induced silencing complex (RISC), that targets messenger RNA to prevent translation. Figure from (Mocellin and Provenzano, 2004).

In *Drosophila*, RNA interference has been implicated in all developmental stages. Injection of dsRNA into *Drosophila* embryos results in specific gene interference during muscle formation (Kennerdell and Carthew, 2000). Disruption of the *EcR* ecdysone receptor gene by RNAi during larval development results in defective larval moulting, metamorphosis and pupariation (Lam and Thummel, 2000) Initially, in adults, the effects of RNAi on gene expression have been inconsistent. Martinek and Young (2000) quantified the suppression of the *period*

gene in transgenic flies and found there was only a 50% reduction in protein levels. This level of suppression is insufficient to produce reliable mutant phenotypes for most genes. A more effective RNAi technique to target genes in adult *Drosophila*, involves genomic and cDNA fusion, which forms hairpin dsRNA molecules following splicing, and effectively suppresses expression of the targeted gene (Kalidas and Smith, 2002). The latter technique was implemented in this study.

Two genes, *CG6480* and *Slh*, which seemed to have significantly reduced expression in aggressive flies (Appendix 1), were selected for further study. Knocking-down these genes in normal flies was expected to elevate aggression. This method can also be applied to genes that display high expression in aggressive flies. In this instance a reduction in aggressive behaviour will be expected, but such a result might also be open to other interpretations (sickness, reduced fitness or fatigue etc).

The gene *CG6480* is located on the left arm of chromosome 3 (cytological map location 77B1). The molecular function and biological processes in which it is involved are not known. One possible function was implied when *CG6480* was found to be included within a group of genes within 77B1, in a screening of P-element insertion lines for mutations affecting the olfactory response in *D. melanogaster* (Anholt *et al.*, 2001).

The gene *Slh* (*SLY-1 homologous)* is located on the left arm of chromosome 2 (cytological map location 22F3-22F4). Its molecular function is described as SNARE binding (Littleton, 2000). SNARE is a protein complex known to play a key role in vesicle–target membrane fusion. The *Slh* gene is related to the neuronal SNARE complex, which is required for synaptic vesicle exocytosis at nerve terminals. The exact biological function of SLH is not fully understood, but it has been suggested to be involved in regulating synaptic transmission as part of the t-SNARE binding complex (Schulze *et al.*, 1995; Deitcher *et al.*, 1998; Littleton *et al.*, 1998).

6.2 Materials and Methods

RNAi construct

The RNAi constructs for *Slh* and *CG6480* were designed using the genomic cDNA RNAi approach (see Introduction). A genomic DNA containing an exon and an intron was fused to an inverted cDNA fragment, encoding the same exon, thus creating a hairpin-shaped palindrome (See Appendix 6).

Preparation of the genomic DNA fragment.

Genomic DNA was extracted from WT Canton-S flies and the required DNA fragment was amplified by PCR using the Expand High Fidelity PCR System (Roche), a polymerase mixture containing Taq DNA polymerase and Tgo DNA polymerase with a proofreading activity that generate high-fidelity, high-specificity PCR products (For primer sequence see Appendix 4). The PCR programme followed the thermal cycling was that recommended for the Expand High Fidelity PCR System (Roche). After DNA amplification, a small sample of the reaction products was run out on 1% agarose gel to verify the expected DNA fragment size. The remainder of the PCR products was purified using the QIAquick PCR Purification Kit and cut directly with the required enzymes (Appendix 6).

Preparation of the cDNA fragment.

Total RNA was extracted from WT Canton-S flies and cDNA was prepared after DNase treatment (see chapter 2). A fragment of cDNA was amplified by PCR using the Expand High Fidelity PCR System (Roche), following the recommended PCR programme. After DNA amplification, a small sample of the reaction products was run out on 1% agarose gel to verify the expected DNA fragment size (Fig 6.2). The remainder of the PCR products was purified using the Qiagen PCR purification kit and cut directly with the required enzymes (Appendix 6).

Once both fragments had been cut by the appropriate restriction enzymes, the genomic DNA fragment was ligated to the inverted cDNA fragment. The joint

fragment was then subcloned into the vector pGEM using the pGEM –T Easy Vectors kit (Promega), to give plasmid pGEMRNAi-*gene* followed by transformation into DH5α *E. coli* competent cells (Appendix 6). Small-scale plasmid preps (~20 μg) were made using the Qiagen DNA Spin Miniprep Kit, and the sequence was verified by Lark Technologies DNA sequencing services (Takeley, UK)

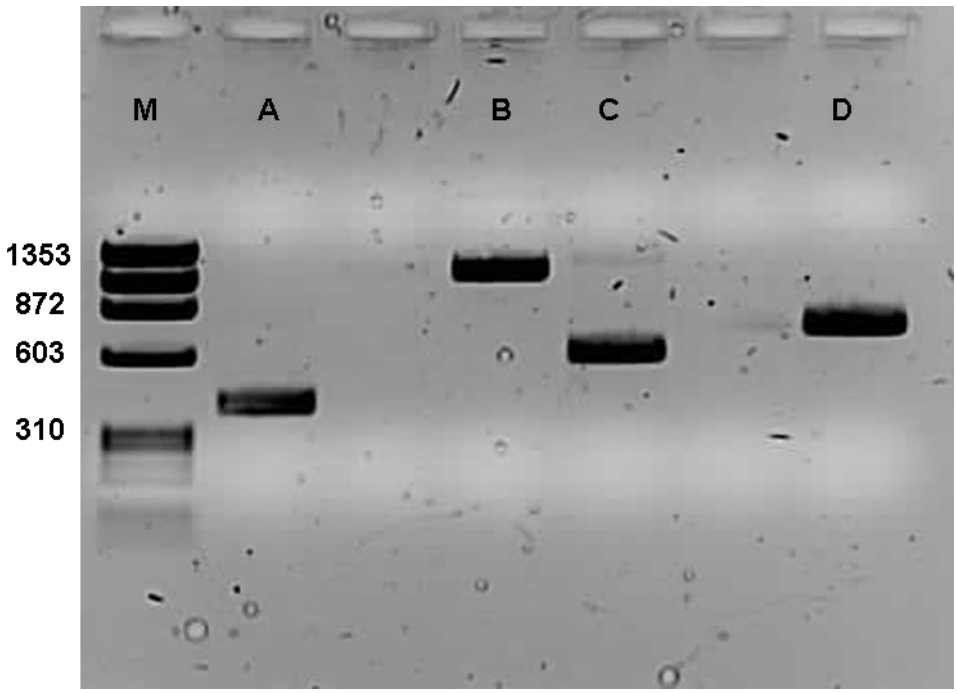


Figure 6.2 : Example of agarose gel showing the *Slh* and *CG6480* PCR products. The amplification of *CG6480* was carried using cDNA template (lane A, expected size 379 bp) and genomic DNA (B, 1052 bp). The fragments of *Slh* amplified from cDNA (577 bp) and from genomic DNA (706 bp) are shown in lane C and D respectively. The cDNA and genomic DNA fragments were purified and ligated to create the RNAi construct (see text).

Once the sequences were verified, the joint fragment was amplified by PCR using the pGEMRNAi-*gene* (pGEM harbouring the joint RNAi fragment either for *CG6480* or *Slh*) as a template. A small sample was loaded onto a 1% agarose gel to confirm fragment size, and the remainder of the PCR products was purified using a QIAquick PCR purification kit and cut with the appropriate

restriction enzymes (see Appendix 6). After restriction, the DNA palindrome fragment was subcloned into the vector pUAST followed by a second transformation into DH5α *E.coli* competent cells (see chapter 2). Small-scale plasmid preps (~20 μg) were made using the Qiagen DNA Spin Miniprep kit and the sequence was partly verified[1] by the PNACL DNA sequencing service at the University of Leicester.

Fly strains

Just after the generation of RNAi constructs, a number of RNAi transgenic strains targeting the whole fly genome were made available from the Vienna Drosophila RNAi Centre (http://stockcenter.vdrc.at/control/main). Thus I did not transform my constructs into flies and all the RNAi transgenic flies used in the experiments in this chapter were obtained from the Vienna centre. The following *UAS* strains were used: For *Slh*: transformant ID: 26223 construct ID: 11003, hairpin length 394bp, inserted in chromosome 2, and transformant ID: 105669 construct ID: 101905, hairpin length 504bp, inserted in chromosome 3. For *CG6480*: transformant ID: 23447 construct ID: 13513, hairpin length 320bp, inserted in chromosome 3. The primers and the hairpin construct sequences can be obtained from the site.

The targeting specificity of the RNAi constructs was assessed by the VDRC by blasting all possible 19-mers sequences included in the construct (the minimum length of perfect match required for RNAi) against the *Drosophila* transcriptome (Dietzl *et al.*, 2007). Genes hit by more than 80% of the 19-mers are considered *on-target* (*off-target* genes hit by fewer than 80%). All RNAi lines used in the current study had a single on-target gene and no off-targets.

The flies from the Vienna centre were first crossed to line w^{1118} to verify the homozygosity of the transgene. All flies were kept in separate glass vials containing sugar/agar medium at 18 ºC or 25 ºC in temperature-controlled rooms or incubators. The flies were subjected to a 12:12 hour light/dark cycle.

[1] The palindromic structure of the constructs hinders sequencing.

Flies for the behaviour experiments were obtained by crossing each homozygous UAS line (chromosome 3) for both *UAS-CG6480* and *UAS-Slh*, to a homozygous *elav-GAL4* line (chromosome 3), to generate males with one copy of each transgene *in trans.* All lines were homozygous for the *w* mutation. As a control, white-eyed females (w^{1118}) were crossed to each UAS-transgene. Behavioural analysis was performed as described in Chapter 3.

6.3 Results

Knockdown of *CG6480*

The aggression level of the transgenic flies carrying the *UAS-RNAi* with the GAL4 driver, and their respective controls, is shown in Figure 6.3. An overall comparison using the non-parametric Kruskal-Wallis one-way ANOVA by ranks test indicated significant difference in aggression between the genotypes tested (χ^2= 8.33, df = 2, p = 0.015). To analyse the aggression of each genotype, a multiple comparison test after Kruskal-Wallis was used for pair-wise group comparison (Siegel and Castellan 1988, pp. 213-214). Flies over-expressing RNAi targeting the gene *CG6480* using the *elav-GAL4* driver, showed a significant increase in the levels of aggression ($p<0.05$) compared with the control groups (flies carrying only the *UAS-RNAi-CG6480*, or *elav-GAL4*). These flies showed an increase in aggression of over 30% compared to the control group, and they also showed an increase aggression of 30% compared with flies expressing only the *elav-GAL4* driver (these flies were tested in a previous experiment described in Chapter 5).

Knockdown of *Slh*

To test the effect of knocking-down the *Slh* gene, the *UAS-RNAi-Slh* transgene was expressed with the same GAL4 drivers used above. Over-expression of the *UAS-RNAi-Slh* (inserted in the 3rd chromosome) using the *elav-GAL4* driver did not result in a significant difference in levels of aggression compared to males

carrying only the *UAS-RNAi-Slh* or the *elav-GAL4* transgenes (Kruskal-Wallis χ^2 = 3.01, df = 2, p = 0.22). Driving another strain of UAS-*RNAi-Slh* (inserted in the 2rd chromosome) with the same GAL4 driver result in developmental arrest and flies did not eclose from pupae. When the *actin-GAL4* driver was used with each of the *UAS-RNAi* lines, development of flies ceased even earlier.

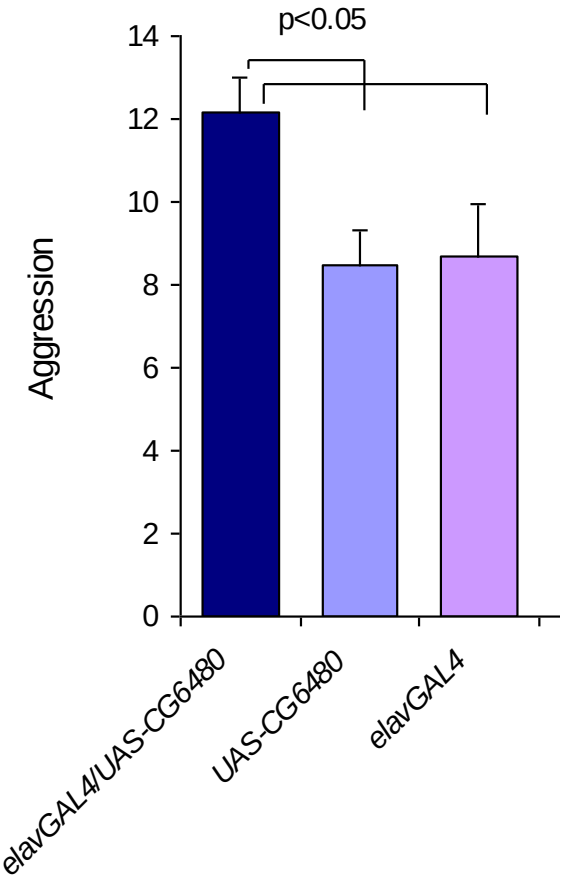


Fig6.3: RNAi knockdown of the gene *CG6480* leads to increased level of aggression. The *UAS-RNAi* transgene was driven with *elav-GAL4* driver (blue) and is compared with control lines expressing either the *UAS* or the *GAL4* transgene only.

6.4 Discussion

The microarray experiments (Chap.4) generated a list of genes that were significantly either up- or down-regulated in the aggressive flies. Here, I have attempted to follow-up the microarray study with a functional analysis of two of these candidate genes, *CG6480* and *Slh* (for which no classical mutants are available) using RNAi. These two transcripts showed the most significant level of downregulation in aggressive flies (Appendix 1). These down-regulated genes were interesting candidate targets for the RNAi approach, since reducing the expression level of these genes in normal flies (i.e. flies that normally show average aggression) is expected in turn to elevate the aggression levels. Experimentally, this design is superior to reducing aggression by knockdown up-regulated genes, because knockdown may lead to non-specific effects that could generate a reduced level of aggression. The RNAi knockdown of *CG6480* (but not of *Slh*) using a pan-neural GAL4 driver (*elav*) resulted in highly aggressive flies. To date, this is the first demonstration of elevating aggressive behaviour by using RNAi of candidate aggression gene.

The UAS-GAL4 system enables the expression of constructs in specific cells by using specific GAL4 drivers. However, when the cellular expression pattern of a gene is unknown, this may lead to expressing the construct (e.g. dsRNA) in locations where the gene is not normally expressed. In the case of driving a UAS-RNAi construct, the targeted transcript for the RNAi will not be present, and no change in the phenotype will occur. This incorrect choice of driver may explain the lack of change in aggression in flies expressing RNAi against *Slh*. It is possible that *Slh* is not normally expressed in neurons (or more precisely, in neurons that are covered by the *elav* driver) but in other brain cells. For example, *ebony*, a gene involved both in aggression and circadian rhythms, was recently found to be expressed in glial cells (not neurons) in the fly's brain (Suh and Jackson, 2007). However, the biological function of SLH is believed to be involved in regulating synaptic transmission as part of the t-SNARE binding complex[2] (Littleton, 2000). This complex is expressed in all neurones, and therefore the *elav*-

[2] Interestingly, SLH binds to Syntaxin (Syx13) to regulate the SNARE complex. *Syx13* was listed as a significantly differentially expressed gene in the microarray experiments (Chapter 4).

GAL4 pan-neural driver is expected to be an effective driver for *Slh-RNAi*. Perhaps *Slh* is a downstream gene, and a reduction in its transcript levels, although induced by aggression, does not change aggression by itself.

RNAi has been recently been successfully used in another study of aggression in *Drosophila* (Chan and Kravitz, 2007). In that study, the neuronal basis for sex-specific aggression was studied by manipulating the levels of *transformer* (*tra*), a gene which is important for sex determination in *Drosophila* (Ashburner, 2005). Expressing RNAi against *tra* in females, using the UAS-GAL4 system[3], resulted in masculinisation of various brain regions (depending on the GAL4 driver used) and in some cases, led to a switch in female behaviour showing a male aggressive pattern (see Introduction).

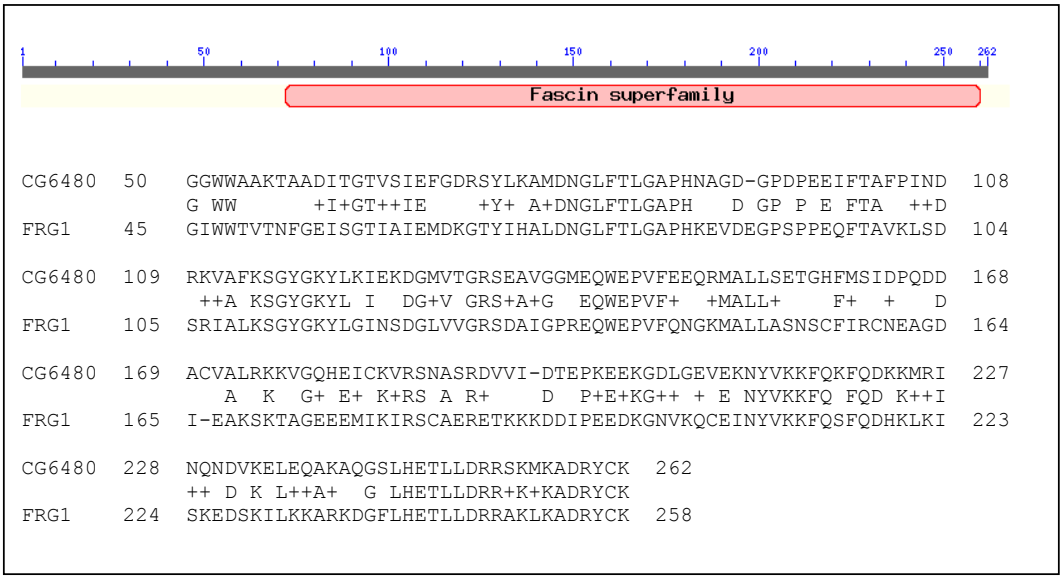
The RNAi approach was also effectively applied in other behavioural studies in *Drosophila*. For example, *Drosophila* females undergo behavioural changes following mating (e.g. unreceptive to courting males), that are induced by the male sex-peptide (SP). By knocking-down the expression of SP receptor (SPR) using RNAi, mated females retained their virginal behaviour and remained receptive towards courting males (Yapici *et al.*, 2008). In another study, the RNAi knock-down of *myospheroid* (*mys*), a gene encoding ßPS (a cell adhesion molecule), driven with mushroom-bodies GAL4 drivers, was found to disrupt olfactory behaviour (Bhandari *et al.*, 2006).

The function of *CG6480* is as yet unknown. To get a better understanding of the gene's role, I have carried out a BLAST search of the Human genome and identified *frg1* as the human ortholog of *CG6480* (Fig. 6.4). FRG1 shows 51% identity (110/215) to *Drosophila* CG6480 (E value = 3e-57) at the protein level. FRG1 and CG6480 share a conserved domain called Fascin (Fig. 6.4). Fascin proteins are histidine-rich and commonly include the repeated motif of HHXH (Yapici *et al.*, 2008; Kureishy *et al.*, 2002). These proteins are evolutionarily conserved and serve in actin cross-linking. Fascins are involved in two forms of actin-based structures: cortical cell extensions and cytoplasm microfilament bundles. The cortical structures, such as filopodia, spikes and lamellipodial ribs,

[3] Similar to the current project, UAS-RNAi strain used in Chan and Kravitz, 2007 were obtained from the Vienna RNAi collection .

have roles in cell-matrix adhesion, cell interactions and cell migration, whereas the cytoplasmic actin bundles appear to participate in cell architecture.

FRG1 is involved in Facioscapulohumeral muscular dystrophy (FSHD), a dominant neuromuscular disorder in humans (Gabellini *et al.*, 2006). Over-expression of *frg1* in muscles of transgenic mice, similar to FSHD patients, causes abnormal alternative splicing of RNAs. This and other evidence might suggest that this protein is a splicing factor (Gabellini *et al.*, 2006). One may speculate that this function of FRG1 might be conserved in the fly, and changes in the *CG6480* transcript levels are followed by changes in alternative-splicing patterns of other genes, such the one involving the *Dat* gene (Chap. 5), resulting in the fly's inclination to initiate aggression.




```
CG6480   50   GGWWAAKTAADITGTVSIEFGDRSYLKAMDNGLFTLGAPHNAGD-GPDPEEIFTAFPIND  108
               G WW      +I+GT++IE    +Y+ A+DNGLFTLGAPH   D GP P E FTA  ++D
FRG1     45   GIWWTVTNFGEISGTIAIEMDKGTYIHALDNGLFTLGAPHKEVDEGPSPPEQFTAVKLSD  104

CG6480  109   RKVAFKSGYGKYLKIEKDGMVTGRSEAVGGMEQWEPVFEEQRMALLSETGHFMSIDPQDD  168
                ++A KSGYGKYL I  DG+V GRS+A+G  EQWEPVF+  +MALL+    F+  +   D
FRG1    105   SRIALKSGYGKYLGINSDGLVVGRSDAIGPREQWEPVFQNGKMALLASNSCFIRCNEAGD  164

CG6480  169   ACVALRKKVGQHEICKVRSNASRDVVI-DTEPKEEKGDLGEVEKNYVKKFQKFQDKKMRI  227
                  A  K  G+ E+ K+RS A R+    D  P+E+KG++ + E NYVKKFQ FQD K++I
FRG1    165   I-EAKSKTAGEEEMIKIRSCAERETKKKDDIPEEDKGNVKQCEINYVKKFQSFQDHKLKI  223

CG6480  228   NQNDVKELEQAKAQGSLHETLLDRRSKMKADRYCK  262
              ++ D K L++A+  G LHETLLDRR+K+KADRYCK
FRG1    224   SKEDSKILKKARKDGFLHETLLDRRAKLKADRYCK  258
```

Fig 6.4 The human ortholog of *CG6480* is *frg1*. Protein alignment of CG6480 with the human FGR1. The Fascin conserved domain is shown at the top.

The RNAi technique has its limitations. Ideally, RNAi should have been verified by demonstrating change in mRNA and protein levels (e.g. by RT-PCR and Western blots, if antibodies are available). However, changes in transcript levels might be too small to be detected, because they may be limited to a small group of neurons. In this case, using *in-situ* hybridization would be a better approach. Defining the expression pattern of these genes would allow the selection of more

appropriate GAL4 drivers. Still, the RNAi was assumed to be effective based on the observed changes in the phenotype (in the case of *CG6480*) or development (failure of eclosion, in the case of *Slh -RNAi*).

In addition to *in-situ* hybridizations, another useful way to follow up the study of these genes would be to find and subclone their promoter region and construct a specific GAL4 driver (e.g. *CG6480-GAL4*). These drivers can then be used together with *UAS-GFP* to map the brain regions that express these genes, or combined with *UAS-RNAi* to drive knockdown of these genes in the relevant cells. In addition, preparation of antibodies against SLH and CG6480 would allow monitoring the aggression changes at the protein level. Furthermore, the use of immunocytochemistry, combined with confocal microscopy, would allow the study of these proteins at the cellular levels and link this to aggressive behaviour in flies. Clearly, this type of study might initiate another PhD project.

Chapter 7

GENERAL DISCUSSION

Studies of behaviour are broadly classified into those addressing the evolutionary aspects (the 'why' questions about ultimate causes), and those aiming to uncover the cellular and molecular mechanisms that generate the motor output (the 'how' questions about proximate causes) (Alcock, 2005). The focus of this thesis was mechanistic: to identify the transcriptional changes associated with aggression in *Drosophila*, which in turn would provide a list of candidate genes that play important role in regulating aggression. Genome-wide microarray profiling combined with large-scale behavioural phenotyping had resulted in a list of genes whose level of expression significantly changed in the fly head following agonistic encounters (Chapter 4) and paved the way for a finer characterisation of these putative aggression genes (e.g. Chapters 5-6).

While this project was underway, several other studies have been published that have taken a similar approach (Edwards *et al.*, 2006; Dierick and Greenspan, 2006; Wang *et al.*, 2008). The concordance between the results is rather small, which might be attributed to various differences in the experimental approach (see Chapter 4: Discussion). For example, while the studies of Dierick *et al.* and Edwards *et al.* were based on capturing genetic variation (by artificial selection) the current study was built on stochastic (environmental) variation in expression levels that would result in identifiable phenotypic variation. It is also possible that the observed overlap between these studies is largely underestimated because of the different ways the data were analysed. It seems that the microarray technology (and the bulk of data it generates) has evolved before the appropriate analysis tools have become available. I have previously discussed in Chapter 4 how the seemingly poor agreement between microarray studies may be resolved in the future by applying new meta-analysis methods (Owens, 2005).

The full potential of the current study and other microarray studies has not been exploited. Some overlap between the microarray studies does exist, which can lead to identifying new aggression genes. For example, in the field of the circadian

clock, the data from several microarray studies in *Drosophila* led to identification of a new core clock gene, *clock-work orange* (Kadener *et al.*, 2007; Matsumoto *et al.*, 2007), and similarly the few genes that are shared between the different aggression studies (Chapter 4) such as *Opb99b* and *CG13794*, may suggest a role for these genes in aggression with some confidence and are thus worth further investigation. But the main power of the microarray approach lies in the simultaneous analysis at the genome level, and goes beyond the identification of individual genes. It is likely that the data from the current studies (and others) will be integrated with other 'omic' databases, such as protein-protein interaction, metabolic and other pathways and transcription factor data, which will generate new insights into aggression. As information on the spatial expression in *Drosophila* is accumulating (for example, the FlyAtlas project, Chintapalli *et al.*, 2007), integration of expression information at the cellular and behavioural level will be particularly informative.

The rationale behind research in *Drosophila* is of reductionism, aiming at identifying molecular components and network principles in a relatively simple system (compact genome, smaller brain) that can later be tracked down in mammalian systems. This was the case in the field of chronobiology, where the identification of first circadian gene (*period*) by Konopoka and Benzer (1971) was instrumental in the later understanding of this system in mammals. The research into aggression in *Drosophila* however, was only launched a few years ago and is rather lagging behind the studies in mammals. Most of the first studies on aggression in *Drosophila* (including this one) were aimed at establishing the experimental set-up and to test candidate genes whose role in aggression was already implicated in mammalian aggression, for example the genes encoding biogenic amines (Hoyer *et al.*, 2008). Aggression research in *Drosophila* is currently entering a new phase where insights from studies in flies might inform the research in mammals.

Three recent technological developments will probably break new ground in aggression research in *Drosophila*. The first two involve the automation of monitoring *Drosophila* behaviour (Dankert *et al.*, 2009; Branson *et al.*, 2009) by video tracking. CADABRA, the system developed by Dankert *et al.* (2009) allows

videotaping the behaviour of pairs of flies. The software detects the position and orientation of the body and wings of each fly, and the motor output (i.e. behaviour) is automatically inferred and recorded. Specific motor outputs defined by the user can be used as a classifier to 'teach' the software to identify specific behaviours. For example, a prolonged single wing extension is associated with courtship (the vibration of the wing for generating a courtship song) while extension of both wings is associated with aggression ('wing threat'). A single camera monitors two separate pairs of flies (in a double chamber), and, with four cameras, four double chambers and two personal computers, the system can accomplish a medium-throughput behavioural screen monitoring eight pairs of flies in a session (this of course can be scaled up). Using this system, Dankert *et al.* (2009) corroborated previous studies showing that silencing of octopaminergic neurons reduces aggression (Hoyer *et al.*, 2008) as does expressing the female isoform of *fruitless* (fru^F) in males (Vrontou *et al.*, 2006).

The system developed by Branson *et al.* (2009) has even a greater potential for generating new insights into aggression. This system can monitor the individual behaviour of a large numbers of flies simultaneously. This will allow the study of behaviour in a more realistic and natural setting, as flies are normally found in high-density populations in the wild (Gromko and Markow, 1993). Previous studies, for example, showed that courtship behaviour (specifically the courtship song), which is normally studied in the laboratory using single male-female pairs, changes dramatically when multiple males are courting the female (Tauber and Eberl, 2001). Similarly, using this system to study aggression may reveal that males and females modify their behaviour in the presence of multiple conspecifics, and this may have bearings both at the mechanistic level, for example how learning and memory process are tied to aggression, and at elucidating the evolutionary forces that shape this behaviour.

The third technological breakthrough in *Drosophila* neurogenetics is the development of 'phototriggers' that allow activation of selected subsets of neurons by light (Lima and Miesenbock, 2005). This technique is based on introducing ATP-gated ionic channel $P2X_2$ into neurons of choice by using the GAL4-UAS system. Flies are then injected with a caged-ATP molecule, and following short UV

light pulses, the ATP is released, activating the $P2X_2$ channels which cause the neuron to fire action potentials. Stimulating the appropriate neurons allows remote activation of specific behaviours, and this was elegantly demonstrated by Clyne and Miesenböck (2008), who were able to elicit quasi-normal courtship songs by photo-activation of thoracic circuits in headless males. Intriguingly, photoactivated females also produced a courtship song (albeit more rudimentary) indicating that the same neural circuit is present, but not active, in females. With the fine description of the motor output of aggression which in now available (Nilsen *et al.*, 2004), one can envision how systematically expressing the phototriggers in various neurons (using different GAL4 and GAL80 drivers) would allow identifying the neural circuits of aggression.

It is likely that aggression is interlinked to other behaviours in *Drosophila*, both at the cellular and the genetic levels. Jordan *et al.* (2007) applied artificial selection and microarray profiling to study various behaviours (locomotion, copulation and aggression) using the same base fly population. The fact that a substantial fraction of the transcriptome was differentially expressed in all of these behaviours (~10%) suggested that many genes are involved in multiple functions (i.e. pleiotropic), which was indeed evident from the overlap between the lists of the differentially expressed genes in the various behaviours. However, the overlapping genes did not always show the same direction of expression change in the different experiments (Jordan *et al.*, 2007), and this ruled out the possibility that a basic behaviour (i.e. locomotion) is the basis of this overlap. Instead, Jordan *et al.* (2007) proposed a modified version of antagonistic pleiotropy where different alleles of a gene (for example, encoding different domains of the protein) serve different biological functions. This may allow natural selection targeting one part of the gene, without changing the function of the gene in other behaviours. For example, in the *per* gene there are several alleles (generated by mutagenesis) that change both circadian rhythmicity (Konopka and Benzer, 1971) and the courtship song (Kyriacou and Hall, 1980). Yet other (natural) variants exist in *per* that encode various threonine–glycine (TG) repeat lengths (e.g. TG_{17}, TG_{20}). The TG polymorphism strongly affects the periodicity of the courtship song, while the circadian rhythm remains largely unchanged (Yu *et al.*, 1987). Thus, the TG polymorphism allows evolutionary divergence in one trait, the courtship song (that

may be used for sexual selection/isolation), without disturbing a more conserved function, the circadian rhythm, of the same gene.

A behaviour that seems to be intimately linked to aggression is male courtship. Although from an evolutionary perspective, these behaviours are opposed (repulsion vs. attraction), at the proximate levels they share many elements in common, suggesting that the underlying neural circuits might be intermingled. Both behaviours involve integration of sensory information about con-specifics, and share some similar motor outputs such as chasing and wing(s) extension (see Chapter 3). Both behaviours involve learning and memory circuits (Yurkovic *et al.*, 2006; Mehren *et al.*, 2004), and both are stimulated by social deprivation (Dankert *et al.*, 2009). A key component in both behaviours is *fru*. The male-specific isoform Fru^M is necessary for execution of both the courtship behaviour (reviewed by Greenspan and Ferveur, 2000) and aggression (Vrontou *et al.*, 2006). A detailed analysis of Fru^M expression reveal subsets of neurons that can act as 'command neurons' for triggering each of the behaviours: To exhibit the complete courtship ritual, a group of about 60 neurons expressing Fru^M in the median bundle in the suboesophageal ganglion (SOG) are required (Manoli and Baker, 2004). Males deprived of Fru^M in these neurons (by expressing RNA interference transgene) show reduced courtship latency and skip the initial elements of courtship, orienting and tapping. Manoli and Baker (2004) concluded that this group of neurons normally inhibit progression of courtship until sufficient sensory information is integrated. Similarly, a small subset of neurons in the SOG which co-express Fru^M and octopamine are necessary for aggression (Certel *et al.*, 2007). Feminisation of these neurons by introducing the *UAS-transformer* transgene resulted in males that showed courtship instead of aggression towards other males (Certel *et al.*, 2007). This may suggest that these neurons are involved in recognition of the sex of con-specifics (one of these neurons does seem to receive input from various sensory modalities), or even represent a switch for male behavioural choice (Certel *et al.*, 2007). The overlap between the subsets of neurons identified in the two studies remains to be examined.

One of the genes that this study focused on was *Dat*, which is involved in dopamine metabolism (Chapter 4, 5). Dopamine has been implicated in aggression

in human and in various model organisms. The role of dopamine in humans and mammals was believed to be mainly connected to craving and reward, motivation and attention (Franken *et al.*, 2005). However, several studies showed evidence for the involvement of dopamine in aggression. For example, studies on Alzheimer patients showed an association between polymorphisms in the dopamine receptor gene *DRD1* and aggressive behaviour (Sweet *et al.*, 1998; Holmes *et al.*, 2001). A single nucleotide polymorphic site (SNP) upstream of the coding region was linked to increased aggression in individuals homozygous for one of the alleles, possibly because of altered expression of the receptor.

Studies in different model-organisms have allowed the testing of the effect of dopamine on aggression in a more direct way. In mice (Couppis and Kennedy, 2008), infusion of dopamine receptor antagonist (competing with dopamine) led to reduction in aggressive-associated behaviours in the resident – intruder paradigm. In rats, dopamine levels were monitored locally and in real-time by using a probe implanted in the nucleus accumbens (NAc, the same brain region that was targeted in the mouse study described above). Dopamine levels were found to increase significantly during aggressive behaviour (van Erp and Miczek, 2000; van Erp and Miczek, 2007). Interestingly, rats trained to fight at specific times showed an elevated level of dopamine at the expected time of fight even in the absence of an intruder male. This increase of dopamine induced by anticipation for confrontation led to the theory of "dopamine reward" in promoting aggression (see Introduction).

Higher dopamine levels were also associated with aggression in invertebrates. In crickets, depletion of dopamine (and octopamine) by injection of a dopamine inhibitor (α-methyl-p-tyrosine AMT) led to a significant reduction in aggression, and in the intensity of fights (Stevenson *et al.*, 2000). Similarly, higher levels of dopamine were measured in shore crab males after winning aggressive encounters, compared with loser males (Sneddon *et al.*, 2000).

The role that this gene (and dopamine in general) plays in regulation of sleep and wakefulness was mentioned earlier (Chapter 5 Discussion). *Dat* was found to be differentially expressed in two sleep microarray studies (Shaw *et al.*, 2000; Cirelli *et al.*, 2005), and flies carry a null mutation (Dat^{lo}) or a deficiency of this locus required a greater rest rebound after sleep deprivation (Shaw *et al.*, 2000).

It is likely that arousal level is intimately linked to propensity for aggression, and that the two systems are associated, probably by dopamine. Rats that are deprived of rapid eye movement (REM) during sleep and also administrated with dopaminergic agonists drugs shows increased tendency for aggression (Tufik, 1981), which was explained by increased sensitivity of dopamine receptors in the brain. In *Drosophila*, administration of methamphetamine (METH), a drug that triggers the release of dopamine (and also inhibits dopamine reuptake) increases wakefulness and behavioural arousal (Andretic *et al.*, 2005). In another study (Kume *et al.*, 2005), a mutation in the *dopamine transporter* gene (*DAT*, not to be confused with *Dat*) called *fumin* has been shown to cause a dramatic increase in wakefulness, and a decrease in arousal threshold in flies. The fact that *Drosophila* is an emerging model system to study sleep (Harbison *et al.*, 2009), alcohol induced behaviours (Heberlein, 2000) and drug addiction (Wolf, 1999) will contribute to elucidating the link between aggression and awareness, and provide a stepping stone to understand these important functions in our own species.

APPENDICES

Appendix 1. List of differentially expressed genes.

	Affy ID	P-value	name	Change	Description
1	153110_at	0.0011	Int6	Up	Translation initiation factor activity.
2	146503_at	0.0012	CG10363	Up	protease inhibitor activity; sugar:hydrogen symporter activity
3	152456_at	0.0016	*Dat*	Up	N-acetyltransferase activity. development
4	142795_at	0.0016	CG8311	Up	dolichol kinase activity.
5	142408_at	0.0017	CG4729	Up	1-acylglycerol-3-phosphate O-acyltransferase activity.
6	150335_at	0.0021	fit	Up	molecular function unknown.
7	146184_at	0.0021	CG17108	Up	acetyl-CoA carboxylase activity.
8	154056_at	0.0022	RpL1	Up	structural constituent of ribosome.
9	147517_at	0.0024	Obp56e	Up	odorant binding. sensory perception of chemical stimulus.
10	141674_at	0.0024	PyK	Up	pyruvate kinase activity.
11	145265_at	0.0025	CG6762	Up	molecular function is unknown.
12	151667_s_at	0.0026	CG6673	Up	glutathione transferase activity .
13	152543_at	0.0027	CG2025	Up	metalloendopeptidase activity. involved in proteolysis
14	153350_at	0.0029	Ef1&agr;48D	Up	
15	155137_at	0.004	CG8067	Up	methyltransferase activity
16	146165_at	0.0043	Lip4	Up	triacylglycerol lipase activity.
17	152988_at	0.0048	CG14969	Up	molecular function unknown.
18	152571_at	0.005	CG7966	Up	selenium binding
19	153791_at	0.0054	l(2)NC136	Up	transcription regulator activity
20	143849_at	0.0056	*Slh*	Down	SNARE binding.
21	142613_at	0.0056	*CG6480*	Down	molecular function unknown.
22	145669_at	0.0057	CG15400	Up	glucose-6-phosphatase activity
23	153449_at	0.0057	eEF1&dgr;	Up	
24	148641_at	0.0058	Adk1	Up	adenylate kinase activity.
25	143205_at	0.0059	janA	Up	molecular function is unknown. sex differentiation.
26	151323_at	0.006	CG15210	Up	molecular function unknown.
27	152287_at	0.0061	mbf1	Up	methyl-CpG binding. central nervous system development.
28	141465_at	0.0062	CG10026	Up	retinal binding transporter activity.
29	153412_at	0.007	wtrw	Up	ion channel activity; calcium channel activity.
30	145974_at	0.007	*CG13794*	Up	neurotransmitter transporter activity.
31	141618_at	0.0071	Kary&bgr;3	Up	

32	148983_at	0.0073	Tsp74F	Up	molecular function is unknown.
33	153007_at	0.0076	Ac76E	Up	adenylate cyclase activity.
34	144700_at	0.0078	CG15347	Up	molecular function is unknown.
35	153028_at	0.0079	guf	Up	molecular function is unknown.
36	149907_r_at	0.0079	CG8066	Up	cysteine protease inhibitor activity
37	147338_at	0.0081	CG6435	Up	molecular function is unknown.
38	154495_at	0.0082	CG6459	Up	molecular function is unknown.
39	149964_at	0.0087	CG14872	Up	transporter activity; binding.
40	142967_at	0.0091	CAP	Up	vinculin binding
41	143949_at	0.0091	scu	Up	steroid dehydrogenase activity.
42	141762_at	0.0093	Cp1	Up	cysteine-type endopeptidase activity. autophagic cell death.
43	143011_at	0.0094	CG4822	Up	ATPase activity.
44	152262_at	0.0095	CG31547	Up	sodium:potassium:chloride symporter activity.
45	150604_at	0.0096	RpL27	Up	structural constituent of ribosome.
46	143904_at	0.0102	RpS20	Up	structural constituent of ribosome.
47	146695_at	0.0102	CG3287	Up	molecular function is unknown.
48	143678_at	0.0103	PebIII	Up	molecular function is unknown.
49	141536_at	0.0104	Sply	Up	sphinganine-1-phosphate aldolase activity
50	146746_at	0.0107	Cyt-b5	Up	electron carrier activity
51	143911_at	0.0107	ATPsyn-&ggr;	Up	
52	154689_at	0.0107	CG7789	Up	5'-bisphosphate nucleotidase activity
53	152350_at	0.0108	CG1927	Up	molecular function is unknown.
54	150984_at	0.0108	CG1732	Up	sodium symporter activity
55	143126_at	0.0112	dm	Up	transcription factor activity
56	147696_at	0.0113	CG13510	Up	molecular function is unknown.
57	153098_at	0.0117	SPE	Up	serine-type endopeptidase activity
58	154581_at	0.0119	CG10632	Up	molecular function is unknown.
59	151143_at	0.012	CG13551	Up	enzyme inhibitor activity
60	153088_at	0.0121	pst	Up	learning and/or memory; olfactory learning.
61	153379_at	0.0122	eys	Down	calcium ion binding
62	146967_s_at	0.0122	RpS15Ab	Up	structural constituent of ribosome
63	142465_at	0.0124	sls	Up	myosin light chain kinase activity
64	149035_at	0.0125	CG14084	Up	molecular function is unknown.
65	143607_at	0.0125	Def	Down	molecular function is unknown.
66	152752_at	0.0126	CG3829	Up	scavenger receptor activity involved in defense response.
67	149162_at	0.0127	CG10584	Up	molecular function is unknown.
68	143365_at	0.0127	sr	Up	RNA polymerase II transcription factor activity
69	147743_at	0.0128	RpL37b	Up	structural constituent of ribosome
70	154701_at	0.0128	Atg5	Up	molecular function is unknown.
71	153774_at	0.0129	lama	Up	molecular function is unknown.
72	151845_at	0.0129	wdp	Up	protein binding.
73	153527_at	0.0131	CG1572	Up	molecular function is unknown.
74	142257_at	0.0131	CG10863	Up	aldehyde reductase activity

75	154383_at	0.0132	CG10602	Up	
76	142310_at	0.0135	Est7	Up	
77	152308_at	0.0137	CG10433	Up	molecular function is unknown.
78	152606_at	0.0138	CG10226	Up	ATPase activity
79	150837_at	0.0138	***Obp99b***	Up	odorant binding
80	141291_at	0.0139	Hcf	Down	transcription factor activity.
81	154545_at	0.0139	FK506-bp1	Down	peptidyl-prolyl cis-trans isomerase activity
82	152521_at	0.0142	CG1441	Up	catalytic activity; binding.
83	142661_at	0.0145	Vha100-2	Up	hydrogen-exporting ATPase activity
84	150281_at	0.0145	RpS30	Up	structural constituent of ribosome
85	152907_at	0.0146	18w	Up	transmembrane receptor activity
86	148647_at	0.0147	Pbgs	Up	porphobilinogen synthase activity.
87	142523_at	0.0149	CG9297	Up	calcium ion binding
88	150964_at	0.015	krz	Up	molecular function is unknown.
89	146868_at	0.015	CG8788	Up	molecular function is unknown.
90	152461_at	0.0151	Adam	Up	translation initiation factor activity.
91	146749_at	0.0152	Corin	Up	serine-type endopeptidase activity
92	152158_at	0.0153	CG3308	Up	deoxyribonuclease activity
93	143369_at	0.0154	sta	Up	structural constituent of ribosome
94	154616_at	0.0154	CG13770	Up	molecular function is unknown.
95	152183_at	0.0156	CG4680	Up	molecular function is unknown.
96	AFFX-BioC-5_at	0.0156		Up	
97	147497_at	0.0157	cer	Up	cysteine protease inhibitor activity
98	142784_at	0.0158	CG9797	Up	zinc ion binding
99	154804_at	0.016	CG8232	Up	poly(A)-specific ribonuclease activity
100	152937_at	0.0162	Cyp4ac1	Up	electron carrier activity; heme binding; iron ion binding
101	149303_at	0.0162	CG14661	Up	molecular function is unknown.
102	151927_at	0.0162	Cpr72Ec	Down	structural constituent of chitin-based cuticle.
103	152629_at	0.0163	CG5455	Down	molecular function is unknown.
104	153163_at	0.0163	Roe1	Up	adenyl-nucleotide exchange factor activity
105	144882_at	0.0163	PGRP-SA	Up	peptidoglycan receptor activity
106	151360_at	0.0165	CG7418	Up	molecular function is unknown.
107	142518_at	0.0168	Nckx30C	Down	calcium, potassium:sodium antiporter activity.
108	153141_at	0.0169	Clc	Up	neurotransmitter transporter activity
109	143143_at	0.0177	Ef2b	Up	GTPase activity.
110	146694_at	0.0179	ubl	Up	molecular function is unknown.
111	148822_at	0.0179	comm2	Down	molecular function is unknown.
112	153280_at	0.018	CG1622	Down	molecular function is unknown.
113	154748_at	0.018	cdm	Up	protein transporter activity
114	153869_at	0.0181	Gas41	Down	general RNA polymerase II transcription factor activity.
115	152196_at	0.0181	CG10345	Up	scavenger receptor activity
116	141685_at	0.0182	Sp7	Up	serine-type peptidase activity
117	143078_at	0.0183	Arr2	Up	metarhodopsin binding

118	143951_at	0.0184	cnk	Up	protein binding;signal transduction
119	152891_at	0.0184	santa-maria	Up	scavenger receptor activity
120	152396_at	0.0184	CG8475	Up	phosphorylase kinase regulator activity
121	152964_at	0.0185	CG8485	Down	SAP kinase activity
122	150576_at	0.0186	CG10514	Up	molecular function is unknown.
123	152538_at	0.0189	Scs-fp	Up	succinate dehydrogenase activity
124	144094_at	0.019	Smox	Down	transforming growth factor beta receptor
125	153376_at	0.0191	skpA	Up	protein binding; centrosome duplication; chromosome condensation
126	141345_at	0.0192	CG5844	Down	dodecenoyl-CoA delta-isomerase activity.
127	153010_at	0.0193	Chmp1	Up	molecular function is unknown.
128	148870_at	0.0197	CG4962	Up	molecular function is unknown.
129	148639_at	0.0198	CG6910	Up	molecular function is unknown.
130	152035_at	0.02	CG10373	Up	molecular function is unknown.
131	149688_at	0.02	CG5281	Down	molecular function is unknown.
132	141414_at	0.02	CG9886	Up	glycerate kinase activity.
133	144219_at	0.0207	l(2)35Di	Up	NADH dehydrogenase activity.
134	154112_at	0.0207	CG12207	Up	molecular function is unknown.
135	148608_at	0.0207	RpL10Ab	Up	structural constituent of ribosome.
136	153994_at	0.0209	RpS24	Up	structural constituent of ribosome
137	153891_at	0.021	CG8569	Up	zinc ion binding
138	151349_at	0.0213	sun	Up	hydrogen-exporting ATPase activity
139	152208_at	0.0213	Fkbp13	Up	FK506 binding
140	153405_at	0.0214	cutlet	Up	DNA binding; ATP binding; cell proliferation.
141	150020_at	0.0215	CG14898	Up	molecular function is unknown.
142	141523_at	0.0226	CG1952	Up	molecular function is unknown.
143	154623_at	0.0228	CG15893	Up	molecular function is unknown.
144	AFFX-CreX-3_at	0.0228		Up	
145	153024_at	0.0229	Ca-P60A	Up	calcium-transporting ATPase activity
146	141810_at	0.0229	Nc73EF	Up	oxoglutarate dehydrogenase (succinyl-transferring) activity
147	153381_at	0.023	CG5973	Up	retinal binding; transporter activity
148	153031_at	0.023	Cyp9f2	Up	electron carrier activity; heme binding; iron ion binding; monooxygenase activity.
149	141589_at	0.0232	Sulf1	Up	N-acetylglucosamine-6-sulfatase activity.
150	148694_at	0.0234	Syx13	Up	SNAP receptor activity
151	152619_at	0.0236	CG15652	Up	molecular function is unknown.
152	153901_at	0.0238	CG6194	Up	cysteine-type endopeptidase activity
153	151918_s_at	0.0239	drpr	Up	molecular function is unknown.
154	147686_at	0.0241	RpS16	Up	structural constituent of ribosome.
155	143018_at	0.0246	tko	Up	structural constituent of ribosome.
156	152371_at	0.0246	Irk2	Up	inward rectifier potassium channel activity;
157	142674_at	0.0246	Snx6	Up	protein binding ;cell communication
158	146919_at	0.0247	mRpL42	Up	structural constituent of ribosome.
159	150866_at	0.0247	CG7911	Up	molecular function is unknown.

160	151958_at	0.0248	Spn1	Up	serine-type endopeptidase inhibitor activity.
161	142164_at	0.0249	CG11601	Up	molecular function is unknown.
162	143788_at	0.0249	Pcmt	Up	protein-L-isoaspartate (D-aspartate) O-methyltransferase activity
163	153887_at	0.0251	Rab4	Up	GTPase activity
164	155147_at	0.0254	CG1307	Up	aminoacyl-tRNA hydrolase activity
165	AFFX-BioDn-3_at	0.0262		Up	
166	142669_at	0.0262	Ost48	Up	dolichyl-diphosphooligosaccharide-protein glycotransferase activity.
167	142829_at	0.0262	CG6961	Down	molecular function is unknown.
168	153784_at	0.0262	a6	Down	odorant binding
169	153500_at	0.0263	CG11984	Up	potassium channel regulator activity
170	141413_at	0.0263	CG4699	Up	aminoacyl-tRNA ligase activity
171	153876_at	0.0263	RpL19	Up	structural constituent of ribosome
172	155042_at	0.0264	scrib	Down	protein binding.
173	146865_at	0.0268	tsu	Up	mRNA binding; microtubule cytoskeleton organization and biogenesis.
174	141298_at	0.0268	CG9186	Up	molecular function is unknown.
175	149933_at	0.027	CG6966	Down	molecular function is unknown.
176	153401_at	0.0271	CG7214	Up	molecular function is unknown.
177	151817_at	0.0271	CG8329	Up	serine-type endopeptidase activity.
178	143390_at	0.0272	&bgr;Tub56D	Up	
179	154226_at	0.0273	ctp	Up	ATPase activity microtubule motor activity .
180	148013_at	0.0274	CG1887	Down	scavenger receptor activity
181	152753_at	0.0276	sec10	Up	molecular function is unknown.
182	143892_at	0.0276	RpS3A	Up	structural constituent of ribosome
183	149106_at	0.0277	CG17233	Down	molecular function is unknown.
184	142891_at	0.028	obst-A	Up	structural constituent of peritrophic membrane; chitin binding
185	143898_at	0.0282	RpL14	Up	structural constituent of ribosome.
186	143265_at	0.0282	Mlc2	Up	calcium ion binding
187	147435_at	0.0285	GstE2	Up	glutathione transferase activity.
188	152020_at	0.0287	CG31150	Up	lipid transporter activity.
189	141486_at	0.0289	Csp	Up	unfolded protein binding; neurotransmitter secretion
190	141647_at	0.0289	CG12016	Up	molecular function is unknown.
191	141784_at	0.029	spir	Up	microtubule binding; actin binding
192	142226_at	0.0291	Sap-r	Up	molecular function is unknown.
193	148529_at	0.0295	CG14153	Up	molecular function is unknown.
194	148912_at	0.0297	CG13026	Up	molecular function is unknown.
195	154368_at	0.03	CG2994	Up	oxidoreductase activity
196	141587_at	0.0302	sec24	Up	zinc ion binding
197	154140_at	0.0303	l(2)gd1	Up	molecular function is unknown.
198	150927_at	0.0307	CG15556	Up	G-protein coupled receptor activity.
199	154434_at	0.0308	CG16972	Up	molecular function is unknown.

200	153843_at	0.031	tacc	Up	microtubule binding
201	143150_at	0.0311	Eno	Up	phosphopyruvate hydratase activity.
202	144100_at	0.0311	CG6421	Up	calcium ion binding
203	145422_at	0.0313	HERC2	Down	guanyl-nucleotide exchange factor activity
204	144147_at	0.0315	BM-40-SPARC	Up	calcium ion binding
205	142505_at	0.0317	eIF-3p40	Up	translation initiation factor activity.
206	152059_at	0.0317	CG1516	Up	pyruvate carboxylase activity
207	150225_at	0.0323	Arc42	Up	RNA polymerase II transcription mediator activity; acyl-CoA dehydrogenase activity
208	155022_at	0.0323	Hem	Up	protein binding,
209	151974_at	0.0324	CG8312	Up	molecular function is unknown.
210	151839_at	0.0324	vir-1	Up	molecular function is unknown. defence response to virus.
211	154280_at	0.0325	Jhebp29	Up	protein binding.
212	145343_at	0.0328	CG7423	Up	molecular function is unknown.
213	154672_at	0.0328	torp4a	Up	ATP binding; unfolded protein binding
214	148352_at	0.033	PGRP-SD	Up	peptidoglycan binding; N-acetylmuramoyl-L-alanine amidase activity
215	152070_at	0.0332	CG10992	Up	cysteine-type endopeptidase activity
216	154959_at	0.0335	CG5728	Up	mRNA binding. regulation of alternative nuclear mRNA splicing via spliceosome.
217	142213_at	0.0339	CSN7b	Up	molecular function is unknown.
218	154993_at	0.0341	Neurochondrin	Down	molecular function is unknown.
219	154269_at	0.0342	Acon	Up	aconitate hydratase activity
220	144234_at	0.0342	sec71	Up	ARF guanyl-nucleotide exchange factor activity.
221	152054_at	0.0343	CG3775	Up	metalloendopeptidase activity
222	151605_at	0.0345		Up	
223	152379_at	0.0345	IRP	Up	iron ion binding
224	141629_at	0.0347	eIF-4E	Up	translation initiation factor activity.
225	141712_at	0.0348	CDase	Down	ceramidase activity.
226	142388_at	0.0349	CG9447	Down	molecular function is unknown.
227	145660_at	0.035	CG31690	Up	UDP-N-acetylglucosamine-peptide N-acetylglucosaminyltransferase activity
228	143239_at	0.035	Lsp2	Down	nutrient reservoir activity; oxygen transporter activity.
229	143263_at	0.0353	Mhc	Up	structural constituent of muscle; ATPase activity
230	149130_at	0.0353	CG5195	Up	DNA polymerase activity
231	151962_at	0.0353	CG5597	Up	molecular function is unknown.
232	145463_at	0.0358	CG34120	Up	transmembrane movement of substances; transporter activity; ATP binding
233	147516_at	0.0358	Obp56d	Up	odorant binding
234	153002_at	0.0359	Timp	Up	metalloendopeptidase inhibitor activity
235	147274_at	0.0362	CG8397	Up	actin binding

236	AFFX-BioC-3_at	0.0363		Up	
237	147561_at	0.0364	maf-S	Up	RNA polymerase II transcription factor activity;
238	146874_at	0.0365	CG2063	Down	molecular function is unknown.
239	153461_at	0.0367	CG1672	Up	phospholipase A2 activity
240	146953_at	0.0367	trsn	Up	sequence-specific DNA binding.
241	145788_at	0.037	CG3008	Up	protein kinase activity
242	152958_at	0.0371	Cyp4d21	Up	electron carrier activity; heme binding; iron ion binding
243	148532_at	0.0372	CG6463	Up	NADH dehydrogenase (ubiquinone) activity
244	148040_at	0.0373	CG8993	Up	disulfide oxidoreductase activity
245	148338_at	0.0373	CG8543	Down	structural constituent of chitin-based cuticle.
246	152303_at	0.0374	CG15309	Up	molecular function is unknown.
247	143550_at	0.0375	RpL7	Up	structural constituent of ribosome
248	155125_at	0.0377	CG7523	Up	molecular function is unknown.
249	154156_at	0.0379	CG5033	Down	ribonucleoprotein binding
250	154768_at	0.0379	CG5482	Up	FK506 binding; peptidyl-prolyl cis-trans isomerase activity, involved in protein folding.
251	152407_at	0.0381	CG11198	Up	acetyl-CoA carboxylase activity
252	151930_at	0.0381	Vha26	Up	hydrogen-exporting ATPase activity,
253	153037_at	0.0383	Ncc69	Up	sodium:chloride symporter activity
254	150567_at	0.0384	RpS27	Up	structural constituent of ribosome
255	142590_at	0.0387	CG9066	Up	transition metal ion binding; heme binding
256	AFFX-BioB-M_at	0.0388		Up	
257	145183_at	0.0393	CG9921	Up	molecular function is unknown.
258	146379_at	0.0395	Ugt36Bc	Up	glucuronosyltransferase activity
259	152579_at	0.0397	daw	Up	transforming growth factor beta receptor binding; growth factor activity
260	152422_at	0.0397	pio	Up	molecular function is unknown.
261	152036_at	0.0401	NP15.6	Up	molecular function is unknown.
262	151937_at	0.0402	CG1213	Up	glucose transmembrane transporter activity.
263	146323_at	0.0402	CG5945	Up	molecular function is unknown.
264	145856_at	0.0403	CG13999	Down	molecular function is unknown.
265	149663_at	0.0404	Ugt86Dd	Up	glucuronosyltransferase activity
266	142583_at	0.0405	CG9090	Up	phosphate transmembrane transporter activity
267	152255_at	0.0407	CG6330	Up	uridine phosphorylase activity.
268	146622_at	0.0408	Tif-IA	Down	RNA polymerase I transcription factor activity
269	151418_at	0.0408	CG13779	Up	peptidase activity
270	152923_at	0.041	CG10585	Down	trans-hexaprenyltranstransferase activity.
271	141768_at	0.0412	Adk2	Up	adenylate kinase activity
272	151664_at	0.0413	Gtp-bp	Up	GTPase activity;
273	153177_at	0.0414	coilin	Up	molecular function is unknown.
274	141583_at	0.0415	Cyp6a20	Up	electron carrier activity; heme binding
275	151429_at	0.0416	CG14933	Up	molecular function is unknown.

276	153468_at	0.0419	Nrv1	Up	potassium-exchanging ATPase activity.
277	145863_at	0.0422	CG9098	Up	SH3/SH2 adaptor activity
278	152591_at	0.0426	CG11395	Up	molecular function is unknown.
279	153344_at	0.0426	CG10664	Up	cytochrome-c oxidase activity
280	141328_at	0.0427	mthl8	Up	G-protein coupled receptor activity
281	151496_s_at	0.0432		Up	
282	151021_at	0.0433	CG11985	Up	molecular function is unknown.
283	152501_at	0.0439	Thiolase	Up	acetyl-CoA C-acyltransferase activity
284	144653_at	0.0442	Rab39	Up	GTPase activity
285	153750_at	0.0443	CG7289	Up	molecular function is unknown.
286	141797_at	0.0443	icln	Down	volume-sensitive anion channel activity.
287	152940_at	0.0444	CG9663	Up	ATPase activity
288	150876_at	0.0445	CG1983	Down	molecular function is unknown.
289	151263_at	0.0446	CG7630	Up	molecular function is unknown.
290	146017_at	0.0448	CG14277	Up	molecular function is unknown.
291	152347_at	0.0448	CG11841	Up	serine-type endopeptidase activity
292	141568_at	0.0448	regucalcin	Up	molecular function is unknown.
293	145182_at	0.0448	CG9919	Down	molecular function is unknown.
294	151301_at	0.0448	CG13315	Up	molecular function is unknown.
295	152194_at	0.0449	Lcch3	Up	GABA-A receptor activity
296	151208_at	0.0449	CG15068	Up	molecular function is unknown.
297	154206_at	0.045	CG5315	Up	hormone binding
298	143059_at	0.0452	Act42A	Up	structural constituent of cytoskeleton
299	145385_at	0.0452	Sec61γ	Up	protein transporter activity
300	145252_at	0.0452	RhoGAPp190	Down	Rho GTPase activator activity
301	154244_at	0.0453	Gapdh1	Up	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity
302	152445_at	0.0453	Pif1A	Up	transcription factor activity
303	142453_at	0.0455	GNBP3	Up	pattern recognition receptor activity (defense response).
304	147379_at	0.0455	CG14483	Up	molecular function is unknown.
305	144063_at	0.0455	CREG	Up	protein binding; transcription repressor activity.
306	143616_at	0.0455	RpL18A	Up	structural constituent of ribosome
307	150780_at	0.0456	CG9989	Up	endonuclease activity
308	150171_at	0.0457	snRNP-U1	Up	mRNA binding; regulation of alternative nuclear mRNA splicing, via spliceosome.
309	153713_at	0.0457	ScpX	Up	sterol carrier protein X-related thiolase activity.
310	142781_at	0.0459	growl	Up	5-formyltetrahydrofolate cyclo-ligase activity.
311	151610_at	0.046		Down	
312	153721_at	0.0462	CG3589	Up	serine-type endopeptidase activity
313	152721_at	0.0462	Idgf1	Up	imaginal disc growth factor activity
314	146974_at	0.0462	Rpb5	Up	DNA-directed RNA polymerase activity
315	152301_at	0.0463	CG12918	Up	molecular function is unknown.

316	148395_at	0.0467	CG7112	Down	Rab GTPase activator activity
317	142295_at	0.0469	tsr	Up	actin binding
318	152312_at	0.047	CG8256	Up	glycerol-3-phosphate dehydrogenase activity
319	145890_at	0.0471	CG9498	Up	molecular function is unknown.
320	141233_at	0.0471	CG5966	Up	triacylglycerol lipase activity
321	147469_at	0.0472	CG15098	Up	molecular function is unknown.
322	153630_at	0.0473	CG2991	Down	molecular function is unknown.
323	144235_at	0.0475	nimC1	Down	Wnt-protein binding
324	155021_at	0.0478	CG42390	Up	molecular function is unknown.
325	154149_at	0.048	CG6686	Up	molecular function is unknown.
326	143620_at	0.048	RpS25	Up	structural constituent of ribosome
327	149957_at	0.0481	CG6125	Up	high affinity sulfate transmembrane transporter activity, transporter activity
328	153155_at	0.0481	TER94	Up	ATPase activity
329	143839_at	0.0482	Mpk2	Up	MAP kinase activity.
330	150482_at	0.0482	CG13607	Up	molecular function is unknown.
331	152038_at	0.0486	l(2)k16918	Up	molecular function is unknown.
332	147544_at	0.0487	Obp57c	Up	odorant binding
333	150181_at	0.0487	Cyp12a4	Up	electron carrier activity; heme binding
334	141329_at	0.0487	poe	Down	calmodulin binding involved in spermatid development; sperm individualization.
335	143726_at	0.0487	Dhc93AB	Up	ATPase activity.
336	152187_at	0.0488	CG3321	Up	hydrogen-exporting ATPase activity
337	142793_at	0.0488	CaMKII	Down	calmodulin-dependent protein kinase activity involved in male courtship behavior; learning and memory.
338	147607_at	0.0491	Glycogenin	Up	glycogenin glucosyltransferase activity
339	146214_at	0.0491	CG16743	Up	molecular function is unknown.
340	149899_at	0.0492	Cyp313a1	Up	electron carrier activity
341	144168_at	0.0493	CG13364	Up	molecular function is unknown.
342	151539_at	0.0494	CG30359	Up	cation binding; catalytic activity, involved in carbohydrate metabolic process.
343	154665_at	0.0495	Pep	Up	DNA binding, involved in regulation of nuclear mRNA splicing, via spliceosome.
344	148103_at	0.0497	dro5	Up	ion channel inhibitor activity involved in defense response.
345	143793_at	0.0498	Srp19	Up	7S RNA binding
346	148218_at	0.0498	CG10635	Up	chaperone binding
347	154674_at	0.0498	inx3	Up	gap junction channel activity

Appendix 2. List of enriched Gene Ontology (GO) terms.

Category[1]	Count	%	PValue	Term
SP_PIR_KEYWORDS	20	5.76%	4.61E-13	ribonucleoprotein
SP_PIR_KEYWORDS	18	5.19%	3.27E-12	ribosomal protein
GOTERM_CC_ALL	18	5.19%	8.12E-10	GO:0005830~cytosolic ribosome (sensu Eukaryota)
GOTERM_CC_ALL	105	30.26%	1.59E-09	GO:0005737~cytoplasm
GOTERM_CC_ALL	20	5.76%	1.65E-09	GO:0044445~cytosolic part
GOTERM_CC_ALL	19	5.48%	3.06E-08	GO:0005811~lipid particle
GOTERM_CC_ALL	88	25.36%	3.17E-08	GO:0044444~cytoplasmic part
GOTERM_MF_ALL	21	6.05%	1.11E-07	GO:0003735~structural constituent of ribosome
KEGG_PATHWAY	17	4.90%	1.80E-07	dme03010:Ribosome
SP_PIR_KEYWORDS	23	6.63%	2.69E-07	signal
GOTERM_CC_ALL	20	5.76%	2.79E-07	GO:0033279~ribosomal subunit
SP_PIR_KEYWORDS	24	6.92%	8.64E-07	alternative splicing
GOTERM_CC_ALL	10	2.88%	1.26E-06	GO:0005843~cytosolic small ribosomal subunit (sensu Eukaryota)
GOTERM_CC_ALL	21	6.05%	2.07E-06	GO:0005840~ribosome
SP_PIR_KEYWORDS	12	3.46%	2.20E-06	endoplasmic reticulum
SP_PIR_KEYWORDS	23	6.63%	2.68E-06	transport
GOTERM_CC_ALL	12	3.46%	4.68E-06	GO:0015935~small ribosomal subunit
GOTERM_CC_ALL	30	8.65%	5.26E-06	GO:0005829~cytosol
SP_PIR_KEYWORDS	14	4.03%	7.96E-06	Secreted
SP_PIR_KEYWORDS	17	4.90%	2.40E-05	cytoplasm
SP_PIR_KEYWORDS	31	8.93%	5.41E-05	membrane
GOTERM_MF_ALL	32	9.22%	1.20E-04	GO:0005198~structural molecule activity
GOTERM_CC_ALL	26	7.49%	1.55E-04	GO:0030529~ribonucleoprotein complex
GOTERM_BP_ALL	67	19.31%	1.88E-04	GO:0006810~transport
GOTERM_BP_ALL	68	19.60%	1.98E-04	GO:0051234~establishment of localization
SP_PIR_KEYWORDS	34	9.80%	3.40E-04	hydrolase
GOTERM_BP_ALL	21	6.05%	3.98E-04	GO:0006952~defense response
GOTERM_BP_ALL	45	12.97%	5.20E-04	GO:0044249~cellular biosynthetic process
GOTERM_CC_ALL	8	2.31%	7.95E-04	GO:0005842~cytosolic large ribosomal subunit (sensu Eukaryota)
GOTERM_BP_ALL	9	2.59%	9.78E-04	GO:0048102~autophagic cell death
GOTERM_BP_ALL	9	2.59%	9.78E-04	GO:0035071~salivary gland cell autophagic cell death
GOTERM_BP_ALL	9	2.59%	9.78E-04	GO:0035070~salivary gland histolysis

[1] Original database or resource where the terms orient (e.g. GOTERM_MF_ALL: GO term molecular function)

GOTERM_BP_ALL	30	8.65%	0.001018	GO:0006412~translation
GOTERM_CC_ALL	71	20.46%	0.001028	GO:0032991~macromolecular complex
GOTERM_BP_ALL	9	2.59%	0.001158	GO:0007559~histolysis
SP_PIR_KEYWORDS	18	5.19%	0.00118	oxidoreductase
GOTERM_BP_ALL	12	3.46%	0.001262	GO:0051707~response to other organism
SP_PIR_KEYWORDS	7	2.02%	0.001775	protein biosynthesis
GOTERM_BP_ALL	6	1.73%	0.003163	GO:0019731~antibacterial humoral response
GOTERM_BP_ALL	47	13.54%	0.003291	GO:0009058~biosynthetic process
GOTERM_BP_ALL	14	4.03%	0.003294	GO:0051704~multi-organism process
GOTERM_BP_ALL	23	6.63%	0.003649	GO:0016192~vesicle-mediated transport
GOTERM_CC_ALL	58	16.71%	0.004358	GO:0043234~protein complex
GOTERM_BP_ALL	12	3.46%	0.005017	GO:0009607~response to biotic stimulus
GOTERM_CC_ALL	67	19.31%	0.005585	GO:0044446~intracellular organelle part
SP_PIR_KEYWORDS	14	4.03%	0.005751	glycoprotein
GOTERM_CC_ALL	67	19.31%	0.005831	GO:0044422~organelle part
SP_PIR_KEYWORDS	24	6.92%	0.006117	transmembrane
GOTERM_BP_ALL	32	9.22%	0.006603	GO:0009059~macromolecule biosynthetic process
SP_PIR_KEYWORDS	7	2.02%	0.006966	heme
GOTERM_MF_ALL	3	0.86%	0.008251	GO:0005528~FK506 binding
GOTERM_MF_ALL	3	0.86%	0.008251	GO:0005527~macrolide binding
GOTERM_MF_ALL	3	0.86%	0.008251	GO:0016885~ligase activity, forming carbon-carbon bonds
GOTERM_CC_ALL	9	2.59%	0.008513	GO:0015934~large ribosomal subunit
GOTERM_BP_ALL	11	3.17%	0.009598	GO:0006955~immune response
GOTERM_BP_ALL	69	19.88%	0.011221	GO:0051179~localization
GOTERM_BP_ALL	4	1.15%	0.011367	GO:0019395~fatty acid oxidation
GOTERM_BP_ALL	4	1.15%	0.011367	GO:0006963~positive regulation of antibacterial peptide biosynthetic process
GOTERM_BP_ALL	4	1.15%	0.011367	GO:0002808~regulation of antibacterial peptide biosynthetic process
GOTERM_BP_ALL	4	1.15%	0.011367	GO:0002780~antibacterial peptide biosynthetic process
GOTERM_BP_ALL	4	1.15%	0.011367	GO:0002778~antibacterial peptide production
SP_PIR_KEYWORDS	6	1.73%	0.011503	transit peptide
GOTERM_BP_ALL	7	2.02%	0.012177	GO:0042742~defense response to bacterium
GOTERM_BP_ALL	42	12.10%	0.012248	GO:0050896~response to stimulus
GOTERM_BP_ALL	20	5.76%	0.01298	GO:0015031~protein transport
GOTERM_BP_ALL	18	5.19%	0.013632	GO:0006886~intracellular protein transport
GOTERM_CC_ALL	136	39.19%	0.014618	GO:0044424~intracellular part
GOTERM_BP_ALL	20	5.76%	0.014631	GO:0045184~establishment of protein localization
GOTERM_MF_ALL	18	5.19%	0.016269	GO:0022804~active transmembrane transporter activity
SP_PIR_KEYWORDS	20	5.76%	0.016275	nucleotide-binding
GOTERM_MF_ALL	35	10.09%	0.016488	GO:0005215~transporter activity
GOTERM_MF_ALL	7	2.02%	0.017103	GO:0015662~ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism

GOTERM_MF_ALL	4	1.15%	0.017479	GO:0005044~scavenger receptor activity
GOTERM_MF_ALL	11	3.17%	0.017514	GO:0015405~P-P-bond-hydrolysis-driven transmembrane transporter activity
GOTERM_MF_ALL	11	3.17%	0.017514	GO:0015399~primary active transmembrane transporter activity
GOTERM_BP_ALL	7	2.02%	0.017562	GO:0009617~response to bacterium
GOTERM_BP_ALL	3	0.86%	0.017841	GO:0006821~chloride transport
GOTERM_BP_ALL	5	1.44%	0.017897	GO:0002252~immune effector process
INTERPRO	3	0.86%	0.017899	IPR002155:Thiolase
GOTERM_MF_ALL	10	2.88%	0.01919	GO:0043492~ATPase activity, coupled to movement of substances
GOTERM_MF_ALL	10	2.88%	0.01919	GO:0042626~ATPase activity, coupled to transmembrane movement of substances
PIR_SUPERFAMILY	4	1.15%	0.019962	PIRSF000052:trichodiene oxygenase
SP_PIR_KEYWORDS	6	1.73%	0.020025	monooxygenase
GOTERM_MF_ALL	10	2.88%	0.020578	GO:0016820~hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
SP_PIR_KEYWORDS	4	1.15%	0.021613	innate immunity
SP_PIR_KEYWORDS	3	0.86%	0.022148	potassium transport
SP_PIR_KEYWORDS	10	2.88%	0.022454	phosphoprotein
GOTERM_MF_ALL	3	0.86%	0.023217	GO:0043021~ribonucleoprotein binding
GOTERM_MF_ALL	7	2.02%	0.024274	GO:0008135~translation factor activity, nucleic acid binding
SP_PIR_KEYWORDS	4	1.15%	0.024501	immune response
GOTERM_CC_ALL	25	7.20%	0.02495	GO:0005739~mitochondrion
GOTERM_BP_ALL	5	1.44%	0.024998	GO:0016049~cell growth
GOTERM_BP_ALL	15	4.32%	0.027445	GO:0010324~membrane invagination
GOTERM_BP_ALL	15	4.32%	0.027445	GO:0006897~endocytosis
SP_PIR_KEYWORDS	3	0.86%	0.027708	potassium
GOTERM_BP_ALL	4	1.15%	0.027958	GO:0002440~production of molecular mediator of immune response
GOTERM_BP_ALL	4	1.15%	0.027958	GO:0002807~positive regulation of antimicrobial peptide biosynthetic process
GOTERM_BP_ALL	4	1.15%	0.027958	GO:0002805~regulation of antimicrobial peptide biosynthetic process
GOTERM_BP_ALL	4	1.15%	0.027958	GO:0002777~antimicrobial peptide biosynthetic process
GOTERM_BP_ALL	4	1.15%	0.027958	GO:0002775~antimicrobial peptide production
SP_PIR_KEYWORDS	5	1.44%	0.028107	microsome
SP_PIR_KEYWORDS	8	2.31%	0.029135	ion transport
GOTERM_BP_ALL	12	3.46%	0.029868	GO:0051186~cofactor metabolic process
SP_PIR_KEYWORDS	19	5.48%	0.030099	transferase
GOTERM_BP_ALL	17	4.90%	0.030466	GO:0016044~membrane organization and biogenesis
GOTERM_MF_ALL	7	2.02%	0.030476	GO:0045182~translation regulator activity
GOTERM_BP_ALL	23	6.63%	0.030615	GO:0033036~macromolecule localization
SP_PIR_KEYWORDS	3	0.86%	0.030676	electron transport
SP_PIR_KEYWORDS	4	1.15%	0.030869	chaperone
GOTERM_BP_ALL	4	1.15%	0.030998	GO:0043043~peptide biosynthetic process

GOTERM_BP_ALL	4	1.15%	0.030998	GO:0050830~defense response to Gram-positive bacterium
GOTERM_BP_ALL	7	2.02%	0.031294	GO:0019730~antimicrobial humoral response
GOTERM_BP_ALL	21	6.05%	0.032523	GO:0008104~protein localization
SP_PIR_KEYWORDS	8	2.31%	0.032646	iron
SP_PIR_KEYWORDS	6	1.73%	0.032882	lyase
SP_PIR_KEYWORDS	7	2.02%	0.032898	Mitochondrion
GOTERM_BP_ALL	5	1.44%	0.033583	GO:0008361~regulation of cell size
SP_PIR_KEYWORDS	3	0.86%	0.033763	calmodulin-binding
GOTERM_CC_ALL	6	1.73%	0.03389	GO:0044432~endoplasmic reticulum part
GOTERM_BP_ALL	26	7.49%	0.03644	GO:0051649~establishment of cellular localization
GOTERM_MF_ALL	5	1.44%	0.036984	GO:0008553~hydrogen-exporting ATPase activity, phosphorylative mechanism
GOTERM_BP_ALL	9	2.59%	0.037308	GO:0035272~exocrine system development
GOTERM_BP_ALL	9	2.59%	0.037308	GO:0007431~salivary gland development
GOTERM_MF_ALL	28	8.07%	0.037804	GO:0022857~transmembrane transporter activity
GOTERM_MF_ALL	27	7.78%	0.038237	GO:0022892~substrate-specific transporter activity
SP_PIR_KEYWORDS	10	2.88%	0.039778	protease
GOTERM_CC_ALL	14	4.03%	0.039938	GO:0005783~endoplasmic reticulum
SP_PIR_KEYWORDS	3	0.86%	0.040281	ribosome
GOTERM_MF_ALL	17	4.90%	0.040819	GO:0030234~enzyme regulator activity
GOTERM_BP_ALL	12	3.46%	0.041555	GO:0002376~immune system process
GOTERM_BP_ALL	11	3.17%	0.041607	GO:0006732~coenzyme metabolic process
GOTERM_BP_ALL	7	2.02%	0.042921	GO:0006959~humoral immune response
GOTERM_BP_ALL	6	1.73%	0.044914	GO:0045087~innate immune response
GOTERM_MF_ALL	7	2.02%	0.045788	GO:0042625~ATPase activity, coupled to transmembrane movement of ions
GOTERM_BP_ALL	26	7.49%	0.045908	GO:0051641~cellular localization
GOTERM_CC_ALL	3	0.86%	0.046072	GO:0030867~rough endoplasmic reticulum membrane
GOTERM_MF_ALL	8	2.31%	0.046173	GO:0004857~enzyme inhibitor activity
GOTERM_CC_ALL	8	2.31%	0.046501	GO:0005624~membrane fraction
INTERPRO	2	0.58%	0.04664	IPR002443:Na-K-Cl co-transporter
INTERPRO	2	0.58%	0.04664	IPR000630:Ribosomal protein S8
INTERPRO	3	0.86%	0.047586	IPR002159:CD36 antigen
GOTERM_BP_ALL	4	1.15%	0.048667	GO:0006816~calcium ion transport
GOTERM_MF_ALL	8	2.31%	0.049294	GO:0020037~heme binding
GOTERM_MF_ALL	8	2.31%	0.049294	GO:0046906~tetrapyrrole binding
GOTERM_CC_ALL	36	10.37%	0.049861	GO:0043232~intracellular non-membrane-bound organelle
GOTERM_CC_ALL	36	10.37%	0.049861	GO:0043228~non-membrane-bound organelle
SP_PIR_KEYWORDS	6	1.73%	0.049958	sensory transduction

Appendix 3. Genes shared between the different aggression microarray studies*

Tauber/Dierick	Tauber/Wang	Tauber/Wang/Dierick
CG10584	CG10026	CG13794
CG11841	CG1441	Obp99b
CG11984	CG15347	
CG13607	CG5195	
CG8311	CG7966	
CG9090	Cyp4d21	
CG9297	Cyp6a20	
Obp57c	**Dat**	
PyK	wdp	
regucalcin		

* Dierick *et al*. 2006, Wang *et al*. 2007, Tauber: this study

Appendix 4. Primer list

Chapter 4: qPCR primers

CG6480-F	TGCACGCATTAAGAAATTGG
CG6480-R	ACACTGTACCGGTGATGTCG
CG6762-F	ATGGACACCACCGTTCACTC
CG6762-R	CACCTCATCTTCGCTGGTTT
Syx13-F	GGCAGGTCGAGCAAATAGAG
Syx13-R	GGCCGTCTGCTCAATACTGT
Rp49-F	CACTTCATCCGCCACCAGT
Rp49-R	CGCTTGTTCGATCCGTAACC
Slh-C-F	TAGCCAAGCCACACAGTACG
Slh-C-R	ATCACCTGCTCGGTGATCTT
DopR2-F	AACTCCTGCGAGCAGACCTA
DopR2-R	GATCTGGTTCACCGAGTGGT
Dat-A F	TCCGAAATTTAACGCTTCGT
Dat-A R	GCGTCCTCCATTTTCTGTGT
Dat-B-F	AGGCAACATGGAAGTGCAGA
Dat-B-R	GCTTCCCAGAGACGGTCAAT

Chapter 5: generating the transgenes overexpressing DatA and DatB

DatRT	ACACTACGTGAATCGAACG
DatA5-N	GTGTTATCGTTGGCC
DatB5	CTGGCATTCATTGTTTGCTC
Dat3	CGCCACCGAACTACAAACTA
Dat-tail 3`-N	GAAAACGCCGGCGTACAGCTTGGTCTG
Dat-tail A5`-N	GAAAGATCTCAACATGGAGGACGCATTGAC
Dat-tail B5`	CGGAATTCCAACATGGAAGTGCAGAAGCT

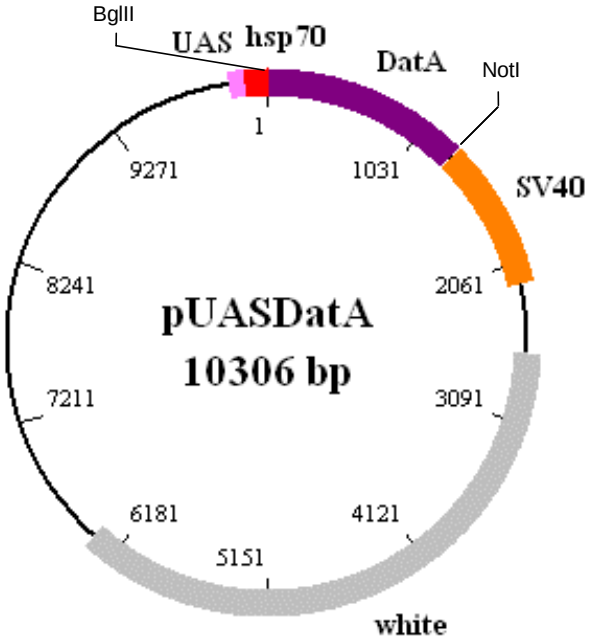
Primers for detecting the transgenes

pUASt-2	CTGCAACTACTGAAATCTGCC
PUAST-R	GTCCAATTATGTCACACC
Dat-tail 3`-N	GAAAACGCCGGCGTACAGCTTGGTCTG
5uasA	TGTCCTCCGAGCGGAGACTCTAG
3uasA	TTCTTGGCAGATTTCAGTAGTTGCAG

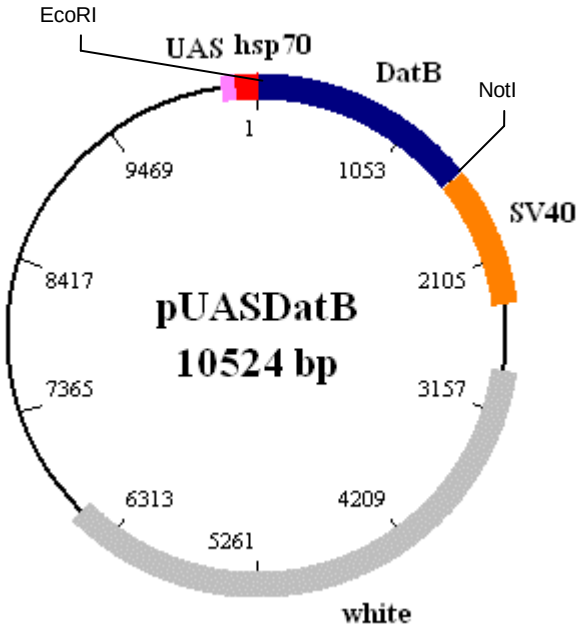
Chapter 6: generating the RNAi constructs

CG6480RNAi-gF	ATAAGAATGCGGCCGCTTACTTGCAATACC
CG6480RNAi-gR	ATCGCGGATCCAGACTACGATCATGCACGC
CG6480RNAi-cF	ATCGCGGATCCTGGTGGGCAGCAAAGACA
CG6480RNAi-cR	ATCCGGAATTCCACCTTCTTTCGCAATGCC
SlhRNAi-gF	ATCCGGAATTCATGCTGACCCTGCGGGAGC
SlhRNAi-gR	ATCGCGGATCCGCGAAGAGCGAGTCTACAA
SlhRNAi-cF	ATAAGAATGCGGCCGCATGCTGACCCTGCG
SlhRNAi-cR	ATGGAGGATCCGCGAAGAGCGAGTCTACAA

Appendix 5. The pUAS-Dat plasmids

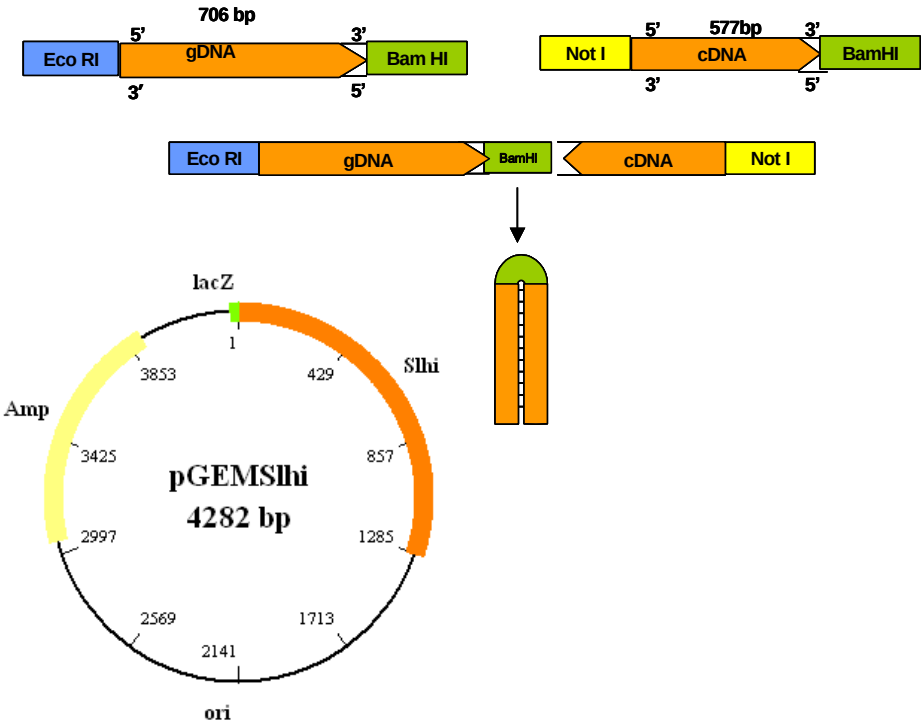


The *DatA* and *DatB* isoforms were PCR amplified and cloned into the pUASt plasmid cloning site following digestion by BglII and NotI (*DatA*) and EcoRI and NotI (*DatB*) sites. These plasmids were then injected into fly embryos to generate transgenic flies.

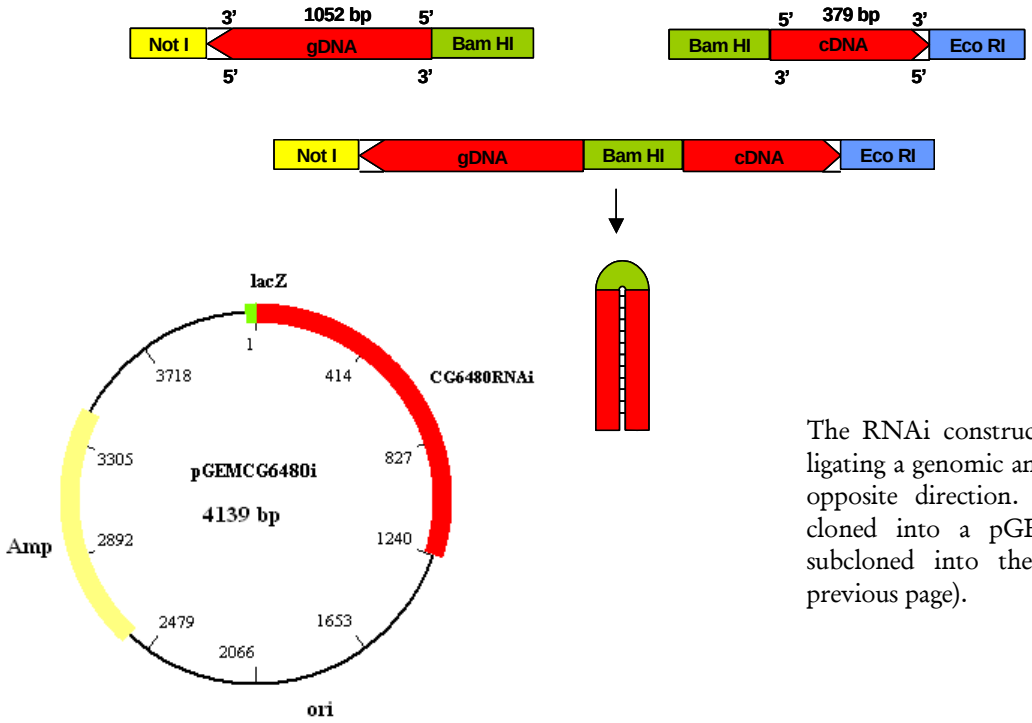


Appendix 6. Generation of the RNAi constructs

Slh-RNAi



CG6480-RNAi



The RNAi constructs were generated by ligating a genomic and cDNA fragments in opposite direction. The constructs were cloned into a pGEM vector and later subcloned into the pUASt vector (see previous page).

Appendix 7. Video recording example of aggressive behaviour in Drosophila

See enclosed DVD for a video showing examples of aggressive behaviour in Drosophila.

REFERENCES

Abrahams, B.S., M.C. Kwok, E. Trinh, S. Budaghzadeh, S.M. Hossain, and E.M. Simpson. 2005. Pathological aggression in "fierce" mice corrected by human nuclear receptor 2E1. *J.Neurosci.* 25:6263-6270.

Adams, M.D., S.E. Celniker, R.A. Holt, C.A. Evans, J.D. Gocayne, P.G. Amanatides, S.E. Scherer, *et al.* 2000. The genome sequence of *Drosophila melanogaster*. *Science.* 287:2185-2195.

Alcock, J. 2005. Animal Behavior : An Evolutionary Approach. Sinauer Associates, Sunderland, Mass.

Amara, S.G., and J.L. Arriza. 1993. Neurotransmitter transporters: three distinct gene families. *Current Opinion in Neurobiology.* 3:337-344.

Andretic, R., B. van Swinderen, and R.J. Greenspan. 2005. Dopaminergic modulation of arousal in Drosophila. *Curr.Biol.* 15:1165-1175.

Anholt, R.R., J.J. Fanara, G.M. Fedorowicz, I. Ganguly, N.H. Kulkarni, T.F. Mackay, and S.M. Rollmann. 2001. Functional genomics of odor-guided behavior in Drosophila melanogaster. *Chem.Senses.* 26:215-221.

Arbeitman, M.N., E.E. Furlong, F. Imam, E. Johnson, B.H. Null, B.S. Baker, M.A. Krasnow, M.P. Scott, R.W. Davis, and K.P. White. 2002. Gene expression during the life cycle of Drosophila melanogaster. *Science.* 297:2270-227

Ashburner, M. 2005. *Drosophila*: A Laboratory Handbook. Cold Spring Harbor Laboratory Press, New York.

Ashburner, M., C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, *et al.* 2000. Gene ontology tool for the unification of biology. The Gene Ontology Consortium. *Nat.Genet.* 25:25-29.

Baier, A., B. Wittek, and B. Brembs. 2002. *Drosophila* as a new model organism for the neurobiology of aggression? *J.Exp.Biol.* 205:1233-1240.

Bandura, A., D. Ross, and S.A. Ross. 1961. Transmission of aggression through imitation of aggressive models. *J.Abnorm.Soc.Psychol.* 63:575-582.

Bandura, A., D. Ross, and S.A. Ross. 1963. Imitation of film-mediated aggressive models. *J.Abnorm.Soc.Psychol.* 66:3-11.

Berkowitz, L. 1989. Frustration-aggression hypothesis: examination and reformulation. *Psychol.Bull.* 106:59-73.

Bhandari, P., J.W. Gargano, M.M. Goddeeris, and M.S. Grotewiel. 2006. Behavioral responses to odorants in *Drosophila* require nervous system expression of the beta integrin gene myospheroid. *Chem.Senses.* 31:627-639.

Birger, M., M. Swartz, D. Cohen, Y. Alesh, C. Grishpan, and M. Kotelr. 2003. Aggression: the testosterone-serotonin link. *Isr.Med.Assoc.J.* 5:653-658.

Blake, J.A., and M.A. Harris. 2008. The Gene Ontology (GO) project: structured vocabularies for molecular biology and their application to genome and expression analysis. *Curr.Protoc.Bioinformatics.* Chapter 7:Unit 7.2.

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 118:401-415.

Branson, K., A.A. Robie, J. Bender, P. Perona, and M.H. Dickinson. 2009. High-throughput ethomics in large groups of Drosophila. *Nat.Methods.* 6:451-457.

Breithaupt, T., and P. Eger. 2002. Urine makes the difference: chemical communication in fighting crayfish made visible. *J.Exp.Biol.* 205:1221-1231.

Brodbeck, D., R. Amherd, P. Callaerts, E. Hintermann, U.A. Meyer, and M. Affolter. 1998. Molecular and biochemical characterization of the aaNAT1 (Dat) locus in Drosophila melanogaster: differential expression of two gene products. *DNA Cell Biol.* 17:621-633.

Brodkin, E.S., S.A. Goforth, A.H. Keene, J.A. Fossella, and L.M. Silver. 2002. Identification of quantitative trait Loci that affect aggressive behavior in mice. *J.Neurosci.* 22:1165-1170.

Brown, G.L., F.K. Goodwin, J.C. Ballenger, P.F. Goyer, and L.F. Major. 1979. Aggression in humans correlates with cerebrospinal fluid amine metabolites. *Psychiatry Res.* 1:131-139.

Brunner, H.G., M. Nelen, X.O. Breakefield, H.H. Ropers, and B.A. van Oost. 1993. Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. *Science.* 262:578-580.

Buckholtz, J.W., and A. Meyer-Lindenberg. 2008. MAOA and the neurogenetic architecture of human aggression. *Trends Neurosci.* 31:120-129.

Budaev, S. 1997. The Statistical Analysis of Behavioural Latency Measures. *ISCP Newsletter*. 14:1-4.

Caramaschi, D., S.F. de Boer, and J.M. Koolhaas. 2007. Differential role of the 5-HT1A receptor in aggressive and non-aggressive mice: an across-strain comparison. *Physiol.Behav.* 90:590-601.

Carney, G.E. 2007. A rapid genome-wide response to Drosophila melanogaster social interactions. *BMC Genomics.* 8:288.

Cases, O., I. Seif, J. Grimsby, P. Gaspar, K. Chen, S. Pournin, U. Muller, M. Aguet, C. Babinet, and J.C. Shih. 1995. Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. *Science.* 268:1763-1766.

Caspi, A., J. McClay, T.E. Moffitt, J. Mill, J. Martin, I.W. Craig, A. Taylor, and R. Poulton. 2002. Role of genotype in the cycle of violence in maltreated children. *Science.* 297:851-854.

Certel, S.J., M.G. Savella, D.C. Schlegel, and E.A. Kravitz. 2007. Modulation of Drosophila male behavioral choice. *Proc.Natl.Acad.Sci.U.S.A.* 104:4706-4711.

Chan, Y.B., and E.A. Kravitz. 2007. Specific subgroups of FruM neurons control sexually dimorphic patterns of aggression in *Drosophila melanogaster*. *Proc.Natl.Acad.Sci.U.S.A.* 104:19577-19582.

Chase, I.D., C. Bartolomeo, and L.A. Dugatkin. 1994. Aggressive interactions and inter-contest interval: how long do winners keep winning? *Anim.Behav*. 48:393-400.

Chen, S., A.Y. Lee, N.M. Bowens, R. Huber, and E.A. Kravitz. 2002. Fighting fruit flies: a model system for the study of aggression. *Proc.Natl.Acad.Sci.U.S.A.* 99:5664-5668.

Chiavegatto, S., G.E. Demas, and R.J. Nelson. 2006. Nitric Oxide and Aggression. *In* Biology of Aggression. R.J. Nelson, editor. Oxford University Press, Oxford. 150-162.

Chiavegatto, S., V.L. Dawson, L.A. Mamounas, V.E. Koliatsos, T.M. Dawson, and R.J. Nelson. 2001. Brain serotonin dysfunction accounts for aggression in male mice lacking neuronal nitric oxide synthase. *Proc.Natl.Acad.Sci.U.S.A.* 98:1277-1281.

Chintapalli, V.R., J. Wang, and J.A. Dow. 2007. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. *Nat.Genet.* 39:715-720.

Cirelli, C., T.M. LaVaute, and G. Tononi. 2005. Sleep and wakefulness modulate gene expression in Drosophila. *J.Neurochem.* 94:1411-1419.

Clyne, J.D., and G. Miesenböck. 2008. Sex-specific control and tuning of the pattern generator for courtship song in Drosophila. *Cell.* 133:354-363.

Coccaro, E.F., R.J. Kavoussi, R.L. Hauger, T.B. Cooper, and C.F. Ferris. 1998. Cerebrospinal fluid vasopressin levels: correlates with aggression and serotonin function in personality-disordered subjects. *Arch.Gen.Psychiatry.* 55:708-714.

Collins, B.H., E. Rosato, and C.P. Kyriacou. 2004. Seasonal behavior in Drosophila melanogaster requires the photoreceptors, the circadian clock, and phospholipase C. *Proc.Natl.Acad.Sci.U.S.A.* 101:1945-1950.

Couppis, M.H., and C.H. Kennedy. 2008. The rewarding effect of aggression is reduced by nucleus accumbens dopamine receptor antagonism in mice. *Psychopharmacology (Berl).* 197:449-456.

Dabbs, J.M., and R. Morris. 1990. Testosterone, social class, and antisocial behavior in a sample of 4,462 men. *Psychol. Sci.* 1:209-211.

Dankert, H., L. Wang, E.D. Hoopfer, D.J. Anderson, and P. Perona. 2009. Automated monitoring and analysis of social behavior in Drosophila. *Nat.Methods.* 6:297-303.

Deitcher, D.L., A. Ueda, B.A. Stewart, R.W. Burgess, Y. Kidokoro, and T.L. Schwarz. 1998. Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the Drosophila gene neuronal-synaptobrevin. *J.Neurosci.* 18:2028-2039.

Demas, G.E., M.J. Eliasson, T.M. Dawson, V.L. Dawson, L.J. Kriegsfeld, R.J. Nelson, and S.H. Snyder. 1997. Inhibition of neuronal nitric oxide synthase increases aggressive behavior in mice. *Mol.Med.* 3:610-616.

Demir, E., and B.J. Dickson. 2005. *fruitless* splicing specifies male courtship behavior in Drosophila. *Cell.* 121:785-794.

Dennis, G.,Jr, B.T. Sherman, D.A. Hosack, J. Yang, W. Gao, H.C. Lane, and R.A. Lempicki. 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* 4:P3.

Dierick, H.A., and R.J. Greenspan. 2006. Molecular analysis of flies selected for aggressive behavior. *Nat.Genet.* 38:1023-1031.

Dierick, H.A., and R.J. Greenspan. 2007. Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat.Genet.* 39:678-682.

Dietzl, G., D. Chen, F. Schnorrer, K.C. Su, Y. Barinova, M. Fellner, B. Gasser, K. Kinsey, S. Oppel, S. Scheiblauer, A. Couto, V. Marra, K. Keleman, and B.J. Dickson. 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature.* 448:151-156.

Dollard, J., L.W. Doob, N.E. Miller, O.H. Maurer, and R.R. Sears. 1939. Frustration and Aggression. Yale University Press, New Haven.

Douglas, S.J., and J.D. Levine. 2006. Sex cells: dissecting the functions of fruitless isoforms. *Curr.Biol.* 16:R405-7.

Dow, M.A., and F. von Schilcher. 1975. Aggression and mating success in *Drosophila melanogaster*. *Nature.* 254:511-512.

Duffield, G.E. 2003. DNA microarray analyses of circadian timing: the genomic basis of biological time. *J.Neuroendocrinol.* 15:991-1002.

Edwards, D.H., and J. Herberholz. 2006. Crustacean models of aggression. *In* Biology of Aggression. R.J. Nelson, editor. Oxford University Press, Oxford. 38-61.

Edwards, A.C., S.M. Rollmann, T.J. Morgan, and T.F. Mackay. 2006. Quantitative genomics of aggressive behavior in Drosophila melanogaster. *PLoS Genet.* 2:e154.

Efron, B., B.B. Turnbull, and B. Narasimhan. 2009. Package 'locfdr': Computation of local false discovery rates. R package version 2.92. http://CRAN.R-project.org/package=locfdr

Engels, W.R., D.M. Johnson-Schlitz, W.B. Eggleston, and J. Sved. 1990. High-frequency P element loss in Drosophila is homolog dependent. *Cell.* 62:515-525.

Ferrari, P.F., A.M. van Erp, W. Tornatzky, and K.A. Miczek. 2003. Accumbal dopamine and serotonin in anticipation of the next aggressive episode in rats. *Eur.J.Neurosci.* 17:371-378.

Ferris, C.F. 2006. Neuroplasticity and aggression: an interaction between vasopressin and serotonin. *In* Biology of Aggression. R.J. Nelson, editor. Oxford University Press, Oxford. 163-175.

Ferris, C.F. 1996. Serotonin diminishes aggression by suppressing the activity of the vasopressin system. *Ann.N.Y.Acad.Sci.* 794:98-103.

Ferris, C.F., R.H. Melloni Jr, G. Koppel, K.W. Perry, R.W. Fuller, and Y. Delville. 1997. Vasopressin/serotonin interactions in the anterior hypothalamus control aggressive behavior in golden hamsters. *J.Neurosci.* 17:4331-4340.

Ferris, C.F., and M. Potegal. 1988. Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters. *Physiol.Behav.* 44:235-239.

Franken, I.H., J. Booij, and W. van den Brink. 2005. The role of dopamine in human addiction: from reward to motivated attention. *Eur.J.Pharmacol.* 526:199-206.

Freud, S. 1961. *Civilization and its Discontents.* W.W. Norton & company Inc., New York.

Fujii, S., and H. Amrein. 2002. Genes expressed in the Drosophila head reveal a role for fat cells in sex-specific physiology. *EMBO J.* 21:5353-5363.

Gabellini, D., G. D'Antona, M. Moggio, A. Prelle, C. Zecca, R. Adami, B. Angeletti, P. Ciscato, M.A. Pellegrino, R. Bottinelli, M.R. Green, and R. Tupler. 2006. Facioscapulohumeral muscular dystrophy in mice overexpressing FRG1. *Nature.* 439:973-977.

Galindo, K., and D.P. Smith. 2001. A large family of divergent Drosophila odorant-binding proteins expressed in gustatory and olfactory sensilla. *Genetics.* 159:1059-1072.

Giacalone, E., M. Tansella, L. Valzelli, and S. Garattini. 1968. Brain serotonin metabolism in isolated aggressive mice. *Biochem.Pharmacol.* 17:1315-1327.

Gloor, G.B., C.R. Preston, D.M. Johnson-Schlitz, N.A. Nassif, R.W. Phillis, W.K. Benz, H.M. Robertson, and W.R. Engels. 1993. Type I repressors of P element mobility. *Genetics.* 135:81-95.

Goessmann, C., C. Hemelrijk, and R. Huber. 2000. The formation and maintenance of crayfish hierarchies: behavioral and self-structuring properties. *Behav. Ecol. Sociobiol.* 48:418-428.

Golub, T.R., D.K. Slonim, P. Tamayo, C. Huard, M. Gaasenbeek, J.P. Mesirov, H. Coller, M.L. Loh, J.R. Downing, M.A. Caligiuri, C.D. Bloomfield, and E.S. Lander. 1999. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science.* 286:531-537.

Goridis, C., and H. Rohrer. 2002. Specification of catecholaminergic and serotonergic neurons. *Nat.Rev.Neurosci.* 3:531-541.

Gromko, M.H., and T.A. Markow. 1993. Courtship and remating in field populations of Drosophila. *Anim.Behav.* 45:253-262.

Greenspan, R.J., and J.F. Ferveur. 2000. Courtship in Drosophila. *Annu.Rev.Genet.* 34:205-232.

Hammond, S.M., A.A. Caudy, and G.J. Hannon. 2001. Post-transcriptional gene silencing by double-stranded RNA. *Nat.Rev.Genet.* 2:110-119.

Hannon, G.J. 2002. RNA interference. *Nature.* 418:244-251.

Harbison, S.T., T.F. Mackay, and R.R. Anholt. 2009. Understanding the neurogenetics of sleep: progress from Drosophila. *Trends Genet.* 25:262-269.

Hazelrigg, T., R. Levis, and G.M. Rubin. 1984. Transformation of white locus DNA in Drosophila: dosage compensation, *zeste* interaction, and position effects. *Cell.* 36:469-481.

Heberlein, U. 2000. Genetics of alcohol-induced behaviors in Drosophila. *Alcohol Res.Health.* 24:185-188.

Hendricks, T.J., D.V. Fyodorov, L.J. Wegman, N.B. Lelutiu, E.A. Pehek, B. Yamamoto, J. Silver, E.J. Weeber, J.D. Sweatt, and E.S. Deneris. 2003. Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. *Neuron.* 37:233-247.

Hilakivi-Clarke, L.A., and R.G. Lister. 1992. Are there preexisting behavioral characteristics that predict the dominant status of male NIH Swiss mice (Mus musculus)? *J.Comp.Psychol.* 106:184-189.

Hintermann, E., N.C. Grieder, R. Amherd, D. Brodbeck, and U.A. Meyer. 1996. Cloning of an arylalkylamine N-acetyltransferase (aaNAT1) from *Drosophila melanogaster* expressed in the nervous system and the gut. *Proc.Natl.Acad.Sci.U.S.A.* 93:12315-12320.

Hock, K., and R. Huber. 2006. Modeling the acquisition of social rank in crayfish: winner and loser effects and self-structuring properties. *Behaviour*. 143:325-346.

Hodgetts, R.B. 1972. Biochemical characterization of mutants affecting the metabolism of -alanine in Drosophila. *J.Insect Physiol.* 18:937-947.

Hodgetts, R.B., and R.J. Konopka. 1973. Tyrosine and catecholamine metabolism in wild-type Drosophila melanogaster and a mutant, ebony. *J.Insect Physiol.* 19:1211-1220.

Hoffmann, A.A. 1988. Heritable variation for territorial success in two *Drosophila melanogaster* populations. *Anim. Behav.* 36:1180-1198.

Hoffmann, A.A., and Z. Cacoyianni. 1990. Territoriality in *Drosophila melanogaster* as a conditional strategy. *Anim.Behav.* 49:526-537.

Hoffmann, A.A. 1994. Genetic analysis of territoriality in *Drosophila melanogaster*. *In* Quantitative Genetic Studies of Behavioral Evolution C.R. Boake, editor. Chicago University Press, Chicago. 188-205.

Hoffmann, A.A. 1990. The influence of age and experience with conspecifics on territorial behavior in *Drosophila melanogaster*. *Journal of Insect Behavior.* 3:1-12.

Hoheisel, J.D. 2006. Microarray technology: beyond transcript profiling and genotype analysis. *Nat.Rev.Genet.* 7:200-210.

Holmes, C., H. Smith, R. Ganderton, M. Arranz, D. Collier, J. Powell, and S. Lovestone. 2001. Psychosis and aggression in Alzheimer's disease: the effect of dopamine receptor gene variation. *J. Neurol. Neurosurg. Psychiatry.* 71:777-779.

Hosack, D.A., G. Dennis Jr, B.T. Sherman, H.C. Lane, and R.A. Lempicki. 2003. Identifying biological themes within lists of genes with EASE. *Genome Biol.* 4:R70.

Hoyer, S.C., A. Eckart, A. Herrel, T. Zars, S.A. Fischer, S.L. Hardie, and M. Heisenberg. 2008. Octopamine in male aggression of Drosophila. *Curr.Biol.* 18:159-167.

Huber, R., M. Orzeszyna, N. Pokorny, and E.A. Kravitz. 1997a. Biogenic amines and aggression: experimental approaches in crustaceans. *Brain Behav.Evol.* 50 Suppl 1:60-68.

Huber, R., K. Smith, A. Delago, K. Isaksson, and E.A. Kravitz. 1997b. Serotonin and aggressive motivation in crustaceans: altering the decision to retreat. *Proc.Natl.Acad.Sci.U.S.A.* 94:5939-5942.

Jacobs, M.E. 1960. Influence of light on mating of *Drosophila melanogaster*. *Ecology.* 14:182-188.

Jacobs, M.E. 1978. Influence of beta-alanine on mating and territorialism in *Drosophila melanogaster*. *Behav.Genet.* 8:487-502.

Jameson, K.A., Appleby, M.C., and L.C. Freeman. 1999. Finding an appropriate order for a hierarchy based on probabilistic dominance. *Anim. Behav*. 57: 991-998.

Jaronczyk, K., J.B. Carmichael, and T.C. Hobman. 2005. Exploring the functions of RNA interference pathway proteins: some functions are more RISCy than others? *Biochem.J.* 387:561-571.

Jones, B.R. 2007. Some basics, Mendelian traits, polygenic traits, complex traits. *In* Neurobehavioral Genetics: Methods and Applications. B.C. Jones and P. Mormède, editors. CRC Press. 31-36.

Jones, B.C., and P. Mormède. 2007. Neurobehavioral genetics : methods and applications. Taylor & Francis, London.

Jordan, K.W., M.A. Carbone, A. Yamamoto, T.J. Morgan, and T.F. Mackay. 2007. Quantitative genomics of locomotor behavior in Drosophila melanogaster. *Genome Biol.* 8:R172.

Kadener, S., D. Stoleru, M. McDonald, P. Nawathean, and M. Rosbash. 2007. Clockwork Orange is a transcriptional repressor and a new Drosophila circadian pacemaker component. *Genes Dev.* 21:1675-1686.

Kahn-Greene, E.T., E.L. Lipizzi, A.K. Conrad, G.H. Kamimori, and W.D.S. Killgore. 2006. Sleep deprivation adversely affects interpersonal responses to frustration. *Pers. Indiv. Differ.* 41:1433-1443.

Kalidas, S., and D.P. Smith. 2002. Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. *Neuron* 33:177-184.

Karl, T., S. Lin, C. Schwarzer, A. Sainsbury, M. Couzens, W. Wittmann, D. Boey, S. von Horsten, and H. Herzog. 2004. Y1 receptors regulate aggressive behavior by modulating serotonin pathways. *Proc.Natl.Acad.Sci.U.S.A.* 101:12742-12747.

Keegan, K.P., S. Pradhan, J.P. Wang, and R. Allada. 2007. Meta-analysis of Drosophila circadian microarray studies identifies a novel set of rhythmically expressed genes. *PLoS Comput.Biol.* 3:e208.

Kennerdell, J.R., and R.W. Carthew. 2000. Heritable gene silencing in Drosophila using double-stranded RNA. *Nat.Biotechnol.* 18:896-898.

Klein, D.C. 2006. Evolution of the vertebrate pineal gland: the AANAT hypothesis. *Chronobiol.Int.* 23:5-20.

Knudsen, S. 2004. Guide to Analysis of DNA Microarray Data. Wiley-Liss, New-Jersey.

Ko, M.S., J.R. Kitchen, X. Wang, T.A. Threat, X. Wang, A. Hasegawa, T. Sun, M.J. Grahovac, G.J. Kargul, M.K. Lim, Y. Cui, Y. Sano, T. Tanaka, Y. Liang, S. Mason, P.D. Paonessa, A.D. Sauls, G.E. DePalma, R. Sharara, L.B. Rowe, J. Eppig, C. Morrell, and H. Doi. 2000. Large-scale cDNA analysis reveals phased gene expression patterns during preimplantation mouse development. *Development.* 127:1737-1749.

Konopka, R.J., and S. Benzer. 1971. Clock mutants of Drosophila melanogaster. *Proc.Natl.Acad.Sci.U.S.A.* 68:2112-2116.

Kravitz, E.A. 2000. Serotonin and aggression: insights gained from a lobster model system and speculations on the role of amine neurons in a complex behavior. *J.Comp.Physiol.[A]*. 186:221-238.

Krebs, J.R. 1993. An Introduction to Behavioural Ecology. Blackwell Scientific, Oxford.

Kume, K., S. Kume, S.K. Park, J. Hirsh, and F.R. Jackson. 2005. Dopamine is a regulator of arousal in the fruit fly. *J.Neurosci.* 25:7377-7384.

Kureishy, N., V. Sapountzi, S. Prag, N. Anilkumar, and J.C. Adams. 2002. Fascins, and their roles in cell structure and function. *Bioessays.* 24:350-361.

Kyriacou, C.P., Burnet B., and K. Connolly. 1978. The behavioural basis of overdominance in competitive mating success at the ebony locus of *Drosophila melanogaster*. 26:1195-1206.

Kyriacou, C.P. and J.C. Hall. 1980. Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. *Proc.Natl.Acad.Sci.U.S.A.* 77:6729-6733.

Lam, G., and C.S. Thummel. 2000. Inducible expression of double-stranded RNA directs specific genetic interference in Drosophila. *Curr.Biol.* 10:957-963.

Lashkari, D.A., J.H. McCusker, and R.W. Davis. 1997. Whole genome analysis: experimental access to all genome sequenced segments through larger-scale efficient oligonucleotide synthesis and PCR. *Proc.Natl.Acad.Sci.U.S.A.* 94:8945-8947.

Laughon, A., and R.F. Gesteland. 1984. Primary structure of the *Saccharomyces cerevisiae GAL4* gene. *Mol.Cell.Biol.* 4:260-267.

Lee, G., and J.C. Hall. 2000. A newly uncovered phenotype associated with the fruitless gene of *Drosophila melanogaster*: aggression-like head interactions between mutant males. *Behav.Genet.* 30:263-275.

Lima, S.Q., and G. Miesenbock. 2005. Remote control of behavior through genetically targeted photostimulation of neurons. *Cell.* 121:141-152.

Lipshutz, R.J., S.P. Fodor, T.R. Gingeras, and D.J. Lockhart. 1999. High density synthetic oligonucleotide arrays. *Nat.Genet.* 21:20-24.

Littleton, J.T. 2000. A genomic analysis of membrane trafficking and neurotransmitter release in Drosophila. *J.Cell Biol.* 150:F77-82.

Littleton, J.T., E.R. Chapman, R. Kreber, M.B. Garment, S.D. Carlson, and B. Ganetzky. 1998. Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly. *Neuron.* 21:401-413.

Lorenz, K. 1963. On Aggression. Harcourt Brace, San Diego.

Majercak, J., W.F. Chen, and I. Edery. 2004. Splicing of the period gene 3'-terminal intron is regulated by light, circadian clock factors, and phospholipase C. *Mol.Cell.Biol.* 24:3359-3372.

Majercak, J., D. Sidote, P.E. Hardin, and I. Edery. 1999. How a circadian clock adapts to seasonal decreases in temperature and day length. *Neuron.* 24:219-230.

Manoli, D.S., and B.S. Baker. 2004. Median bundle neurons coordinate behaviours during Drosophila male courtship. *Nature.* 430:564-569.

Martinek, S., and M.W. Young. 2000. Specific genetic interference with behavioral rhythms in Drosophila by expression of inverted repeats. *Genetics.* 156:1717-1725.

Maruniak, J.A., C.J. Wysocki, and J.A. Taylor. 1986. Mediation of male mouse urine marking and aggression by the vomeronasal organ. *Physiol.Behav.* 37:655-657.

Matsukage, A., F. Hirose, Y. Hayashi, K. Hamada, and M. Yamaguchi. 1995. The DRE sequence TATCGATA, a putative promoter-activating element for Drosophila melanogaster cell-proliferation-related genes. *Gene.* 166:233-236.

Matsumoto, A., M. Ukai-Tadenuma, R.G. Yamada, J. Houl, K.D. Uno, T. Kasukawa, B. Dauwalder, T.Q. Itoh, K. Takahashi, R. Ueda, P.E. Hardin, T. Tanimura, and H.R. Ueda. 2007. A functional genomics strategy reveals clockwork orange as a transcriptional regulator in the Drosophila circadian clock. *Genes Dev.* 21:1687-1700.

Maynard Smith, J. 1982. Evolution and the Theory of Games. Cambridge University Press, Cambridge.

Maynard-Smith, J., and G.R. Price. 1973. The logic of animal conflict. *Nature.* 246:15-18.

McDermott, R., D. Tingley, J. Cowden, G. Frazzetto, and D.D. Johnson. 2009. Monoamine oxidase A gene (MAOA) predicts behavioral aggression following provocation. *Proc. Natl. Acad. Sci.U.S.A.* 106:2118-2123.

McDonald, M.J., and M. Rosbash. 2001. Microarray analysis and organization of circadian gene expression in Drosophila. *Cell.* 107:567-578.

Mehren, J.E., A. Ejima, and L.C. Griffith. 2004. Unconventional sex: fresh approaches to courtship learning. *Curr.Opin.Neurobiol.* 14:745-750.

Miczek, M.A., and E.W. Fish. 2006. Monoamines, GABA, glutamate, and aggression. *In* Biology of Aggression. R.J. Nelson, editor. Oxford University Press, Oxford. 114-149.

Mocellin, S., and M. Provenzano. 2004. RNA interference: learning gene knock-down from cell physiology. *J.Transl.Med.* 2:39.

Molina, V., L. Ciesielski, S. Gobaille, F. Isel, and P. Mandel. 1987. Inhibition of mouse killing behavior by serotonin-mimetic drugs: effects of partial alterations of serotonin neurotransmission. *Pharmacol. Biochem. Behav.* 27:123-131.

Muller, C.A., and M.B. Manser. 2007. 'Nasty neighbours' rather than 'dear enemies' in a social carnivore. *Proc.Biol.Sci.* 274:959-965.

Mullins, M.C., D.C. Rio, and G.M. Rubin. 1989. cis-acting DNA sequence requirements for P-element transposition. *Genes Dev.* 3:729-738.

Nelson, R.J. 2006. Biology of Aggression. Oxford University Press. Oxford.

Nelson, R.J., G.E. Demas, P.L. Huang, M.C. Fishman, V.L. Dawson, T.M. Dawson, and S.H. Snyder. 1995. Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. *Nature.* 378:383-386.

Neuwald, A.F., and D. Landsman. 1997. GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. *Trends Biochem.Sci.* 22:154-155.

Nilsen, S.P., Y.B. Chan, R. Huber, and E.A. Kravitz. 2004. Gender-selective patterns of aggressive behavior in *Drosophila melanogaster*. *Proc.Natl.Acad.Sci.U.S.A.* 101:12342-12347.

Nolan, T., R.E. Hands, and S.A. Bustin. 2006. Quantification of mRNA using real-time RT-PCR. *Nat.Protoc.* 1:1559-1582.

Obsil, T., R. Ghirlando, D.C. Klein, S. Ganguly, and F. Dyda. 2001. Crystal structure of the 14-3-3zeta:serotonin N-acetyltransferase complex. a role for scaffolding in enzyme regulation. *Cell.* 105:257-267.

Ochman, H., A.S. Gerber, and D.L. Hartl. 1988. Genetic applications of an inverse polymerase chain reaction. *Genetics.* 120:621-623.

Olivier, B. 2004. Serotonin and aggression. *Ann.N.Y.Acad.Sci.* 1036:382-392.

Owens, J. 2005. Gene expression: Do microarrays match up? *Nature Reviews Drug Discovery*. 4:459.

Pan, Z., D.W. Voehringer, and R.E. Meyn. 1999. Analysis of redox regulation of cytochrome c-induced apoptosis in a cell-free system. *Cell Death Differ.* 6:683-688.

Partridge, L., A. Green, and K. Fowler. 1987. Effects of egg production and of exposure to males on female survival in *Drosophila melanogaster. J Insect Physiol*. 33:745-749.

Primrose, S., and R. Twyman. 2003. Principles of Genome Analysis and Genomics. Wiley-Blackwell, Oxford, UK.

Punzo, F., and T. Punzo. 2001. Monoamines in the brain of tarantulas (*APHONOPELMA HENTZI)* (ARANEAE, THERAPHOSIDAE): differences associated with male agonistic interactions. *J. Arachnol*. 29:388-395.

Purves, D., G.J. Augustine, D. Fitzpatrick, L.C. Katz, A. LaMantia, J.O. McNamara, and S.M. Williams. 2001. Neuroscience (2nd edition). Sinauer Associates, Sunderland, MA.

R Development Core Team. 2007. R: A language and environment for statistical computing. R Foundation for Statistical Computing: Vienna, Austria.

Raab, A., R. Dantzer, B. Michaud, P. Mormede, K. Taghzouti, H. Simon, and M. Le Moal. 1986. Behavioural, physiological and immunological consequences of social status and aggression in chronically coexisting resident-intruder dyads of male rats. *Physiol.Behav.* 36:223-228.

Reis, D.J., and K. Fuxe. 1968. Depletion of noradrenaline in brainstem neurons during sham rage behaviour produced by acute brainstem transaction in cat. *Brain Res.* 4:448-451.

Reiter, L.T., L. Potocki, S. Chien, M. Gribskov, and E. Bier. 2001. A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. *Genome Res.* 11:1114-1125.

Rozen, S., and H.J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *In* Bioinformatics Methods and Protocols: Methods in Molecular Biology. S. Krawetz and S. Misener, editors. Humana Press, Totowa, NJ. 365-386.

Saito, Y., and K. Mori. 2005. Where does male-to-male "aggression" compromise "cooperation"? *Pop. Ecol.* 47:159-166.

Sanchez-Martin, J.R., E. Fano, L. Ahedo, J. Cardas, P.F. Brain, and A. Azpiroz. 2000. Relating testosterone levels and free play social behavior in male and female preschool children. *Psychoneuroendocrinology.* 25:773-783.

Saudou, F., D.A. Amara, A. Dierich, M. LeMeur, S. Ramboz, L. Segu, M.C. Buhot, and R. Hen. 1994. Enhanced aggressive behavior in mice lacking 5-HT1B receptor. *Science.* 265:1875-1878.

Schaik, C.P., and C.H. Janson. 2000. Infanticide by males and its implications. Cambridge University Press, Cambridge.

Schena, M., D. Shalon, R.W. Davis, and P.O. Brown. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science.* 270:467-470.

Schulze, K.L., K. Broadie, M.S. Perin, and H.J. Bellen. 1995. Genetic and electrophysiological studies of Drosophila syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission. *Cell.* 80:311-320.

Shaw, P.J., C. Cirelli, R.J. Greenspan, and G. Tononi. 2000. Correlates of sleep and waking in *Drosophila melanogaster*. *Science.* 287:1834-1837.

Siegel, S. and N.J. Castellan 1988. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York.

Sneddon, L.U., A.C. Taylor, F.A. Huntingford, and D.G. Watson. 2000. Agonistic behaviour and biogenic amines in shore crabs *Carcinus maenas*. *J.Exp.Biol.* 203:537-545.

Sokolowski, M.B. 2001. Drosophila: genetics meets behaviour. *Nat.Rev.Genet.* 2:879-890.

Spieth, H.T. 1968. Evolutionary implications of sexual behavior in Drosophila. *Evol. Biol.* 2:157-193.

Spradling, A.C., and G.M. Rubin. 1982. Transposition of cloned P elements into Drosophila germ line chromosomes. *Science.* 218:341-347.

Stevenson, P.A., H.A. Hofmann, K. Schoch, and K. Schildberger. 2000. The fight and flight responses of crickets depleted of biogenic amines. *J.Neurobiol.* 43:107-120.

Suh, J., and F.R. Jackson. 2007. Drosophila *ebony* activity is required in glia for the circadian regulation of locomotor activity. *Neuron.* 55:435-447.

Sweet, R.A., V.L. Nimgaonkar, M.I. Kamboh, O.L. Lopez, F. Zhang, and S.T. DeKosky. 1998. Dopamine receptor genetic variation, psychosis, and aggression in Alzheimer disease. *Arch.Neurol.* 55:1335-1340.

Tauber, E., and D.F. Eberl. 2002. The effect of male competition on the courtship song of *Drosophila melanogaster*. *J. Insect Behav.* 15:109-120.

Tei, H., H. Okamura, Y. Shigeyoshi, C. Fukuhara, R. Ozawa, M. Hirose, and Y. Sakaki. 1997. Circadian oscillation of a mammalian homologue of the Drosophila *period* gene. *Nature.* 389:512-516.

Tinbergen, N. 1951. The Study of Instinct. Clarendon Press, New York, NY.

Toh, K.L., C.R. Jones, Y. He, E.J. Eide, W.A. Hinz, D.M. Virshup, L.J. Ptacek, and Y.H. Fu. 2001. An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science.* 291:1040-1043.

Toma, D.P., K.P. White, J. Hirsch, and R.J. Greenspan. 2002. Identification of genes involved in Drosophila melanogaster geotaxis, a complex behavioral trait. *Nat.Genet.* 31:349-353.

Trainor, B.C., K.M. Greiwe, and R.J. Nelson. 2006. Individual differences in estrogen receptor alpha in select brain nuclei are associated with individual differences in aggression. *Horm.Behav.* 50:338-345.

Trainor, B.C., S. Lin, M.S. Finy, M.R. Rowland, and R.J. Nelson. 2007a. Photoperiod reverses the effects of estrogens on male aggression via genomic and nongenomic pathways. *Proc.Natl.Acad.Sci.U.S.A.* 104:9840-9845.

Trainor, B.C., M.R. Rowland, and R.J. Nelson. 2007b. Photoperiod affects estrogen receptor alpha, estrogen receptor beta and aggressive behavior. *Eur.J.Neurosci.* 26:207-218.

Trivers, R. 2008. Extract from Social Evolution [1985]. *In* The Oxford Book of Modern Science Writing. R. Dawkins, editor. Oxford University Press, Oxford. p. 124.

Tufik, S. 1981. Changes of response to dopaminergic drugs in rats submitted to REM-sleep deprivation. *Psychopharmacology (Berl).* 72:257-260.

Ueda, A., and Y. Kidokoro. 2002. Aggressive behaviours of female *Drosophila melanogaster* are influenced by their social experience and food resources. *Physiol.Entomol.* 27:21-28.

Ueda, H.R., A. Matsumoto, M. Kawamura, M. Iino, T. Tanimura, and S. Hashimoto. 2002. Genome-wide transcriptional orchestration of circadian rhythms in Drosophila. *J.Biol.Chem.* 277:14048-14052.

van Erp, A.M., and K.A. Miczek. 2000. Aggressive behavior, increased accumbal dopamine, and decreased cortical serotonin in rats. *J.Neurosci.* 20:9320-9325.

van Erp, A.M., and K.A. Miczek. 2007. Increased accumbal dopamine during daily alcohol consumption and subsequent aggressive behavior in rats. *Psychopharmacology (Berl).* 191:679-688.

Vergnes, M., A. Depaulis, and A. Boehrer. 1986. Parachlorophenylalanine-induced serotonin depletion increases offensive but not defensive aggression in male rats. *Physiol.Behav.* 36:653-658.

Vrontou, E., S.P. Nilsen, E. Demir, E.A. Kravitz, and B.J. Dickson. 2006. *fruitless* regulates aggression and dominance in *Drosophila*. *Nat.Neurosci.* 9:1469-1471.

Wang, D., L. Qian, H. Xiong, J. Liu, W.S. Neckameyer, S. Oldham, K. Xia, J. Wang, R. Bodmer, and Z. Zhang. 2006. Antioxidants protect PINK1-dependent dopaminergic neurons in Drosophila. *Proc.Natl.Acad.Sci.U.S.A.* 103:13520-13525.

Wang, L., H. Dankert, P. Perona, and D.J. Anderson. 2008. A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. *Proc.Natl.Acad.Sci.U.S.A.* 105:5657-5663.

Wang, P., R.F. Lyman, S.A. Shabalina, T.F. Mackay, and R.R. Anholt. 2007. Association of polymorphisms in odorant-binding protein genes with variation in olfactory response to benzaldehyde in Drosophila. *Genetics.* 177:1655-1665.

Wersinger, S.R., E.I. Ginns, A.M. O'Carroll, S.J. Lolait, and W.S. Young 3rd. 2002. Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. *Mol.Psychiatry.* 7:975-984.

West, S.A., M.G. Murray, C.A. Machado, A.S. Griffin, and E.A. Herre. 2001. Testing Hamilton's rule with competition between relatives. *Nature.* 409:510-513.

Whitfield, C.W., A.M. Cziko, and G.E. Robinson. 2003. Gene expression profiles in the brain predict behavior in individual honey bees. *Science.* 302:296-299.

Wisor, J.P., S. Nishino, I. Sora, G.H. Uhl, E. Mignot, and D.M. Edgar. 2001. Dopaminergic role in stimulant-induced wakefulness. *J.Neurosci.* 21:1787-1794.

Wolf, M.E. 1999. Cocaine addiction: clues from Drosophila on drugs. *Curr.Biol.* 9:R770-2.

Wright, T.R. 1987. The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. *Adv.Genet.* 24:127-222.

Yapici, N., Y.J. Kim, C. Ribeiro, and B.J. Dickson. 2008. A receptor that mediates the post-mating switch in Drosophila reproductive behaviour. *Nature.* 451:33-37.

Ydenberg, R.C., L.A. Giraldeau, and J.B. Falls. 1988. Neighbors, strangers, and the asymmetric war of attrition. *Anim.Behav.* 36:343-347.

Yeh, S.R., R.A. Fricke, and D.H. Edwards. 1996. The effect of social experience on serotonergic modulation of the escape circuit of crayfish. *Science.* 271:366-369.

Yoshimura, H., and N. Ogawa. 1982. Pharmaco-ethological analysis of agonistic behavior between resident and intruder mice: effect of anticholinergic drugs. *Jpn.J.Pharmacol.* 32:1111-1116.

Young, K.A., M.L. Berry, C.L. Mahaffey, J.R. Saionz, N.L. Hawes, B. Chang, Q.Y. Zheng, R.S. Smith, R.T. Bronson, R.J. Nelson, and E.M. Simpson. 2002. Fierce: a new mouse deletion of Nr2e1; violent behaviour and ocular abnormalities are background-dependent. *Behav.Brain Res.* 132:145-158.

Yu Q, H.V. Colot., C.P. Kyriacou, J.C. Hall, and M. Rosbash. 1987. Behaviour modification by in vitro mutagenesis of a variable region within the *period* gene of Drosophila. *Nature*. 326:765-769.

Yurkovic, A., O. Wang, A.C. Basu, and E.A. Kravitz. 2006. Learning and memory associated with aggression in *Drosophila melanogaster*. *Proc.Natl.Acad.Sci.U.S.A.* 103:17519-17524.

Zhuang, X., C. Gross, L. Santarelli, V. Compan, A.C. Trillat, and R. Hen. 1999. Altered emotional states in knockout mice lacking 5-HT1A or 5-HT1B receptors. *Neuropsychopharmacology.* 21:52S-60S.

Zirlinger, M., G. Kreiman, and D.J. Anderson. 2001. Amygdala-enriched genes identified by microarray technology are restricted to specific amygdaloid subnuclei. *Proc.Natl.Acad.Sci.U.S.A.* 98:5270-5275.