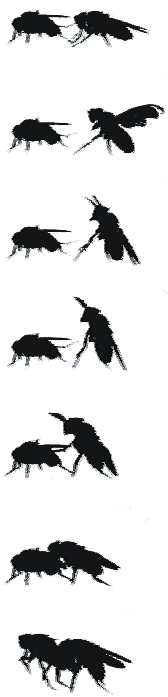
NEURONAL CORRELATES OF AGGRESSION IN *DROSOPHILA MELANOGASTER*



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1 INTRODUCTION

Aggression is a widespread and strikingly multi-faceted phenomenon. In humans, it manifests itself diversely, ranging from criminal offences such as murder, robbery, and verbal assault, to more moderate forms such as talking behind someone's back and deliberately failing to perform a task requested.

Accordingly, there have been diverse definitions of (human) aggression. From a purely behaviouristic viewpoint, and hence avoiding reference to intention, Arnold Buss defined aggression as "the delivery of noxious stimuli to another" (Buss, 1961). However, there are situations in which a noxious stimulus is delivered unintentionally, for example when the dentist has to drill a tooth. These situations are excluded if one classifies only those actions as aggressive when the person violates accepted rules of society (Bandura, 1973) or when a motivational assumption is considered: Dollard and Miller (Dollard et al., 1939) defined aggression as "such sequence of behaviour, the goal-response to which is the injury of the person toward whom it is directed". Along the same line Robert Baron (1979) declared aggression as "any form of behaviour directed toward the goal of harming or injuring another living being who is motivated to avoid such treatment". In contrast to Bandura, here the focus in not on the society at large but on the victim. As discussed by Werbik (1981), definitions with and without underlying motivational assumptions bear problems, for example concerning the need for a generally accepted definition of 'harm' (german: Schädigung).

Since the term aggression unifies numerous aggressive behaviours, there are several approaches to classify its manifestations into subcategories. Focussing on the goal of aggression, aggressive acts with the only intention to hurt are called *hostile (affective/angry/emotional) aggression*, whereas acts with a purpose other than hurting the victim, for example ascertain the aggressor's dominant status, are called *instrumental aggression* (e.g. Berkowitz, 1993). Similarly, Siegel (2004) suggests for nonprimate aggression to distinguish *affective defence* versus *predatory attack*. Affective defence comprises six of seven forms of aggression formerly operationally classified by (Moyer, 1968) on the basis of experimental methodologies applied:

1. fear-induced aggression (animal would like to escape, but escape is denied)
2. maternal aggression (aggression is triggered by an organism near to the young individual)

3. inter-male aggression (presence of a male elicits aggression in another male)
4. irritable aggression (triggered e.g. by electric shock)
5. sex-related aggression (sexual arousal associated with increased levels of hostility),
6. territorial aggression (intruder enters territory of other male).

According to Siegel (2004) these six forms of aggression "share a similar common feature, namely, an aggressive response based on the presence of elements of fear and/or threat". Predatory aggression is regarded as distinct in most classification schemes.

It is still in discussion whether the various manifestations of aggressive behaviour within a single species – such as aggression occurring in association with predation or defence – have different neurochemical and neuroanatomical correlates (Johansson, 1974; Eichelman, 1987; Albert et al., 1993). If this were indeed true, it is not surprising that, despite the omnipresence of aggression, the link between aggression and neuronal substrates is yet to be resolved.

1.1 AGGRESSION IN *DROSOPHILA*

Drosophila melanogaster due to the armada of experimental techniques it provides can serve as an excellent model system to gain insight into the organization and regulation of aggression and its neuronal substrates – a fact, that is reflected in the wealth of recent studies dealing with *Drosophila* aggression (Dierick and Greenspan, 2006; Edwards et al., 2006; Vrontou et al., 2006; Yurkovic et al., 2006; Certel et al., 2007; Dierick and Greenspan, 2007) and their echo (Miller, 2007; Robin et al., 2007; Simon and Krantz, 2007).

Fighting behaviour in *Drosophila* has been known for a long time. The first report dates back to 1915, when Sturtevant reported that males 'apparently butt heads' (Sturtevant, 1915). Almost fifty years later, a more elaborate description of aggressive interactions was provided by Jacobs (1960), who observed "'charging', i.e. a sudden dash at another male" among others. Over the years extensive studies were performed on the rich repertoire of aggressive behavioural patterns displayed by *Drosophila* (e.g. Skrzipek et al., 1979; Hoffmann, 1987a; Chen et al., 2002; Nilsen et al., 2004). In *Drosophila*, agonistic encounters in both genders are composed of a variety of both offensive and defensive components, some of which are displayed more often in one sex than in the other (Chen et al., 2002; Ueda and Kidokoro, 2002; Nilsen et al., 2004). For example, a characteristic behavioural component

performed mainly by females is the 'head butt'. As reported by Sturtevant (1915) males also display head butts, but less frequently than females. Here, the body is thrust forward towards the opponent thereby appearing to strike the other with the head. Males, instead, engage more frequently in 'boxing', in which males rear up on their hind-legs and strike the opponent with their forelegs. A common behaviour to both genders is 'low-posture fencing'. Here, the opponents push each other with their legs, while remaining in normal standing posture (Chen et al., 2002; Nilsen et al., 2004). Whether a fly fights like a female or a male is influenced by the sex-specific splicing of the *fruitless* gene which is part of the *Drosophila* sex determination hierarchy (Vrontou et al., 2006). An ethogram of behavioural patterns characteristic for male aggression is illustrated in Table 1.

Table 1: **Ethogram of offensive and defensive actions of male flies during agonistic encounters** (from Chen et al., 2002).

Component	Description
Offensive actions	
Approach	One fly lowers body, then advances in the direction of the other
Low-Level fencing	Both flies extend one leg and tap opponent's leg
Wing threat	One fly quickly raises both wings to a 45° angle towards opponent
High-level fencing	One or both flies face each other, extend leg forward and push opponent
Chasing	One fly runs after the other
Lunging	One fly rears up on hind legs and snaps down on the other
Holding	One fly grasps the opponent with forelegs and tries to immobilize
Boxing	Both flies rear up on hind legs and strike the opponent with forelegs
Tussling	Both flies tumble over each other, sometimes leaving food surface
Defensive actions	
Walk away	Loser turns and retreats slowly from advance of winner
Defensive wing threat	Loser flicks wings at 45° angle while facing away from opponent
Run away/being chased	Loser runs away quickly from advance of winner
Fly away	Loser flies off food surface

Within each category the order of the components is roughly in increasing levels of intensity.

Agonistic interactions between *Drosophila* males have also been documented in the field, where some *Drosophila* species form leks on leaves of trees and shrubs (Parsons and Bock, 1976; Parsons, 1977; Parsons and Bock, 1977; Shelly, 1987, 1988, 1990; Bell and Kipp, 1994). Leks are male aggregations, where each male defends its own territory, which contains no resource other than the space where mating takes place. Thus, females receive only gametes but no resource such as food or oviposition substrate (Baker, 1983).

Under laboratory conditions, *Drosophila* aggression can be easily elicited. In earlier studies several males were put together in an arena containing a food patch (and females depending on the study) (e.g. Dow and von Schilcher, 1975; Skrzipek et al., 1979; Hoffmann,

1987a). Lately, set-ups to elicit *Drosophila* aggression were simplified by observing only a single 'same-gender' dyad in an arena containing food as the only resource (e.g. Dierick and Greenspan, 2006; Vrontou et al., 2006; Certel et al., 2007). In one study, the set-up was even further simplified by eliminating any resource (Dierick and Greenspan, 2007).

1.2 AGE, EXPERIENCE, BODY SIZE

A characteristic feature of *Drosophila* aggression is its enormous variability (e.g. Archer, 1988), indicating that numerous factors influence this complex behaviour. Two such factors are age and experience, i.e. contact with conspecifics, as demonstrated by Hoffmann (1990). Within the first hours after eclosion, males do not fight; in fact, mating precedes territorial aggression by 14 h on average. From then on, territorial success increases until males are 3 days old. How fast a territory is established, depends on previous experience with conspecifics. Isolated males establish their territories faster than 'socialised' males, i.e. males that were kept together with other males. Notably, this effect is not evident, if the 'socialised' male was kept with females instead of males. However, it appears as if isolation only alters the short-term territorial success of males since after 5 h of observation, territories were held in equal shares by isolated males and males with prior contact to conspecifics. A short-term effect of isolation is also reported for females, where social experience decreased the frequency of aggressive display (Ueda and Kidokoro, 2002).

Another well-known factor affecting aggressive behaviour is body size. In *Drosophila*, field studies indicate that larger males win more aggressive encounters than their smaller opponents (Partridge et al., 1987; Shelly, 1987; Bell and Kipp, 1994). For *Drosophila melanogaster*, this size effect was also found under laboratory conditions (Partridge and Farquhar, 1983; Hoffmann, 1987b; Hoffmann, 1987a). The effect was most obvious when the weight differences between the opponents were pronounced with about 50% due to raising conditions (Hoffmann, 1987b).

There are theoretical models trying to explain why in many species body size has a pronounced impact on the course of a fight. Body size is presumably the most important indicator of an animal's fighting ability in game theory models of aggression (Archer, 1988). Game theory models of aggression deal with the costs and benefits of fighting and the resulting strategies adopted by the contestants. In most real fights, the opponents will differ with respect to either the fighting ability – the so-called 'resource holding power' (RHP) – or in prior possession of a resource. It is argued that RHP disparity, for example due to size

differences, and holder:attacker imbalance should shape the course of a fight (Parker, 1974; Smith and Parker, 1976). Accordingly, competitors of a fight would assess their opponent's RHP relative to their own and would adjust their behaviour according to this assessment. Escalation should be therefore restricted to opponents with similar RHP. Where there is a strong difference in RHP, the animal with the largest RHP should win since the opponent withdraws after a conventional display. In accordance with this hypothesis, size influences initiation, duration and outcome of aggressive encounters in numerous species throughout the animal kingdom with the larger opponent being likely to win (reviewed in Archer, 1988; Polak, 1994; Nowbahari et al., 1999).

1.3 SENSORY MODALITIES AND *DROSOPHILA* AGGRESSION

So far, how sensory modalities influence *Drosophila* aggression is an uncharted territory. In courtship, both partners exchange signals belonging to various modalities, in particular acoustic signals, pheromones and visual stimuli (reviewed in Greenspan and Ferveur, 2000). Similarly, male aggression consists of a variety of behavioural patterns that could convey information to the diverse sensory organs of the opponent. For example, wing threat, a posture where one male raises up its wings for an extended period of time, might be mainly a visual signal, as there is no direct contact between the two contestants. While *Drosophila* males extend their legs and tap/push each other ('fencing'), a behavioural pattern which is frequently accompanied by wing flicks, pheromones and acoustic signals could be exchanged. In the following the effects of acoustic, chemical and visual signals with respect to *Drosophila* male-male interactions will be briefly described.

Acoustic signals: In courtship, another complex behaviour displayed by *Drosophila*, acoustic signals are of major importance. Here, males produce a species-specific courtship song by vibrating their wings. This courtship song consists of two characteristic features: a series of pulses, the pulse song, interrupted by occasional periods of humming, the sine song (Shorey, 1962; Schilcher, 1976). A small pilot study focused on the acoustic signals transmitted during agonistic interactions (Jónsson, 2005). In contrast to the highly stereotypical courtship song, very short (< 5 msec) and erratic sounds (inter-pulse interval > 100 msec) are characteristics of male aggression, that are mainly produced during defence situations. Whether the competitors indeed perceive these sounds as signals needs to be further elucidated.

Chemical stimuli: In many species, olfaction has a pronounced impact on aggression. For example, when rats marked with colony male urine were placed in established colonies (colony-intruder paradigm), they were ignored by the alpha-male. Animals marked with strange male urine, however, were always attacked by the alpha male (Garciabrull et al., 1993). Along this line, resident mice deficient for an ion channel specific for neurons of the mouse vomeronasal organ, TRP2, display no aggression towards castrated male intruders swabbed with strange male urine in contrast to heterozygote and wild-type residents (Stowers et al., 2002). In insects, cuticular hydrocarbons constitute an essential recognition signal between two or more individuals (reviewed in Howard and Blomquist, 2005). In ants, they serve as colony-specific nestmate recognition cues among others: cotton balls soaked with mixtures of hydrocarbons differing to the nest-specific hydrocarbons elicit aggression (Greene and Gordon, 2007). In *Drosophila*, information about the impact of chemical stimuli on male aggression is still lacking. However, in another type of male-male interaction, courtship dominance, chemical stimuli play an important role. Courtship dominance takes place when one male directs intense courtship towards another male, which remains passive (Svetec et al., 2005). By using males lacking the characteristic adult unsaturated hydrocarbons 7-tricosene and 7-pentacosene, Svetec et al. (2005) demonstrated that courtship dominance occurs when the dominant male, but not the subordinate one, had previous contact to live adult males carrying male unsaturated hydrocarbons. Concerning *Drosophila* aggression, as mentioned above, previous contact to conspecific males altered short-term territorial success (Hoffmann, 1990). It remains to be elucidated whether this effect is partially due to chemical stimuli and how pheromones generally contribute to wild-type aggression.

Visual stimuli: Information is lacking regarding the influence of vision on *Drosophila* aggression. This is surprising in the light of the *white* mutation which is present in the genetic background of many transgenic flies and affects contrast perception (Hengstenberg and Gotz, 1967; Wehner et al., 1969). In courtship, a behaviour which requires visual stimuli for normal execution (reviewed in Tompkins, 1984), the *white* mutation diminishes the courtship index, i.e. the fractional amount of the total observation time spent in courtship activity, compared to wild-type flies if tested under white light. In contrast, there is no difference in performance under dim red light (Joiner and Griffith, 1997) in which the flies see poorly, if at all.

1.4 THE *WHITE* GENE

The *white* gene encodes a half transporter belonging to the ATP-binding cassette (ABC) transporter superfamily (O'Hare et al., 1984). ABC transporters are fundamental for numerous processes in the cell and mutations in the corresponding genes cause or contribute to several human genetic disorders such as cystic fibrosis and retinal degeneration (reviewed in Dean and Allikmets, 2001). In *Drosophila*, the White protein forms a heterodimer with either of two other ABC transporter proteins: presumably, White and Scarlet together form a tryptophan transporter, whereas White and Brown form a guanine transporter (Sullivan and Sullivan, 1975; Sullivan et al., 1979, 1980; Bingham et al., 1981; O'Hare et al., 1984; Dreesen et al., 1988; Tearle et al., 1989) Guanine and tryptophan are precursors of the red pigments (drosopterins) and brown pigments (xanthommatin), respectively. These pigments are stored in granules of specialized pigment cells in each ommatidium of the compound eye (review concerning eye pigmentation: Summers et al., 1982). Consequently, *white* null mutant flies lack eye pigmentation resulting in the eponymous white eyes. As mentioned above, *white* null mutant flies are impaired in contrast perception (Hengstenberg and Gotz, 1967; Wehner et al., 1969) and consequently in behaviours that require accurate vision, such as courtship (Tompkins, 1984).

Surprisingly, *white* mutant flies have altered memory in an operant learning paradigm even though tested in complete darkness (Diegelmann et al., 2006). This finding is surprising insofar, as to my knowledge there is so far only one indication that White is indeed present in the central brain: Campbell and Nash (2001) detected *white* messenger RNA in heads of *sine oculis*^{1} (*so*^{1}) flies by using RT-PCR. *so*^{1} flies have neither eyes nor ocelli and should consequently lack pigment producing cells. However, immunohistochemical staining using a White antibody detected White only in pigment cells of the retina and subretinal glia, not in the central brain (I. Meinertzhagen, personal communication). Using the same anti-White antibody and an additional anti-Scarlet antibody, Mackenzie and colleagues localized White and Scarlet in the membranes of pigment granules within pigment cells and retinula cells of the compound eye, but not in the plasma membrane (Mackenzie et al., 2000). The authors therefore suggest that a transporter other than White/Scarlet transports tryptophan across the plasma membrane, whereas White/Scarlet mediates the transport of an intermediate in the xanthommatin biosynthetic pathway from the cytoplasm into pigment granules.

1.5 BIOGENIC AMINES

Both vertebrates and invertebrates have several biogenic amines, the major being dopamine, serotonin and histamine, which exist in both phylogenetic groups, whereas noradrenaline and adrenaline occur only in vertebrates (Blenau and Baumann, 2001). The presumed invertebrate counterparts of the adrenergic transmitters are octopamine and tyramine (Roeder, 1999). There is ample evidence that amine neuron systems are key players in regulating aggression in a variety of animal species (reviewed in Nelson and Trainor, 2007). The most extensively studied amine system with respect to aggression is the serotonergic system: it appears that in vertebrates reduced activity of the serotonergic system is associated with enhanced aggression (e.g. reviewed in Lesch, 2003; Underwood and Mann, 2003). In contrast, in *Drosophila*, serotonin is not necessary for aggression, but an increased serotonin level can enhance it (Dierick and Greenspan, 2007), thereby suggesting that serotonin might function differently in modulating aggression in vertebrates and invertebrates.

Drosophila provides an excellent model system to study the impact of biogenic amines on aggression due to the availability of various mutant lines, driver lines and effector lines specific for amine systems. Driver lines and effector lines are the two essential components making up the binary GAL4/UAS system (Brand and Perrimon, 1993). With the GAL4/UAS technique genes can be expressed in a temporally and spatially restricted manner.

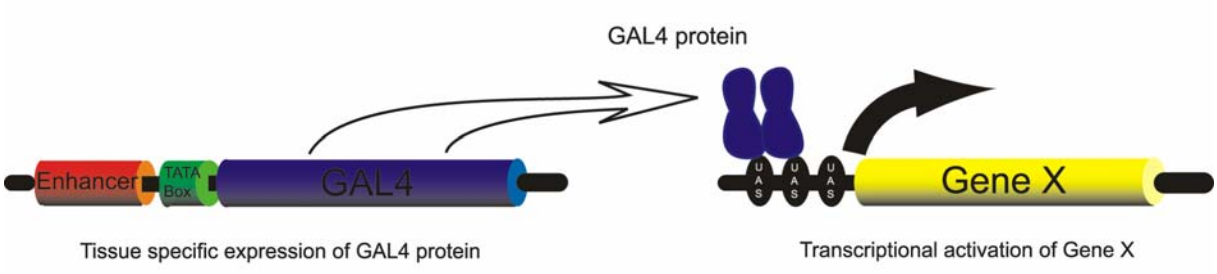


Figure 1: GAL4/UAS technique (modified from Brand and Dormand, 1995)

For this purpose a construct carrying the gene of the yeast transcription factor GAL4 is inserted in the *Drosophila* genome. In this so-called driver-line, GAL4 expression is driven by a group of endogenous promoters/enhancers in a spatially and temporally controlled manner ('enhancer trap'), or by a group of promoters/enhancers previously cloned upstream of the GAL4 gene and subsequently inserted into the *Drosophila* genome. On a second construct and inserted in another strain, the GAL4-binding site (UAS) and downstream of it a

gene of one's own choice are encoded (effector line). The effector gene will now be expressed in a GAL4 dependent manner, that is, only in those cells and at that particular time at which GAL4 is expressed (Figure 1).

The following figure summarizes the biosynthesis of tyramine, octopamine, dopamine and serotonin. It also illustrates some of the tools used in this study; however, it is not a complete overview of the tools available to target the *Drosophila* amine systems.

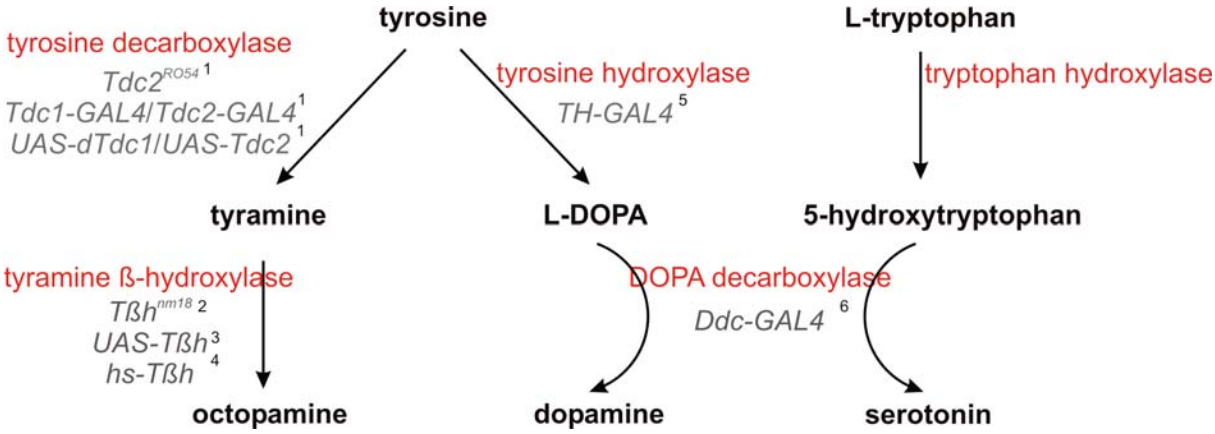


Figure 2: Biosynthesis of biogenic amines in *Drosophila* with some tools specific for the enzymes involved in the biosynthesis: [1] (Cole et al., 2005); [2] (Monastirioti et al., 1996); [3] (Monastirioti, 2003); [4] (Schwaerzel et al., 2003); [5] (Friggi-Grelin et al., 2003); [6] (Li et al., 2000)

1.5.1 Noradrenaline/adrenaline

The adrenergic system plays a pivotal role in modulating aggressive behaviour. However, how it exerts its influence is still under debate.

In vertebrates, monoamine oxidase A (MAOA) inactivates serotonin, dopamine, and noradrenaline. Adult male mice lacking MAOA display enhanced aggression (Cases et al., 1995). Interestingly, in MAOA deficient mice the brain serotonin level is highly elevated in pups, but declines to wild-type level in adult mice, whereas the noradrenaline level remains elevated (even though there is a pronounced drop in noradrenaline level at the last measurement, 210 days). Therefore, one might hypothesize that the high level of noradrenaline during adulthood leads to enhanced aggression. In accordance, dopamine beta-hydroxylase knockout mice that lack noradrenergic function show hardly any aggressive behaviour (Marino et al., 2005). In general, it appears as if noradrenaline enhances aggression, even though there might be a dose-dependent biphasic effect (Haller et al., 1998).

For example, in mice antidepressants stimulating noradrenaline release or blocking re-uptake lead to an increase of aggression at low doses whereas a decrease at large doses. These effects might be influenced by serotonin, however, since the applied antidepressants also affect serotonin levels (Cai et al., 1993).

Noradrenaline exerts its action via three classes of noradrenergic receptors: α_1-adrenoceptors, α_2-adrenoceptors and β-adrenoceptors (reviewed in Hein, 2006). Whereas α_1-adrenoceptors are localized postsynaptically, α_2-adrenoceptors and β-adrenoceptors are situated both postsynaptically and presynaptically. The increase in aggression due to low doses of antidepressants (Matsumoto et al., 1991) can be blocked by β_2-adrenoceptor antagonists (Matsumoto et al., 1994). However, mice with targeted disruption of the α_{2C}-adrenoceptor gene have shortened attack latency, whereas tissue specific α_{2C}-adrenoceptor overexpression results in increased latency to attack (Sallinen et al., 1998). Thus, a complex picture emerges concerning the interrelation of noradrenaline and aggression.

A working model concerning the function of the adrenergic system on aggression must consider the extensive distribution of noradrenaline and adrenaline synthesis sites. They are located within the central nervous system with the greatest concentration of noradrenergic neurons within the *Locus coereleus*, the sympathetic nervous system (noradrenaline) and the adrenal medulla (mainly adrenaline). Consequently, the adrenergic system can modulate aggression at various levels with some evoked effects influencing aggression only indirectly, for example by altering energy metabolism or pain perception. An overview concerning the complex effects of the adrenergic system on aggression is given in Haller et al. (1998), Haller and Kruk (2003) and Siegel (2004).

1.5.2 Octopamine/tyramine

In invertebrates, the interrelation of octopamine and aggression is equally complex. In crustaceans, octopamine injection leads to a submissive looking body posture (Livingstone et al., 1980; Antonsen and Paul, 1997). In crickets, somewhat surprisingly, depletion of dopamine, octopamine, and serotonin by reserpine leaves the major components of normal aggression intact, even though animals are extremely lethargic. A more refined depletion of dopamine and octopamine by AMT (α-methyl-p-tyrosine), however, decreases the duration and the intensity of aggressive encounters (Stevenson et al., 2000). This condition can be reversed by injecting the octopamine agonist chlordimeform (Stevenson et al., 2005), thereby

suggesting that the depletion of octopamine, but not dopamine causes the decrease in aggression. Chlordimeform injection into naïve animals does not alter spontaneous aggression, but normally submissive losers re-engage in fights faster (Stevenson et al., 2005). Similarly, flying also renews willingness to fight (Hofmann and Stevenson, 2000; Stevenson et al., 2005). Treating crickets with either AMT or epinastine, a neuronal octopamine receptor antagonist, abolishes this effect of flying (Stevenson et al., 2005). The authors therefore suggest that "this selective modulation of aggression by flying is achieved by the restricted release of octopamine as a neuromodulator in the nervous system".

In *Drosophila*, two studies using mutants for tyramine beta-hydroxylase ($T\beta h^{nM18}$) investigate the role of octopamine in modulating male aggression. Tβh converts tyramine to octopamine, therefore $T\beta h^{nM18}$ flies lack octopamine, but show ~10fold increased brain tyramine levels. Baier et al. (2002) observed in $T\beta h^{nM18}$ males an overall strong decrease of various components of aggressive behaviour when fighting against wild-type males. In contrast, focussing on the males' behavioural choice between courtship and aggression, Certel et al. (2007) observed no impairment for $T\beta h^{nM18}$ males when fighting against each other (S. Certel and E.A. Kravitz, personal communication). Only when a male approaches another male by vibrating its wing(s), occurring in about three encounters per 30 min recording period, $T\beta h^{nM18}$ males show less often a transition to aggressive behaviour than wild-type males, instead they transition to courtship behavioural patterns, i.e. licking and abdomen bending. This discrepancy might be due to differences in genetic background and behavioural analysis.

When studying the effects of octopamine on invertebrate aggression, one has to keep in mind that similarly to the situation in vertebrates, octopamine's influence on aggression might be manifold. In *Drosophila*, there are ~100 octopamine-immunoreactive cells in the brain innervating most major neuropiles, such as the optic lobes, the antennal lobe glomeruli and the mushroom body (mainly the calyces) (Sinakevitch and Strausfeld, 2006). Additionally, octopaminergic cells are localized in neurons of the thoraco-abdominal nervous system supplying peripheral effector organs among others (Monastirioti, 2003). As a neurohormone octopamine can exert long-range effects (reviewed in Roeder, 1999). It is suggested that octopamine primes the organism for energy-demanding behaviours. For example, octopamine is released before a flight to stimulate muscle glycolysis during take-off in locusts. During flight, octopamine-containing neurons are shut-down presumably to decrease glycolysis and to allow fat metabolism as the major energy resource (Duch and Pfluger, 1999; Mentel et al., 2003). Moreover, octopamine is thought to control the

maintenance of various rhythmic behaviours. Depending on the location, iontophoretical release of octopamine elicits motor activity similar to either tibia stepping movements or flight or it suppresses ongoing oviposition digging previously evoked by severing the ventral nerve cord in females (Sombati and Hoyle, 1984). Consistent with these results, octopamine triggers activity in isolated thoracic nerve cord preparations of the locust resembling the motor program elicited during flight (Stevenson and Kutsch, 1987). Furthermore, decapitated *Drosophila* show highly increased locomotion and hind leg grooming after octopamine application on the exposed nerve cord (Yellman et al., 1997). Additionally, octopamine might alter invertebrate aggression, by affecting hearing, touch, vision, taste and smell (reviewed in Farooqui, 2007). For example, in *Bombyx mori* males, octopamine alters the response of receptor neurons sensitive to the pheromone components bombykol and bombykal (Pophof, 2002). Thus, in invertebrates octopamine might affect aggression on various levels, which might work in concert to modulate a behaviour as complex as aggression.

Lately, it is discussed that tyramine is not merely the precursor of octopamine, but also a signalling molecule. Tyramine inhibits excitatory junctional potentials of larval body-wall muscle (Kutsukake et al., 2000; Nagaya et al., 2002), regulates the chloride permeability of Malpighian tubules (Blumenthal, 2003) and is suggested to modulate larval locomotion (Saraswati et al., 2004). Moreover, immunohistochemical data indicate that there are neurons expressing tyramine but not octopamine (Nagaya et al., 2002). Therefore, not only octopamine but also its precursor tyramine might contribute in modulating *Drosophila* aggressive behaviour.

1.6 NEURONAL CORRELATES OF AGGRESSION – AN OUTLOOK

The various experimental techniques provided by *Drosophila melanogaster* enable the investigator to interfere selectively with the highly sensitive system of aggression. A drawback, however, is the rich repertoire of aggressive behavioural patterns *Drosophila* displays, since its manual analysis is a time-consuming and demanding task. To simplify the analysis and to standardize evaluation, I chose to focus on a single indicator of aggression: the lunge, a striking feature unique to *Drosophila* male aggression. I developed in cooperation with **Andreas Eckart** (University of Würzburg) an automated, video-based analysis of *Drosophila* male aggression, by evaluating the lunge. While I was responsible for developing

the underlying ideas as well as checking the software's functionality, Andreas Eckart wrote all software scripts.

Here I demonstrate that (1) body size differences of 8% and higher influence the outcome of a fight in favour of the larger male; (2) walking activity alters lunge frequency with more lunges performed by the more active pair of males; (3) flies mutant for the *white* gene are profoundly impaired in aggression, which is not solely due to reduced visual performance. (4) Either knocking-down *white* in various brain regions or chemically ablating the mushroom body located in the central brain by deleting its neuroblast precursors diminishes aggression, indicating that integrity of various neural circuits/brain regions is required for wild-type aggression to occur. Furthermore, I show that (5) flies lacking octopamine signalling but having altered tyramine signalling display hardly any lunges. A quantitative high-speed analysis revealed that lunge execution is almost indistinguishable from wild-type males. The underlying high-speed movies were generated in cooperation with **Anthony Herrel** (University of Antwerp), who gave equipment and advice. The results from the experiments in which octopamine levels and/or tyramine levels were restored suggest that an elaborate pattern of octopamine levels in time and space is required to enable flies to express wild-type aggressive behaviour.

2 MATERIAL AND METHODS

2.1 FLY STOCKS AND MAINTENANCE

Flies were raised at 25°C and 60% relative humidity in a 14/10 hr light/dark cycle on standard *Drosophila* medium (cornmeal, agar, molasses, yeast, nipagin). Only flies of the UAS-RNAi-*white* experiments were raised in a 17/7hr light/dark cycle. The w^{+};;UAS-RNAi-*white* line and the w^{1118};(;)GMR-*white* line were obtained from T. Zars (University of Missouri-Columbia) and S. Fischer (University of Regensburg), respectively. The w^{1118} stock, the *dTdc2*-GAL4 (Cole et al., 2005) stock and the *Appl*-GAL4 (Torroja et al., 1999) stock were cantonized for at least six generations. For the outcrossing 50 males and females were used in each generation. The UAS-*kir2.1* line, the UAS-shi^{ts1} line and the $Tdc2^{RO54}$ line were crossed into a w^{+} background. The $T\beta h^{nM18}$ line had already been crossed into a w^{+} background by H. Scholz (University of Würzburg, Germany) and independently by S. Certel (Harvard Medical School, USA).

Newly emerged male flies were collected and individually kept for six days in 22 cm^3 vials containing food. On day 5 all flies were anesthetised by cooling down (4°C) for less than 2 min and every other male was marked with a white dot (AeroColor, colour 101, Schmincke, Germany) on the thorax. They were put back into the food vials for at least 12 hr. On day six two males were aspirated into the arena.

2.2 HIGH-SPEED ANALYSIS

The arena measured 2 cm x 3 cm x 12 cm. Clips were recorded at 500 frames per second using a Redlake MotionPro 2000 digital high speed camera equipped with a Sigma Macro lens. For CS males, videos were taken from lateral and dorsal views, whereas for $T\beta h^{nM18}$ males only videos in dorsal view were captured. Anatomical landmarks were digitised frame-by-frame using Didge (version 2.2.0, Alistair Cullum, Creighton University, Ohama, USA). To analyse clips recorded in lateral and dorsal view, eleven and eighteen points were digitized, respectively, to track movements of the body, the legs and the position of the legs relative to each other (Figure 3A and C). Based on these points for lateral and dorsal view thirteen and twelve variables, respectively, were calculated describing the displacements of body and limb segments (Figure 3B and D). Displacement profiles were

smoothed using a fourth order zero phase shift butterworth filter with user defined cut-off frequency set at 150 Hz (Winter 2005). Velocities and accelerations were then calculated by differentiation of the displacement profiles. Although displacements were calculated for both limb pairs regarding the dorsal view, only the greater of the two was retained for statistical analysis to reduce the number of variables.

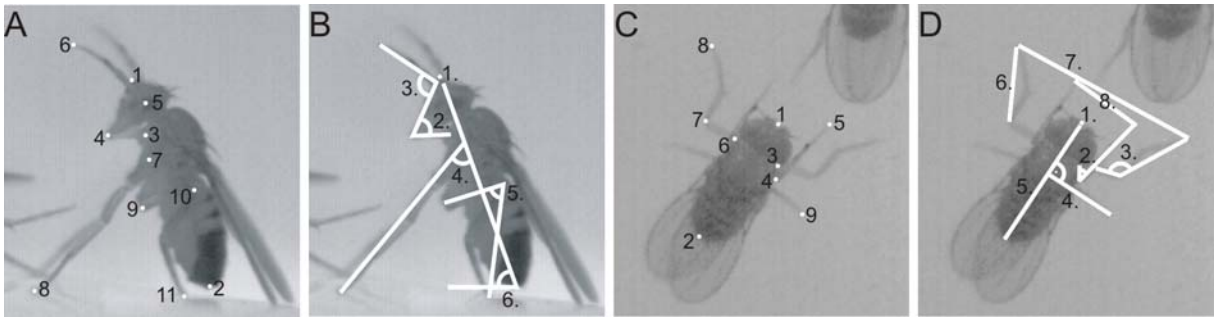


Figure 3: Details to the kinematic analysis of lunges. (A) Eleven points were digitized (1) tip of the head; (2) tip of the abdomen; (3) intersection of the front leg with the body; (4) coxa-trochanteric joint of the front leg; (5) femorotibial joint of the front leg; (6) tip of the front leg; (7) intersection of the middle leg with the body; (8) tip of the middle leg; (9) coxa-trochanteric joint of the hind leg; (10) femorotibial joint of the hind leg; (11) tip of the hind leg. **(B)** Based on the coordinates, head velocity, head acceleration, five angles and one distance were calculated. For each angle maximal displacement and maximal positive and negative velocity were determined. Angles, displacements, velocities and acceleration calculated were (1) head velocity/head acceleration; (2) the proximal angle front leg as determined by markers 3,4,5; (3) the distal angle front leg as determined by markers 4,5,6; (4) the angle enclosed by the two lines given by the markers 7/8 and 1/2; (5) the angle hind leg as determined by markers 9,10,11; (6) the angle enclosed by the line 1/2 to the horizontal and (7) the distance of 1 to the ground (not illustrated). **(C)** 18 points were digitized. For simplicity, points characterizing limbs are illustrated for one leg only even though they were digitized for both: (1) tip of the head; (2) tip of the abdomen; (3) intersection of the front leg with the body; (4) femorotibial joint of the front leg; (5) tip of the front leg; (6) intersection of the middle leg with the body; (7) femorotibial joint of middle leg; (8) tip of the middle leg; (9) tip of the hind leg. **(D)** Based on the coordinates, head velocity, head acceleration, two angles and five distances were calculated. For each angle maximal displacement and maximal positive and negative velocity were determined. Angles, displacements and velocities calculated were (1) head velocity; head acceleration (2) the angle front leg as determined by markers 3,4,5; (3) angle of the middle leg as determined by markers 6,7,8; (4) distance of the tip of the hind leg to the long axis of the body; (5) body length; (6) middle leg length (distance between markers 7 and 8); (7) distance between the middle limbs; (8) distance between the front limbs.

2.3 OA/TA ENRICHED FOOD

TDC2^{RO54} flies received food supplemented with 0.3 mg/ml tyramine hydrochloride (T2879; Sigma) and 3 mg/ml octopamine hydrochloride (O0250; Sigma) for the time period between eclosion and test. Food was melted in a microwave. Shortly before it solidified, TA and/or OA were mixed into the food. Food for *Tβh*^{nM18} flies was supplemented with 5 mg/ml OA. Here, in one group, the eggs were already laid on OA-enriched food, in the other group, treatment started after eclosion and ended with the start of the fight.

2.4 HEAT-SHOCK PROTOCOL

Males were treated as described in Schwaerzel et al. (2003). Males were heat-shocked at 37°C for 30 min 18 hr and 12 hr before the fight. For the heat-shock, males were aspirated into a new vial containing only a moist filter paper. These vials had been preheated at 37°C for 30 min. After each heat-shock, males were aspirated back into the original vial. Females were treated as described in Monastirioti (2003). Females were heat-shocked twice for 60 min with a 3 hr break in between. This heat-shock regime was not applied for males, as it led to a high mortality rate in males. Otherwise, the treatment was the same as that of males.

2.5 *SHIBIRE*^{TS1}-EXPERIMENTS

Flies were raised at 25°C. The fight arena was preheated for 30 min, before the first fight of the day was set up. Flies were directly aspirated into the fight arena, without prior heating. Three different sets of experiments were performed at 33°C, 31.5°C, or 30.5°C, in order to find a temperature, which affects aggression of control males the least. However, each genotype was affected to the same extent independent of how much the temperature was elevated; therefore data were pooled for each genotype.

2.6 HU TREATMENT

Flies were treated as described in Debelle and Heisenberg (1994). Briefly, equally-aged larvae of less than 1 hr old were collected. Larvae were first transferred to heat-killed yeast suspension containing hydroxyurea (60 mg/ml), whereas control larvae were transferred to yeast only. After 4 hr larvae were washed in distilled water and transferred into vials containing standard medium.

2.7 STATISTICAL ANALYSES

When not otherwise stated, Kruskal-Wallis-ANOVA was applied to detect overall differences among several unpaired groups. When differences between groups occurred, the significantly different groups were filtered out by pairwise comparisons using Mann-Whitney-U-tests. Differences between two genotypes concerning the percentage of egg-laying females were determined with Fisher's Exact test. In all figures, one, two and three asterisks

indicate a α-level of 0.05, 0.01 and 0.001. For all multiple comparisons, Bonferroni-correction was applied. However, even those differences are indicated, which failed to pass the significance criterion after Bonferroni correction, but were significant without it. In these cases asterisks are given in parentheses.

To compare the kinematics lunges performed by CS males with lunges executed by $T\beta h^{nM18}$ males twelve variables were calculated. As $T\beta h^{nM18}$ males were significantly smaller than CS males, a MANCOVA was applied including all twelve variables. Since only the front leg variables correlated with size, an ANCOVA was run for each of them. For each of the other variables, a t-test was used.

Statistical Analyses were performed with STATISTICA, version 7.1 (StatSoft, Tulsa, OK, USA) and JMP IN software, version 4.02 (SAS Institute, Cary, NC, USA).

3 RESULTS

3.1 *DROSOPHILA* MALE AGGRESSION: THE LUNGE

Drosophila male aggression is composed of various aggressive behavioural patterns. 'Fencing', i.e. pushing the opponent with one's legs, is regarded as a low-intensity aggressive behaviour (Chen et al., 2002); however, it has been questioned, whether it is indeed aggressive in nature (Dierick and Greenspan, 2006). During a 'wing threat' a male raises up its wings for an extended period of time, thereby mostly facing its opponent. The most frequent aggressive behaviour to displace an opponent from the territory are 'lunges' (Hoffmann, 1987a). They occur alone or in the context of tussling, a high-intensity aggressive interaction, which characterizes escalated encounters. Here, both males 'box' at each other, that is, they stand on their hind legs and strike each other with their forelegs, frequently interrupted by mutual lunges.

A lunge is composed of three distinct phases, although the third phase may not always be present. During phase 1 the attacking male raises up on his hind-legs. He then snaps down on its opponent, whom he tries to grab with his forelegs (phase 2). If successful, he holds on while the attacked fly tries to escape (phase 3). Phase 1 and 2 take only 46 msec in total (median; n = 25). Figure 4A and H show representative stills of lunges as recorded from lateral view and top view, respectively.

For each perspective separately, five lunges performed by the same male were recorded at a frame rate of 500 frames per second. Several parameters were calculated over normalized successive time bins with the full length of phase 1 and 2 being defined as 100% (Figure 4 B-G). Means based on clips from lateral and dorsal view are given in red and green, respectively. The raising of the fly's long body axis during phase 1 followed by its snapping down in phase 2 are represented in lateral view as an increase of body angle during the first phase and a decrease during the latter phase. From top view, the projected body size sharply decreases during phase 1 and abruptly increases during phase 2 (Figure 4D). In both perspectives there is a pronounced increase in velocity during phase 2 with peak velocities of 254 ± 11.8 mm/sec calculated from lateral view (Figure 4B). The changes in acceleration over time are also similar in both perspectives reaching accelerations of 31.84 ± 1.97 m/sec^2 from lateral view (means ± SEMs) (Figure 4C).

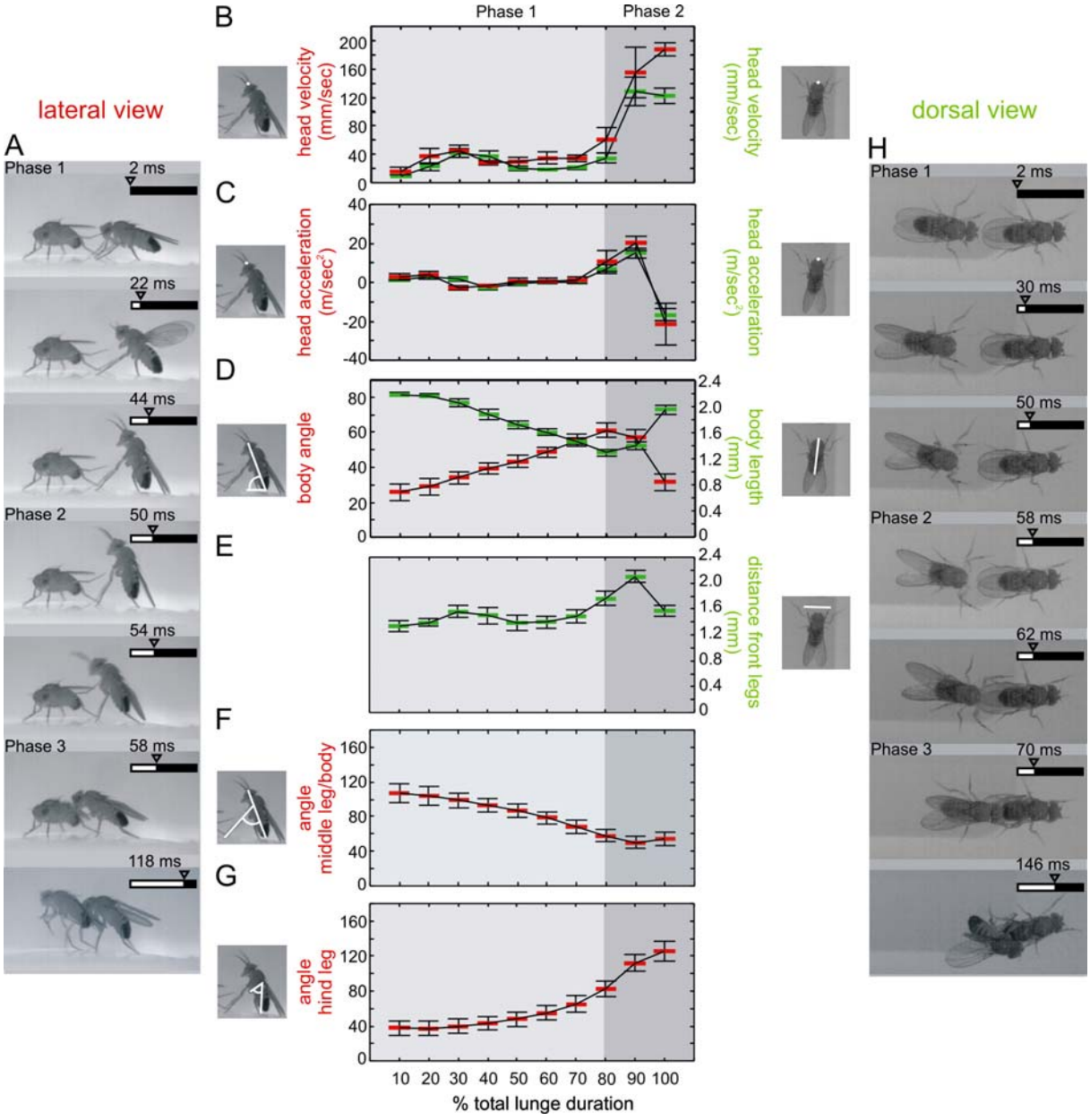


Figure 4: **The lunge (dorsal and lateral view)**. **(A)** still series of a lunge taken from lateral view showing all three phases of a lunge; **(B – G)** time course of various parameters with the full length of phase 1 and 2 taken as 100%. The respective parameter illustrated in each graph is depicted in the picture to the left (lateral view) and to the right (dorsal view). Medians from lateral and dorsal view are given in red and green, respectively. **(B)** head velocity; **(C)** head acceleration; **(D)** body angle (lateral) and body length (dorsal); **(E)** distance front legs; **(F)** angle middle leg/body; **(G)** angle hind leg. **(H)** still series of a lunge taken from top view showing all three phases of a lunge

Figure 4 E-G illustrate characteristic movements of front legs, middle legs and hind legs, respectively. With the front legs, the attacking fly tries to grab his opponent, which is best seen from dorsal view. While snapping down on the opponent, the attacking fly opens its front legs (measured as the distance between the tips of the front legs), which are immediately closed when touching the opponent (Figure 4E). Right before the onset of a lunge, middle legs are positioned remarkably anterior of the fly's body centre of gravity resulting in a rather

large angle enclosed by the two lines given by the middle leg and the fly's long body axis. During phase 1 the fly straightens its middle legs, so that the long body axis elevates; accordingly the angle between middle leg and long body axis decreases (Figure 4F). Notably, the position of middle and hind leg ground contact is mostly constant throughout phase 1 and 2. The hind-legs appear to give the momentum for the lunge: the angle enclosed by the femur and the line connecting the tibiofemural joint and the hind leg tip steadily increases during the lunge (Figure 4G).

To simplify the analysis of *Drosophila* aggression and to standardize evaluation, I chose to focus on a single indicator of aggression: the lunge. The lunge is unique to aggression, it occurs frequently and it has characteristic features which make its evaluation amenable to automated software analysis. I therefore developed in cooperation with Andreas Eckart for the first time an automated, video-based analysis of *Drosophila* male aggression, relying on the assumption that the number of lunges reflects the overall aggression of the competitors.

3.2 AUTOMATED ANALYSIS OF *DROSOPHILA* MALE AGGRESSION

This section is aimed at providing *Drosophila* researchers interested in applying the automated analysis of *Drosophila* male aggression in their laboratories with all the information needed. It describes the requirements for the set-up (3.2.1) and the movies (3.2.2). Also, it includes for each software a detailed manual (3.2.3 – 3.2.8).

The automated analysis of *Drosophila* male aggression is a multi-step process:

3.2.1 Setting up the arena.

3.2.2 Generating a video using Adobe Premiere.

Processing the generated video to extract relevant information such as the area covered by each fly:

3.2.3 Adjusting the basic conditions (properties) of each video using **CalcProp**. These conditions are stored in a so-called property file.

3.2.4 Processing of property files by **LoopRun**, which generates output files containing all relevant information for each single frame.

3.2.5 Extracting the number of lunges each fly performed based on the LoopRun output file using **LungeCount**.

3.2.6 Calculating information such as the distance each fly walked based on the LoopRun output file using **FlySpeedHisto**.

3.2.7 Calculating the size of each fly based on a slightly different LoopRun output file ('fill holes within objects') using **FlySize**.

3.2.8 if preferred: Checking of lunges given by the LungeCount software using **LungeView**.

The program environment of CalcProp, LoopRun and LungeView is Lab View. Therefore, Lab View has to be installed prior to the application of any of the six programs mentioned above. The six programs themselves need no separate installation.

3.2.1 Setting up the arena

To enable the software to extract common features of lunges, it is essential to keep the flies' body position and their distance to the camera constant throughout the experiment. Hence, the camera (Panasonic NV-GS 400) equipped with a 4+ lens (Hoya; Tokina Co.; Japan) is positioned above the 4 cm x 5 cm x 12 cm arena. The floor of the arena is made of polyoxymethylen. In its centre, a hollow of 1 cm x 1 cm x 0.5 cm filled with an aliquot of a mixture of 67 ml apple juice, 1.5 g agarose and 1.7 g sucrose is surrounded by a 0.5 cm-wide moat of 2% agarose. The food must be of light colour, so that it strongly contrasts with the flies' bodies. The glass walls are covered by Fluon (FluonGP1, Whitford GmbH, Germany). This tetrafluoroethylene co-polymer results in a slippery layer on the glass wall to prevent the insects from sitting on the wall. In addition, the height of the walls prevents most flies from landing on the lid. Thus, the flies are always in focus of the camera, an essential prerequisite for the automated analysis.

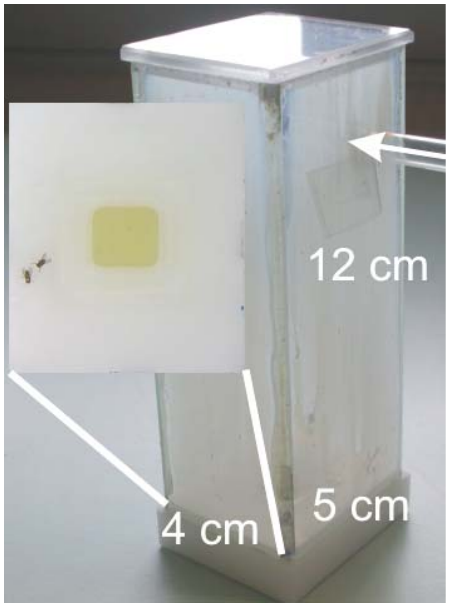
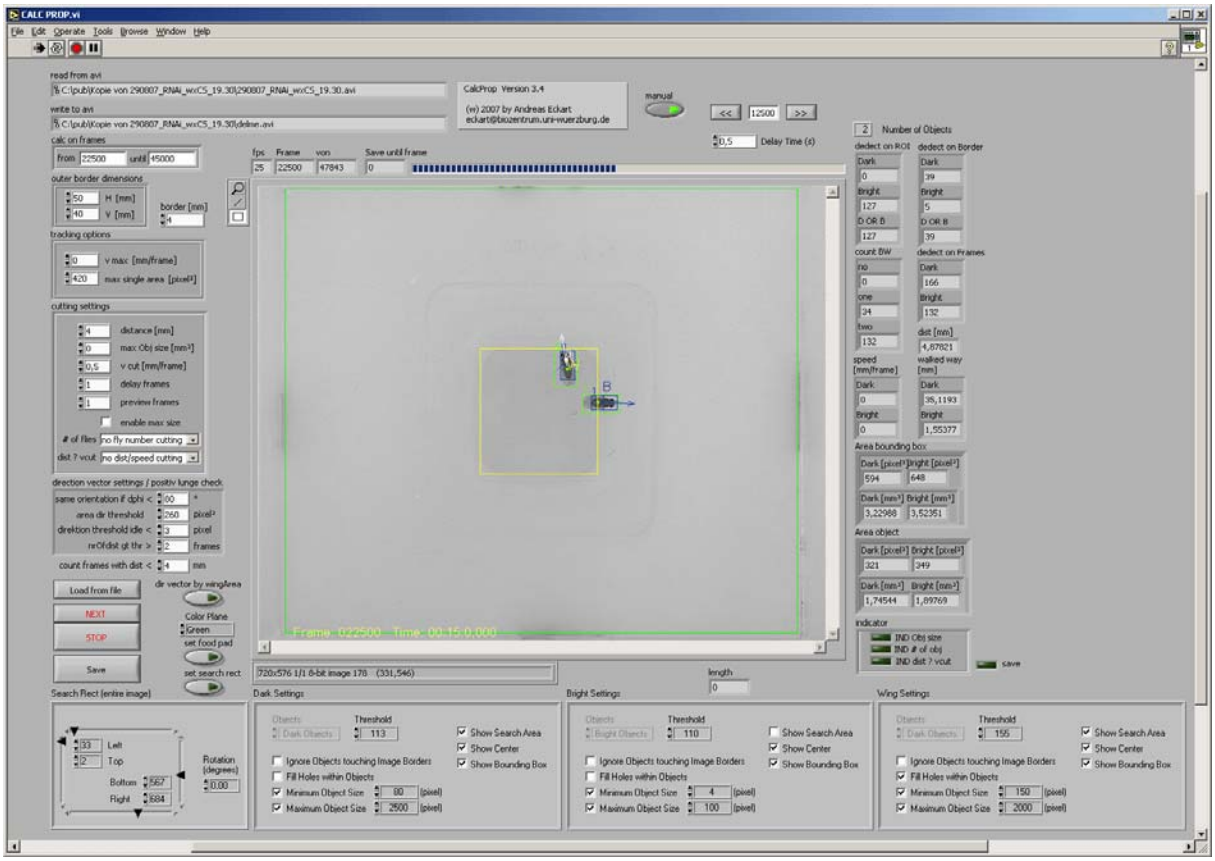


Figure 5: **The experimental setup**. Flies are confined to the 4 x 5 cm^2 floor and thus to the focal plane of the camera positioned above the arena due to coating the glass walls with Fluon. In the middle of the floor a 1 cm^2 hole is filled with a mixture of agarose, apple juice and sugar.

3.2.2 Recording videos

Videos were recorded using Adobe Premiere 6.0 (Adobe Systems Incorporated, San Jose, CA, USA). Videos must be recorded in progressive scan modus with a resolution of 720 x 576 pixel^2 (pixel aspect ratio 1.067) and a frame rate of 25 frames per second (a frame rate of 30 frames per second was never tested). The aperture of the camera was set at 3.4 and the shutter speed at 1/250 of a second. Each morning before the start of the experiments the white-balance levels were set to ensure optimal contrast levels.

3.2.3 CalcProp Version 3.4

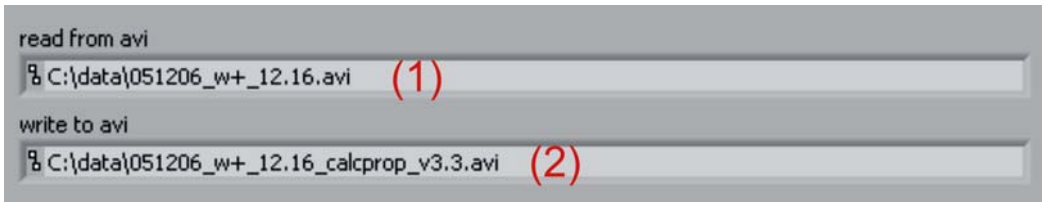


The user interface looks identical for CalcProp and LoopRun. In CalcProp the investigator has to determine basic properties of each video such as the area in which to look for objects (i.e. the arena dimensions), how these objects should look like (size and colour of flies) and how to distinguish the two objects (size and colour of white dot). CalcProp detects the two flies as dark objects on a white surface (the floor). The white dot on the thorax of one of the two flies makes the two dark objects distinguishable. It is essential to find the correct

settings, so that CalcProp and subsequently LoopRun are able to precisely detect and distinguish each fly. LoopRun uses all the settings specified in CalcProp to extract essential information for LungeCount, however it is not accessible to changes in settings, which can be only done in CalcProp.

3.2.3.1 Select a video

As soon as you open CalcProp, a dialog will automatically appear asking you which video you would like to process. Select a video in .avi -format and click 'ok'. CalcProp will then ask you where you would like to store the output file and under which name. Select both and click 'ok'. The video will appear in the inner window starting with the first frame. The name of the selected video (1) and prospective output video (2) – once processed by LoopRun – will be given here:



3.2.3.2 Determine the settings for each video

It is recommendable to save a demo-file containing most of the relevant settings, for example regarding the two objects and the white dot on the fly's thorax. This demo-file saves not only a lot of time, but also guarantees standardized evaluation. How to find the correct settings for a demo-file will be explained below. Once a demo-file exists, you have to click on 'load from file' (3) and open the respective demo file, which has the suffix .prp. Subsequently, you only have to adjust the 'search rectangle' (i.e. in which area should LoopRun look for objects) and the area of the food patch, since both vary from video to video.

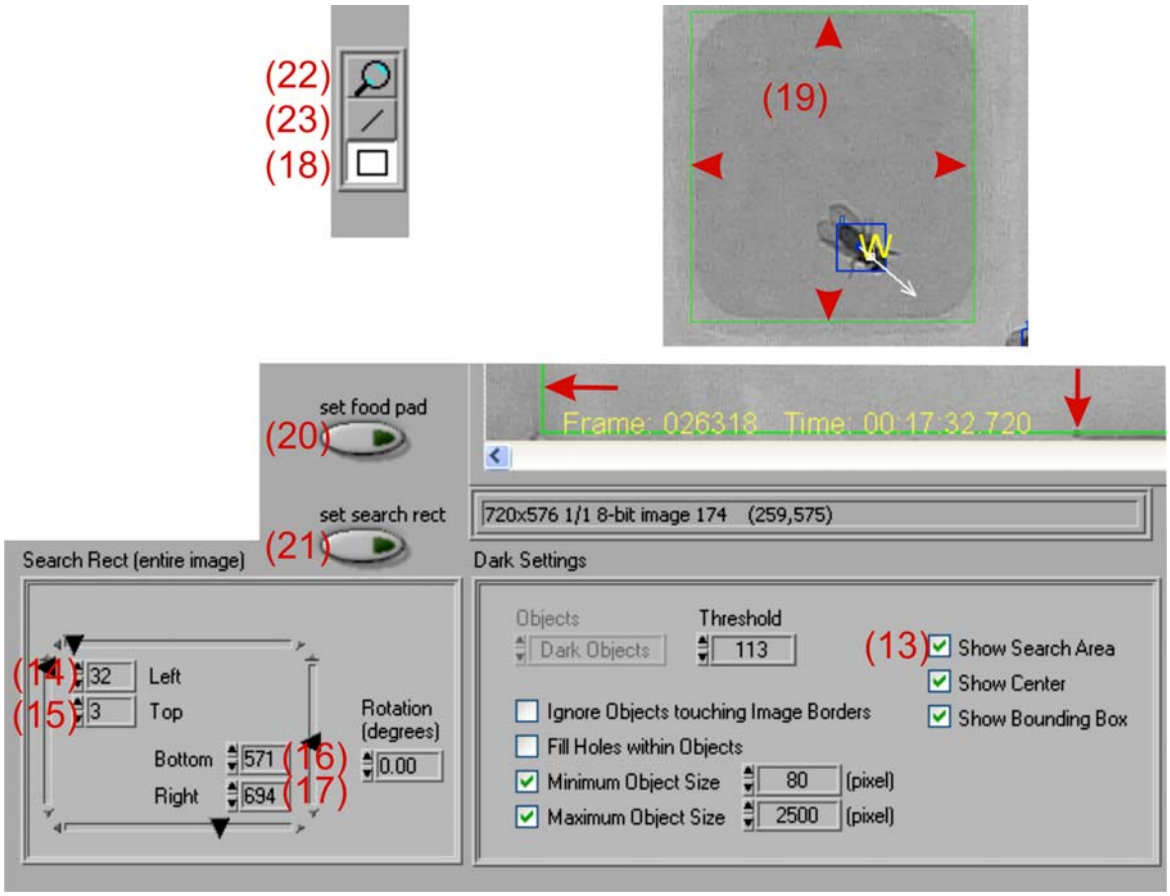
If the selected time window of the analysis, i.e. the first frame and the last frame included in the analysis given in (4) und (5), does not start with frame 1, you should jump to the first frame of your selected time window, where you can then fix the settings for both the arena and the food patch. For this purpose, you have to press 'manual' (6), which lights up. You can then determine how many frames (7) you would like to jump ahead (8) or backwards (9). There might be a delay before the software reacts, depending on your settings in (10).

'Delay time' means the time period before the next frame is presented in the inner window. In addition, the reaction time of some of the buttons (e.g. 'load from file' (3)) is dependent on the delay time given in (10). It is therefore recommended to set the delay time as short as possible. But please be aware, that the delay time in (10) should not be set too short, as the computer must be able to still display the object dimensions, as illustrated in the picture below (the blue frame around the fly). When you press 'manual' (6), the last frame being displayed will freeze in the inner window. (11) and (12) list which frame is presented in the inner window and of how many frames the video consists, respectively.

To adjust the search rectangle and the food patch for each video, you have to use the icons illustrated in the figure below:

Check 'show search area' (13) to exhibit the search area given in a coloured rectangle (arrows). The colour of the rectangle might vary from computer to computer (green in the figure). You can alter the left, top, bottom and right boundary by changing the pixel number in (14), (15), (16) and (17), respectively. The search rectangle, also called search area, determines, in which area the software will look for objects. If you do not have a demo-file yet, do not be confused if there is no difference whether you hit 'show search area' (13) or not. In both cases, there should be a rectangle corresponding to the number given in (14), (15), (16) and (17).

The food patch can be selected by first clicking on the tool represented in (18). You can then drag a rectangle around the food patch with your cursor (19; arrow heads). Press 'set food pad' (20). The food patch is selected. In contrast to the search rectangle, there is NO visual representation of the location of the food patch given in pixel. With the same tool (18), you can also specify the search rectangle. This method is an alternative method of determining the search rectangle, but it is less precise than the one explained above. In that case, after dragging the respective rectangle for the search area, press 'set search rect' (21), instead of 'set food pad' (20). Here, the area location will be displayed in (14), (15), (16) and (17).



After having determined the search rectangle and the food patch, you can click on 'save' and choose a location and name for this 'property-file'. The file will have the suffix .prp. By clicking next, you can select the next video. Repeat 'select a video' and 'determine the settings for video processing' for each video that you would like to be processed.

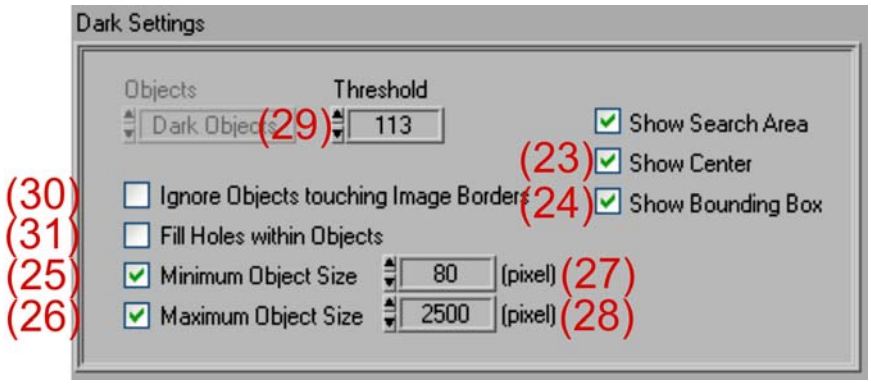
With (10) you can zoom into the window. To zoom out, you must press Shift, while hitting (10). (11) enables you to measure a distance within the represented frame. The distance is given in mm (location: under the inner window on the right side).

3.2.3.3 Produce a demo-file

Select a video and determine the search rectangle and food patch as described above. All settings to detect objects are based on the green colour plane (22).

Dark settings (located below the inner window, left):

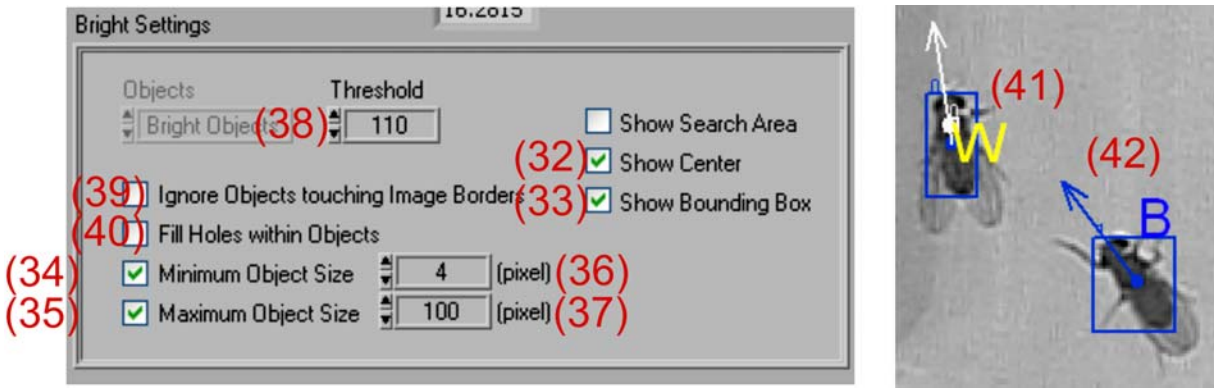
These settings specify the features of an object, i.e. the fly. Be aware that BOTH flies are detected by using the settings specified under 'dark settings'!



Check 'show centre'(23) and 'show bounding box' (24) to monitor whether the software detects the flies correctly. The bounding box is the rectangle enclosing the fly, in other words, it is not exclusively the area covered by the fly's body. Check 'Minimum Object Size' (25) and 'Maximum Object Size' (26) and enter specific values for each of them ((27) and (28)). 80 and 2500 pixel^2 proved to be optimal to track flies independent of the behavioural pattern displayed. Furthermore, set a threshold specifying how dark your objects are (29). The threshold should be selected in such a way that only the fly's body, but not its wings make up the object. A threshold of 113 proved to be optimal, since higher values tend to include the wings, while lower values impede the flies' detection. To choose the correct threshold is essential for a proper functioning of the LungeCount software. Do not check 'Ignore Objects touching Image Borders' (30), as the arena dimensions might include the image borders. Do not check 'Fill Holes within Objects' (31) for the output file used for LungeCount and FlySpeedHisto. However, this command is essential for the output file used for FlySize. Thus, you have to process every video twice, once with 'Fill Holes within Objects' (31) being checked in 'dark settings' and once without. All other properties stay constant.

Bright settings (located below the inner window, right):

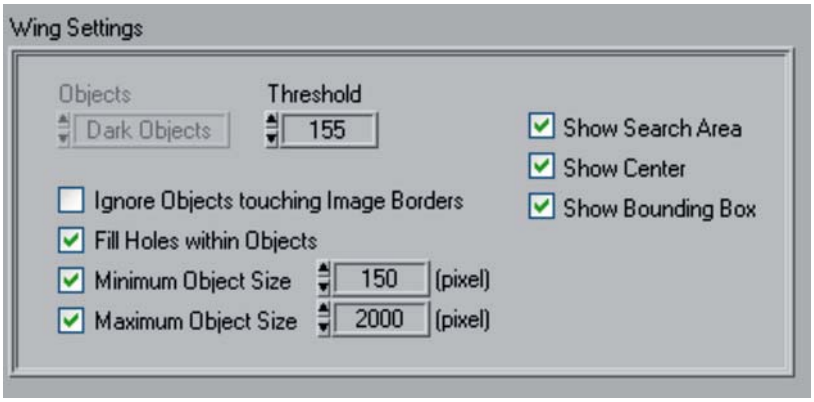
These settings do NOT detect the bright fly, but ONLY the white dot on the thorax of one fly. Both flies are detected as dark objects (specified under 'dark settings'). However, one dark objects includes a white object, the white dot. The dark object with the white dot in its centre is called the bright fly. In the displayed window, the bright fly carries a W for white (41), whereas the dark fly carries a B for black (42).



The white object (the dot) is specified the same way as the dark object. First, check 'show centre' (32) and 'show bounding box' (33) to monitor whether the software detects the object, i.e. the white dot, correctly. Check 'Minimum Object Size' (34) and 'Maximum Object Size' (35) and enter specific values for each of them ((36) and (37)). Since the white dot is very small, 4 and 100 pixel^2 proved to be optimal. Furthermore, you have to set a threshold, of how dark your object is (38). Here, you have to be cautious with the background noise. A threshold of 110 was optimal. NEVER check 'Ignore Objects touching Image Border' (39) or 'Fill Holes within Object' (40) for the bright settings.

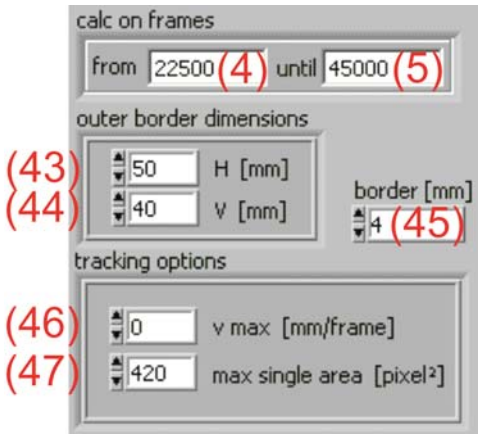
Wing settings:

These settings are a leftover of other versions and are not fully functional. Even though there is no current function, SETTINGS HAVE TO BE DETERMINED, otherwise the calculations performed by LoopRun will be slowed down. Thus, please adopt the settings illustrated in the figure below for your demo-file (don't forget to check the boxes!) and read the idea behind 'wing settings' given below under 'Additional options'/'Direction'/'Wing settings'. If the settings given here do not fit to your clip, please take the time and find the correct settings for your clip.



Calc on frames, outer border dimensions, and tracking options:

Calc on frames: To determine the time window of the video, for which you would like to analyse the number of lunges, type in from which frame (4) until which frame (5), you would like the video to be processed.



Outer border dimensions: To give a distance reference, you have to list the length of the horizontal (43) and the vertical line (44) of the search arena/rectangle, consequently according to our set-up 50 mm x 40 mm. You can define a so-called border zone. In our experiments we defined the outer 4 mm as the border-zone (45).

Tracking options: If the two flies are very close to each other, the software cannot distinguish the dark objects as separate objects; therefore they merge to one object. However, for the analysis it is essential to keep track that indeed both flies are present on the arena. Therefore, there is a fixed maximum size for a single fly: 420 pixel^2 (47). If this threshold is exceeded, the one object is regarded as consisting of two objects. The maximal velocity did not prove to be beneficial to track animals (46).

Calc frames with distance:

The software counts all those frames, where a certain distance between the two flies falls below a specified value 

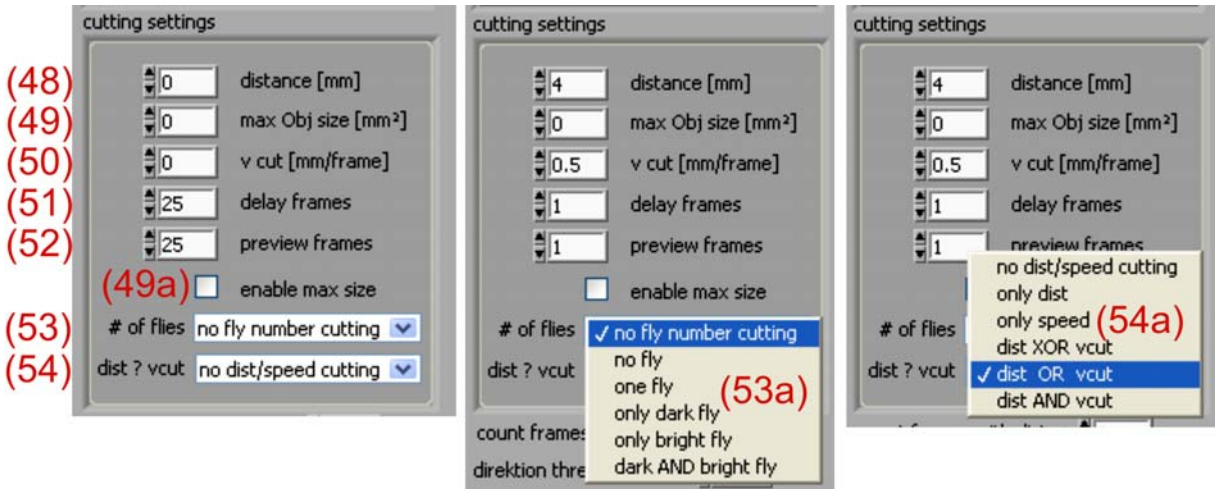
given in (right above 'load from file (3)).

After selecting all settings introduced so far, the demo-file is ready to save. Click on 'save' and give the demo-file a name and a location to be stored.

3.2.3.4 Additional options

Cutting settings:

If you would like to count the lunges yourself, but focussed only on those frame sequences, where a lunge is likely to occur, CalcProp/LoopRun can minimize the video to those frame sequences of interest. Following cutting options exist:



(48) *distance of the two flies to each other*: All frames, in which the flies are closer than e.g. 4 mm away, are included into the short video version.

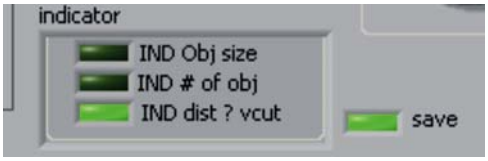
(49) *maximum Object size*: Frames, in which the selected object size is exceeded, will be included in the short video version. You might want to set the object size so that it is only exceeded if two flies are so close to each other that they merge to one object. If you would like to use 'max obj size' as criterion for cutting your original video, check the box 'enable max size' (49a).

(50) *velocity for cutting*: Since many lunges are accompanied by high velocities, you might want to focus on those scenes where a high speed occurs.

(51) / (52) *delay /preview frames*: You can select delay (51) and preview frames (52), i.e. frames that come after and before frames that meet the criteria selected above. To include the adjacent frames often clarifies whether a lunge indeed occurred.

(52) *number of flies*: Furthermore, you can decide to cut your original video based on the number of flies present in the frame. For this purpose, click on the text field next to '# of flies' (53), a list will open, from which you can choose the preferred selection criterion (53a).

(53) To enable criterion (48) and/or (50), click on the text field right to 'dist ? vcut' (54). A list appears, from which you can choose the preferred combination of the two selection criteria (54a).

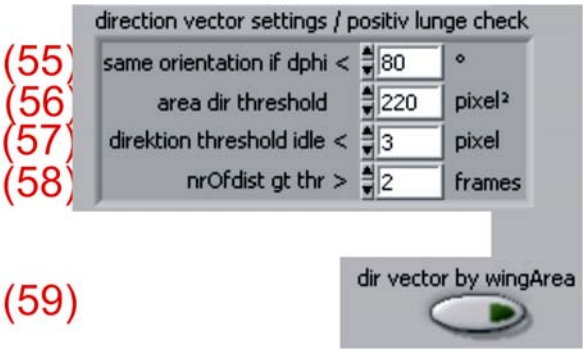


There is an indicator of whether the presented frame will be included in the short version of the video (located to the lower right of the inner window): if one of the three fields lightens up, the presented frame will be selected for the short video version. In the example given, the frame meets the condition of falling below the required distance between the two flies. Thus, the respective field lights up and the frame will be saved.

Direction:

Forward motion:

The orientation of the fly's head and abdomen is identified in LungeCount, not in CalcProp. However, to get a visual impression whether the orientation is identified correctly in most frames (LungeCount offers no visual representation), CalcProp offers all features LungeCount uses (independently) to detect the fly's orientation. The underlying idea is that flies hardly walk backwards.



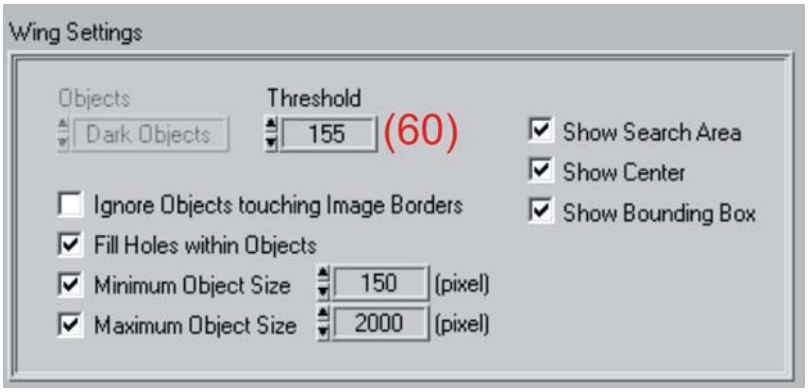
Therefore, the head is defined as that end of the object, which corresponds with the flow of forward motion. A forward motion is defined as a shift of the fly's body centre of gravity of more than 3 pixels (57) in two subsequent frames (58). To exclude other behaviours than walking to influence the direction settings, e.g. slipping down the wall or lunging (strong backward motion can occur if the attacking fly misses its opponent), the area of the fly has to be at least 220 pixel^2 (56), which assures ground contact of all legs. The direction will be kept, as long as the fly's change in direction is less than 80° in one frame (55).

Each fly's orientation will be depicted in the inner window based on these criteria, as long as (59) was not hit and therefore does not light up. Otherwise the direction will be based on the following settings:

Wing settings:

An alternative approach to detect the body orientation is based on the idea that the flies' wings are normally pointing to the fly's back. Therefore, an object is specified, which

only recognizes the dark body of the fly, and additionally a second object is specified that detects both the fly's body and its wings. The centre of gravity of the latter object (the 'wing object') should be closer to the abdomen than the centre of gravity of the first object (the pure 'body object'). Thus, the body orientation can be determined by comparing the location of the two centres of gravity. To detect not only the body, but also the wings, requires to make the threshold weaker, for example 150 (60). All other settings can be adopted from the dark object settings. However, to identify the body orientation using the wings does not function properly on the food patch, which contrasts less with the flies' wings than the white floor. Therefore, this function should not be used.



3.2.3.5 Basic properties of frames shown by CalcProp

LoopRun, but not CalcProp, produces a file containing all the relevant features of every frame. However, CalcProp gives you already an impression of the features, before the whole video is processed. CalcProp shows the following features:

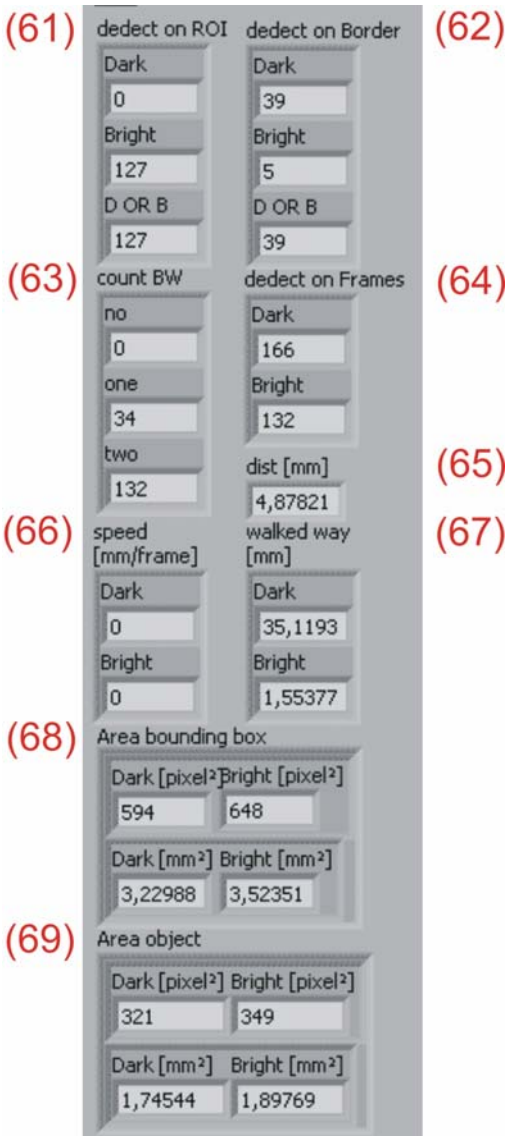
(61) ROI stands for region of interest. The ROI is specified, when pressing 'set food pad'. The program lists in how many frames the dark fly, the bright fly, or both flies were detected on the ROI, i.e., the food patch.

(62) The program lists in how many frames the dark fly, the bright fly, or both flies were detected within the border zone, which is defined by the investigator.

(63) 'count BW' states in how many frames no fly, one fly or two flies were detected, whereas

(64) 'detect on frames' states in how many frames the dark fly and in how many frames the white fly could be detected.

(65) CalcProp gives you the distance of the flies to each other in the particular frame depicted.



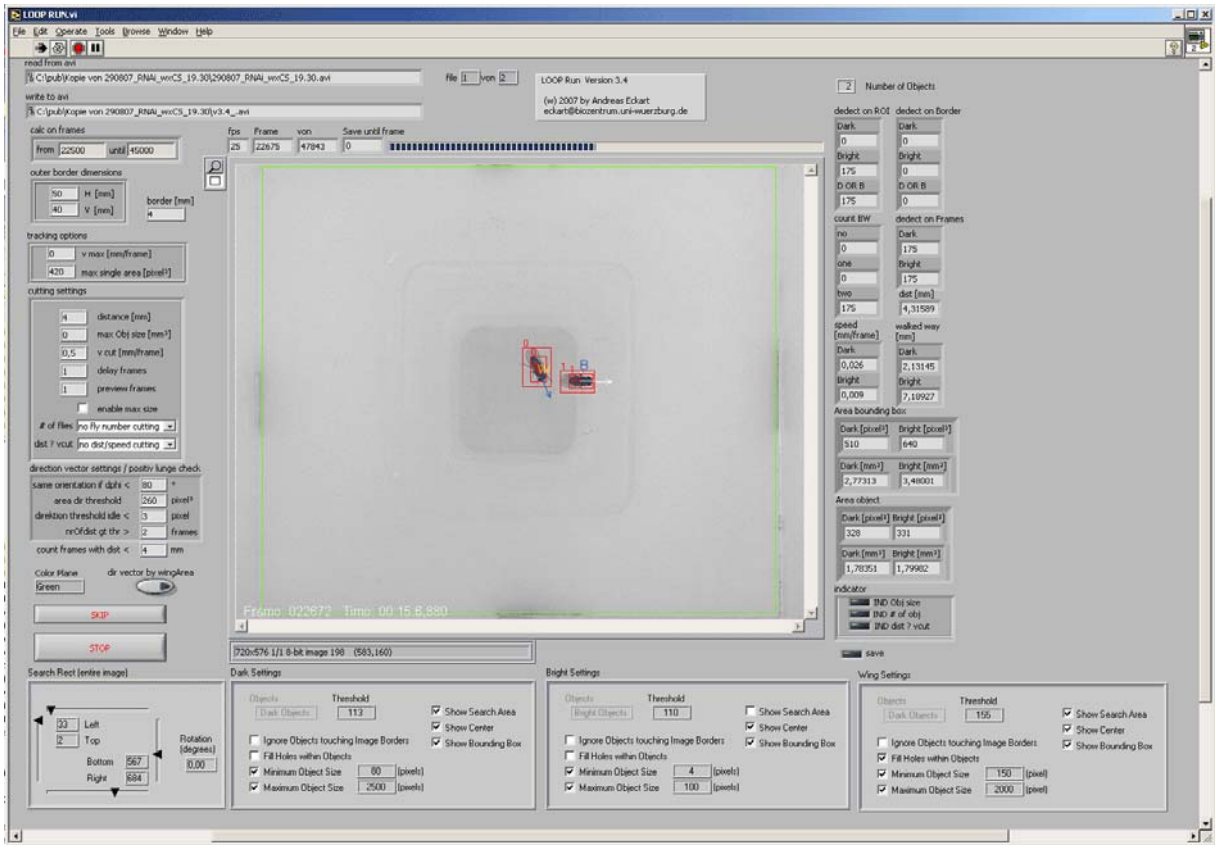
(66) It also lists the speed of each fly separately in the particular frame shown in the window.

(67) Walked way reflects the distance each fly walked in total including the presented frame.

(68) The area of the bounding box is the area of the rectangle including the fly. The area is once given in pixel^2 and once given in mm^2.

(69) The area of the object is the area covered by those pixels that are below the threshold specified in 'dark settings'. Hence, this area should contain only the dark body of the fly, and should be smaller than the area of the bounding box. The LungeCount program uses the 'area object', which is again given in pixel^2 and in mm^2.

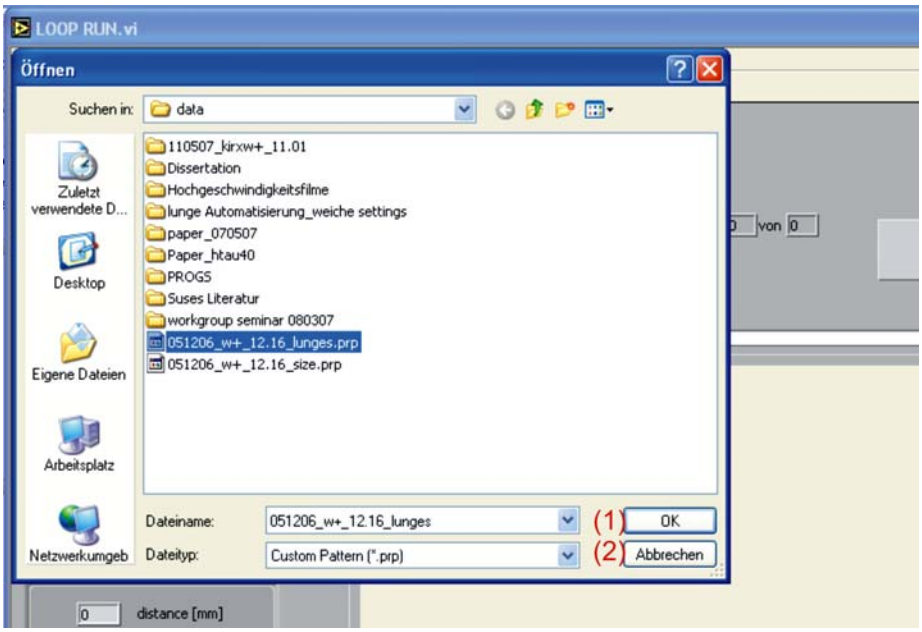
3.2.4 LoopRun Version 3.4



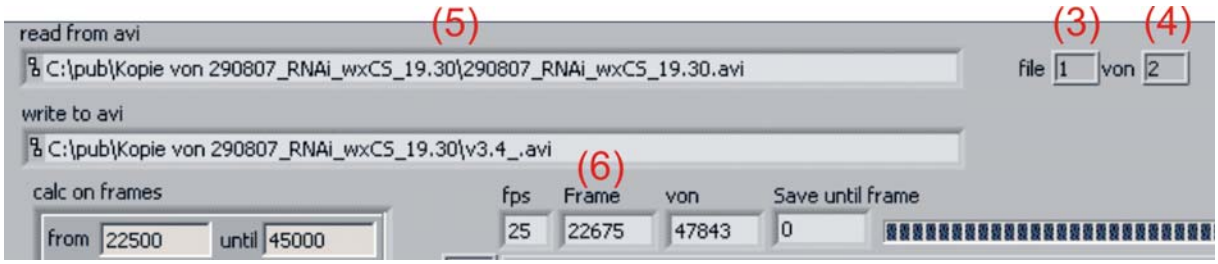
LoopRun uses all settings specified in CalcProp to extract relevant information for every video frame. LoopRun is not accessible to any changes in settings. The investigator can only determine which property files previously created with CalcProp will be processed by LoopRun. Once property files are selected, LoopRun will automatically process the according videos one after another.

3.2.4.1 Select and run property files

Open Loop Run. A dialog appears, asking you, which property-files (having the suffix .prp) to open. Select a file, click ‘ok’ (1). If desired, select a second file, click ‘ok’ (1). Repeat the procedure for all property-files that you would like to be successively processed. After selecting the last property-file click ‘**abbrechen**’ (2), NOT ‘ok’.



LoopRun starts to process the videos one after another. You can monitor which video (number (3) and name (5)) of how many videos (4) in the list is currently processed and how far the processing of the current video is advanced (6):



3.2.4.2 LoopRun output files

For every video, LoopRun is creating one video and three text files, all carrying the name selected in CalcProp, but having different suffices. You find all text files and the video at the location you specified in CalcProp under (2). The video is only of importance, if you selected cutting options. Otherwise you can delete the video, which will consist of a single frame. If you set cutting options, you can watch the short video version frame by frame by using VirtualDub. The other three files are all text files, e.g.

1. 051206_w+_12.16_lunges.avi (name.**avi**)
2. 051206_w+_12.16_lunges.avi_cutgraph (name.**avi_cutgraph**)
3. 051206_w+_12.16_lunges.avi_graph (name.**avi_graph**)

1. name.avi

The first file contains the following information:

```
File read:   C:\data\051206_w+_12.16.avi (1)
File out:    C:\data\051206_w+_12.16_lunges.avi

Frames with no BW dedected fly:      0     frames      0.000000    sec.
(2) Frames with one BW dedected fly:    20    frames      0.800000    sec.
Frames with two BW dedected flies:  22481 frames      899.240000  sec.
(3) 22489 frames with dark fly
22493 frames with bright fly
1529  frames with dark fly on ROI
(4) 3803  frames with bright fly on ROI
4342  frames with dark OR bright fly on ROI
11675 frames with dark fly on border
(5) 2279  frames with bright fly on border
12239 frames with dark OR bright fly on border
(6) dark walked distance:   10838.387695
bright walked distance: 9698.630859
indication counts:
        IND Obj size=     0
(7)     IND # of obj=     0
        IND dist ? vcut=  0
(8) frames with dist underflow:   4321
```

(1) The original video is the ‘file read’.

(2) The number of frames with either no fly, one fly or both flies are listed,

(3) as well as the number of frames, in which either the dark fly or the bright fly was present.

(4) The food patch is normally the region of interest (ROI). Thus, (4) shows you the number of frames, in which either the dark fly or the bright fly or both flies were on the food patch.

(5) Accordingly, (5) shows you the number of frames, in which either the dark fly or the bright fly or both flies were detected within the border zone.

(6) Do not use the walked distance given in this output file, as it is too imprecise.

(7) If you set cutting options, (7) shows you how often which cutting option was applied.

(8) Do not use the information given here, since it is too imprecise. (6) and (8) are calculated by the software ‘FlySpeedHisto’.

2. name.avi_cutgraph

This text file contains no relevant information if you did not select cutting options. In that case, you can delete it. If you selected cutting options, this text file will be a shorter version of file 3 (name.avi_graph) containing only those frames included in the short video version.

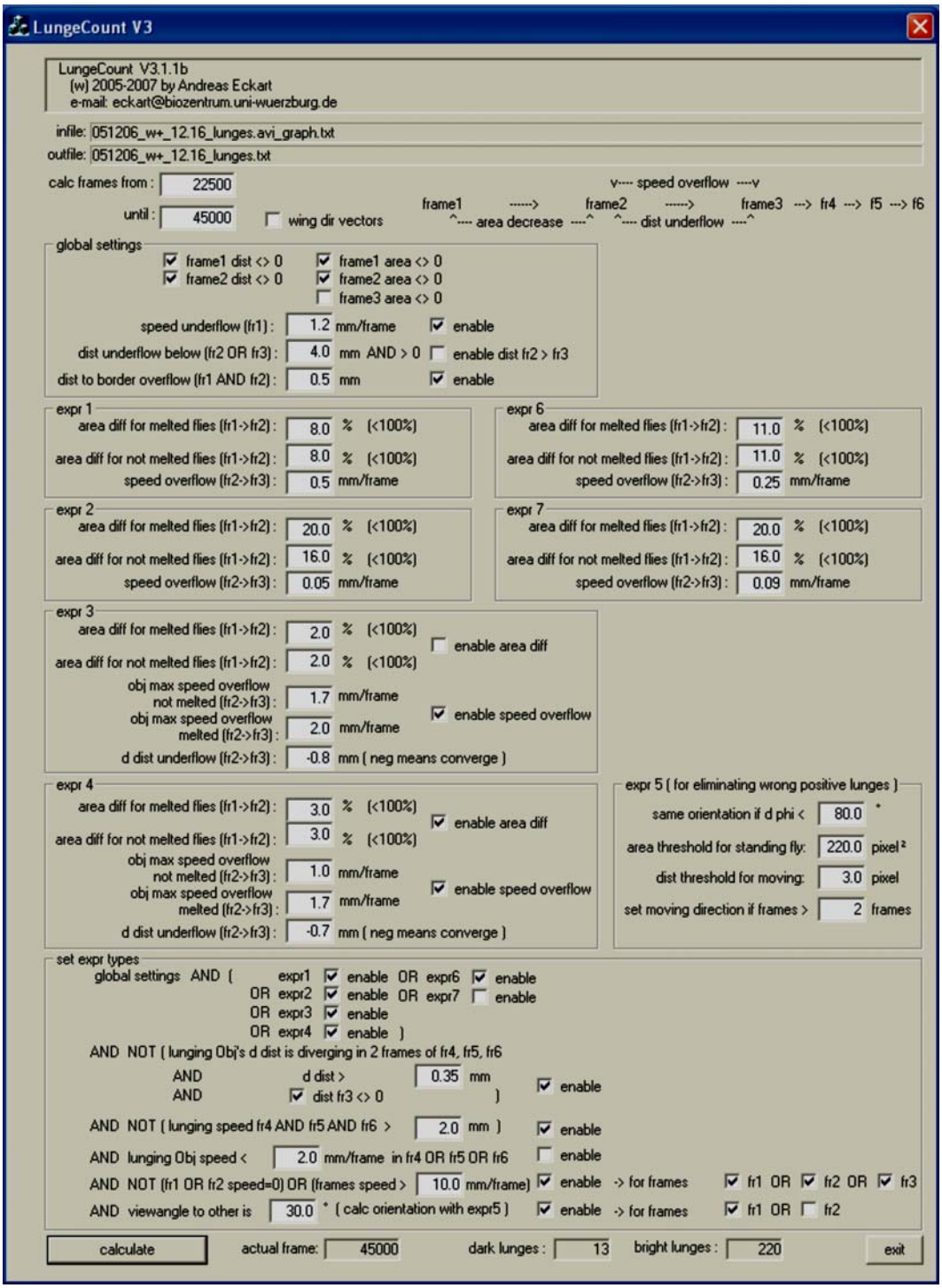
3. name.avi_graph

The third file lists for every frame the relevant information used in LungeCount, FlySpeedHisto and FlySize. The first column lists the frame number. To the right of every frame number, there are 32 columns, each listing one piece of information concerning the respective frame number ('dark' stands for 'dark fly', 'bright' stands for 'bright fly'):

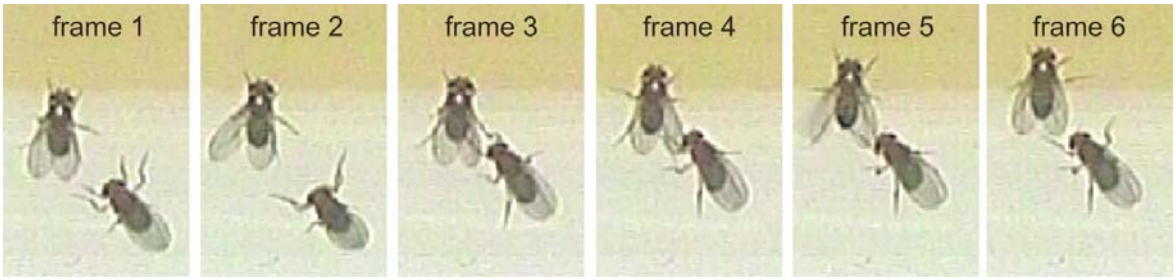
1	frame	18	bright aspect ratio
2	dark speed (mm/frame)	19	dark center (mm): location
3	bright speed (mm/frame)	20	bright center (mm): location
4	distance (mm): of flies to each other	21	walked dark distance (mm)
5	melted flies: 1, if flies' body centres of gravity are fused, otherwise 0	22	walked bright distance (mm)
6	dark area (mm^2)	23	dark wing area (mm^2)
7	bright area (mm^2)	24	dark wing centre (mm): location
8	dark area ($pixel^2$)	25	bright wing area (mm^2)
9	bright area ($pixel^2$)	26	bright wing center (mm)
10	dark min dist to border (mm): distance of dark fly to nearest wall	27	dark wing area ($pixel^2$)
11	bright min dist to border (mm)	28	dark wing center (pixel)
12	d dist: difference in distance (4) between the previous and the recent frame	29	bright wing area ($pixel^2$)
13	dark center (pixel): location	30	bright wing center (pixel)
14	bright center (pixel): location	31	melted wings? 1, if the two objects specified under wing settings are fused, otherwise 0
15	dark orientation	32	dark on ROI: 1, if on ROI, otherwise 0
16	bright orientation	33	bright on ROI
17	dark aspect ratio: ratio body width to body length		

Columns 23 to 31 are only of importance, if the fly's orientation shall be determined based on the location of the wings, which is NOT recommended.

3.2.5 LungeCount Version 3.1.1

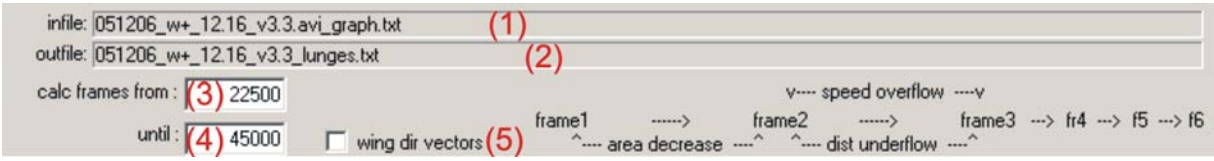


‚LungeCount' is based on detecting six subsequent frames, each of them fulfilling several criteria. If all criteria are met, the frame sequence is counted as one lunge and this lunge is accounted to one fly, either the dark fly or the bright fly. The criteria to be fulfilled will be described below. A characteristic sequence of six frames that would be counted as one lunge of the dark fly is illustrated here:



3.2.5.1 Running an analysis

1. Select a file to be analysed:



Click on the text field to the right of ‚infile' (1): A dialog appears from which you can select the file to be analysed. The text file's suffix must be .avi_graph. Click 'open'. The file to be analysed is selected. Click then on the text field to the right of 'outfile' (2) and select where you want to store the output file and under which name. Click 'save'.

To select the time window, for which you would like to run the lunge analysis, type in the first frame (3) and the last frame (4) of the time window, e.g. 22500 for the first frame of the 15th min and 45000 for the first frame of the 30th min. Do NOT check 'wing dir vectors' (5). This function is NOT working properly in combination with the set-up. For further information refer to 'CalcProp'/'Additional options'/'Direction'/'Wing settings'.

2. Run the analysis:

If you do not want to change the criteria for lunges, click 'calculate' (6) to run the analysis. The program runs through the frames, as reflected in 'actual frame' (7). It gives you the number of lunges for the dark (8) and the bright fly (9), separately.

Repeat step 1 (Select a file to be analysed) and step 2 (Run the analysis) for each file that you would like to process.

3.2.5.2 LungeCount output files

For each analysis you get an output file:

```
File= 051206_w+_12.16_calcprop_3.3.avi_graph.txt (1)

File read:  C:\data\051206_w+_12.16.avi (2)
File out:   C:\data\051206_w+_12.16_ausprobieren.avi
(3)   (4)                                                              (7)
frame of lunge    dark/bright
22663 dark   fly  fr(22663)   D_view(YES) W_view( NO) D_Center(489.742/290.725)     W_Center(452.261/321.808)
      D_phi(139.682)    W_phi(178.626)    D_dphi(0.648421)  W_dphi(141.705)   why: expr1, expr4, expr6,
22717 dark   fly  fr(22717)   D_view(YES) W_view( NO) D_Center(401.684/304.339)     W_Center(371.111/335.181)
      D_phi(143.454)    W_phi(198.263)    D_dphi(8.705)     W_dphi(116.486)   why: expr1, expr2, expr4, expr6,
22724 dark   fly  fr(22724)   D_view(YES) W_view( NO) D_Center(386.827/287.368)     W_Center(371.146/321.878)
      D_phi(121.363)    W_phi(188.124)    D_dphi(6.92629)   W_dphi(106.313)   why: expr1, expr2, expr6,
22792 bright fly  fr(22792)   D_view( NO) W_view(YES) D_Center(354.956/287.661)     W_Center(313.732/299.656)
      D_phi(205.75)     W_phi(338.791)    D_dphi(41.9735)   W_dphi(4.98621)   why: expr1, expr2, expr6,

  •
  •
  •
  •
  •
  •

44918 bright fly  fr(44918)   D_view( NO) W_view(YES) D_Center(44.5156/102.528)     W_Center(88.9548/115.184)
      D_phi(163.258)    W_phi(170.126)    D_dphi(147.362)   W_dphi(25.7706)   why: expr6,

calculation ist done from frame 22500 until frame 45000 (5)
total  dark lunges:     13
total white lunges:     220 (6)

nr of  dark obj moving frames:      1464
nr of white obj moving frames:      840

 dark obj walked way [mm]:    10838.4
white obj walked way[mm]:     9695.13

End of File
```

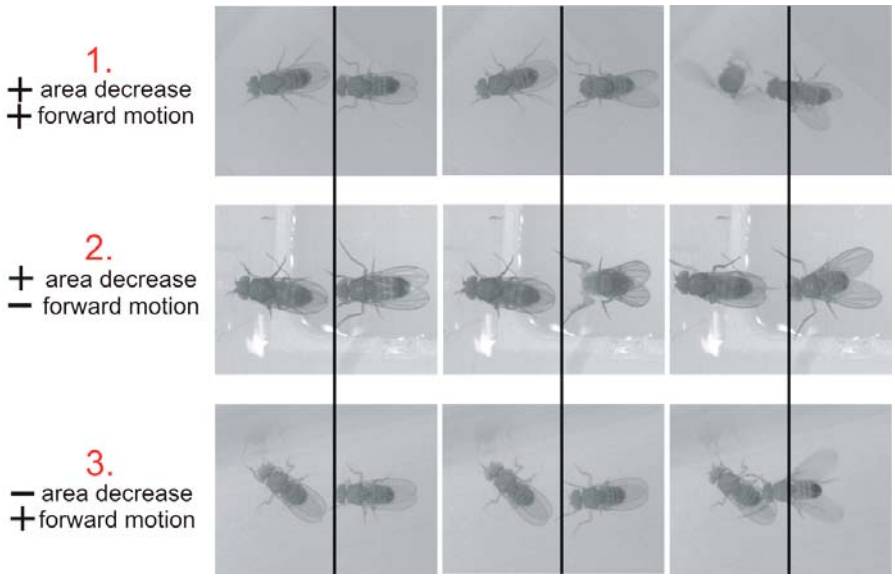
The output file gives the file (1) and video (2), on which it is based. Additionally, it lists at which video frame (3) which fly performed a lunge (4). At the end, it states which frames were processed for the analysis (5) and how often which fly lunged in total (6). DO NOT USE THE LOCOMOTION INFORMATION GIVEN IN THAT FILE, AS IT IS TOO INPRECISE. In addition, it will provide further information, why this frame sequences was selected to be a lunge (7). The various so-called ‘expressions’ that each define a certain type of lunge will be explained below. If you are not interested in changing the conditions for a lunge, you can ignore this information.

A second output file has the suffix txt._debug. This file is of no importance if you are not interested in changing the settings. It lists information for each frame separately for the dark and the bright fly. The only valuable information is given in the column before the last column, which lists the orientation of the fly in relation to the opponent’s body centre of gravity, e.g. ‘d_phi (18.1688)’.

3.2.5.3 Details of the program

In case you are interested in altering the conditions for a lunge, here, every criterion is described in detail and how you can change it.

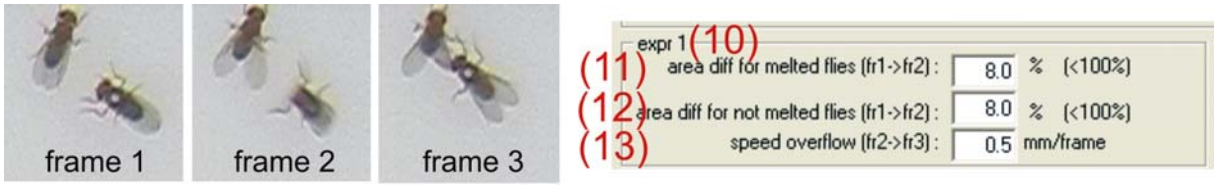
The software extracts characteristic features of lunges, such as the sharp decrease in body size during phase 1 and remarkable high head velocity during phase 2. The briefness of a lunge is a challenging feature, though. The lunge (phase 1 and 2) takes only 48.32 ± 1.75 msec (mean ± SEM), however, a regular Digital Camera captures a picture only every 40 msec (25 frame per seconds). Therefore, only a single frame represents the lunge itself. The figure below illustrates the frame right before the lunge (frame 1), during the lunge (frame 2) and the one right after the lunge (frame 3) from left to right. Due to the briefness of a lunge, the software had to be adjusted to also detect frame sequence where the sharp decrease of the body size is present ('+'), but no forward motion is recognizable ('-') as illustrated in 2. Also the software had to detect frame sequences, where the strong forward motion can be detected ('+'), but not the area decrease ('-'), an example given in 3. 1. illustrates a lunge frame sequence, where both features, area decrease ('+') and forward motion ('+'), were captured. The black lines are given as a reference for the initial head position at the first frame.



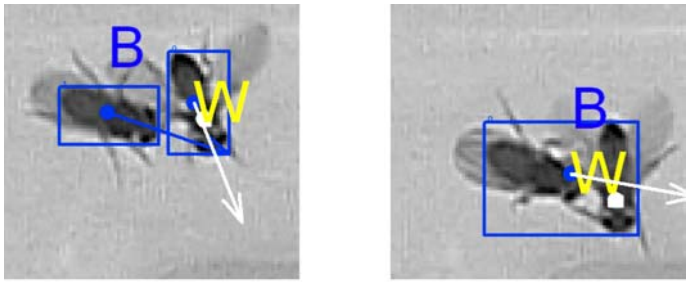
Expressions

Every type of lunge needs to be separately defined for the software; therefore several so-called 'expressions' are used, each defining a particular type of lunge. Each expression will be described separately.

Expression 1:



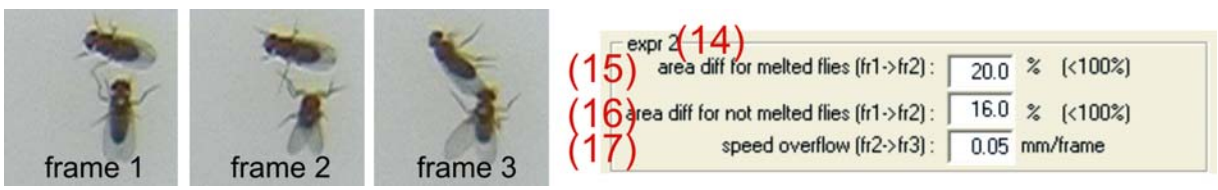
Expression 1 (10) defines a 'classic' lunge: the area covered by the attacking fly decreases from frame 1 to frame 2 by at least 8% (11/12), followed by the attacking fly snapping down on its opponent, which is reflected in a high velocity of the fly's centre of gravity with at least 0.5 mm/frame (13). The software distinguishes between pairs of flies, which centres of gravity are fused (11), so called 'melted' flies and pairs of flies, which centres of gravity are not fused (12), so called 'not melted' flies.



'not melted' 'melted'

Problematic are the transitions from 'not melted' to 'melted' and reverse, as they result firstly in strong shifts of the flies' body centre of gravity and secondly in pronounced increases/decreases of the flies' body area (the overall area covered by both flies is assigned to each fly during the 'melted' status). Expression 1 distinguishes between pairs of flies, which body centres of gravity are fused in frame 3 and those that are not. As this condition 'melted/unmelted' of frame 3 should have no influence on the area decrease between frame 1 and frame 2, there is actually no need to give separate values for 'melted' and 'not melted' flies.

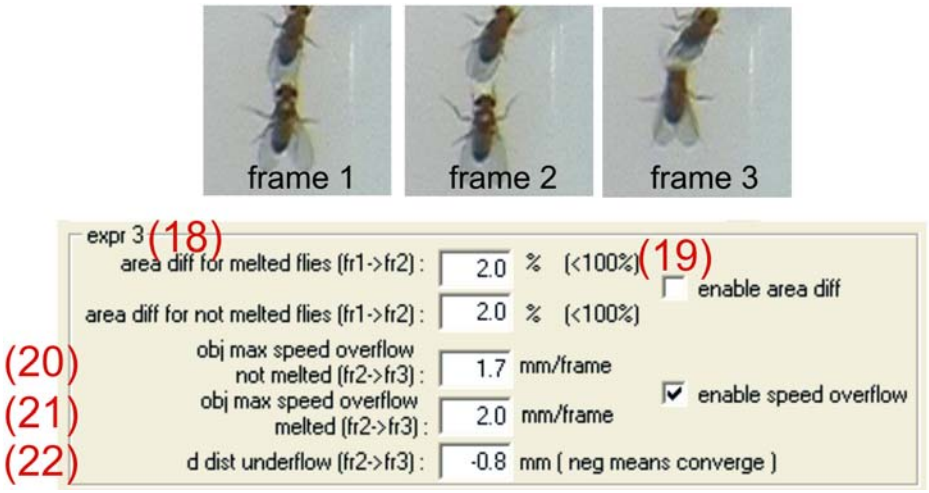
Expression 2:



Expression 2 (14) defines a lunge, for which the camera captured only the strong decrease in area, but no forward motion. Consequently, the attacking fly is rising up on its

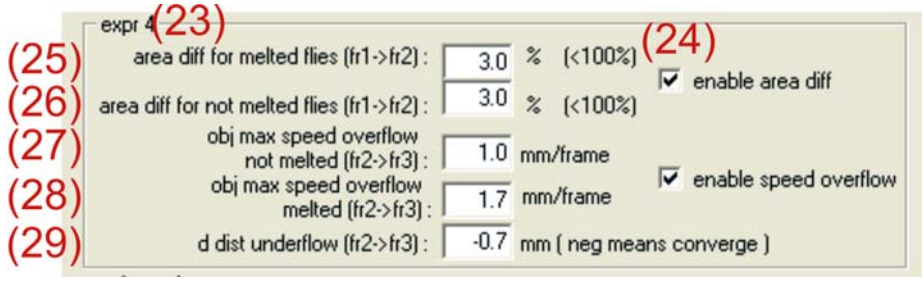
hind-legs resulting in a pronounced area decrease of 16% (15/16); however, the forward motion is almost missing with 0.05 mm/frame (17).

Expression 3:



Expression 3 (18) reflects the other extreme: There is no area decrease, but a strong forward motion. Therefore, there is no need for a decrease in area (area diff is NOT checked (19)), but there has to be a strong forward motion with 1.7 and 2.0 mm/frame for pairs of flies, for which the body centres of gravity are not merged (20) and merged (21) in frame 3, respectively. Here, the distinction is meaningful, since the shift in the body centre of gravity of the attacking fly will be more pronounced if the two flies fuse in frame 3. As a new criterion, the motion of the attacking fly has to be directed towards the opponent; therefore, the difference in distance ('d dist') between the two flies has to decrease by 0.8 mm (22).

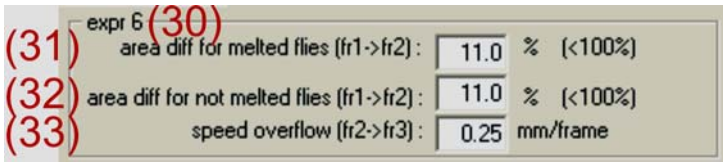
Expression 4:



Expression 4 (23) is the intermediate form between expression 3 and expression 1. The area of the attacking fly has to decrease by 3% ((25)/(26) and (24) checked), but simultaneously the forward motion has to be less pronounced compared to expression 3 with 1.7 or 1.0 mm/frame, depending of whether the two flies merge (28) in frame 3 or not (29).

Again, the distance of the two flies to each other must converge; however, it has to decrease slightly less than in expression 3 with only 0.7 mm (29).

Expression 6:

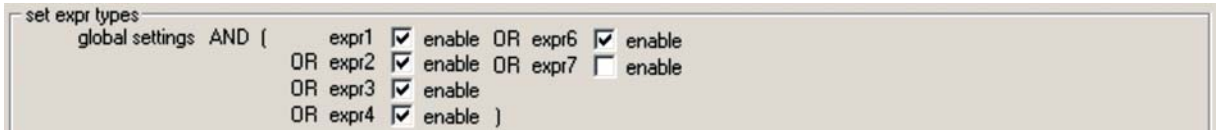


Expression 6 (30) is an intermediate form between expression 2 and expression 1: The area decrease is fairly pronounced with 11% (31/32), but the speed is not as high as in expression 1 with 0.25 mm/frame (33).

Expression 7:

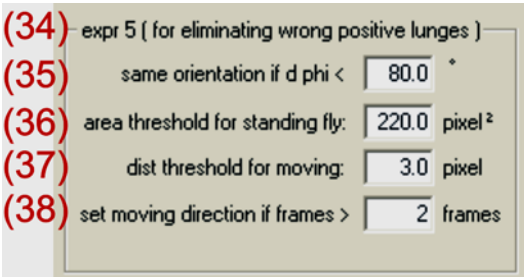
was not used for the regular experiments; however, it provides the option to include another intermediate stage.

If you want to include/exclude a certain type of lunge, you can enable/disable the according expressions here:



The flies' orientation

Expression 5:

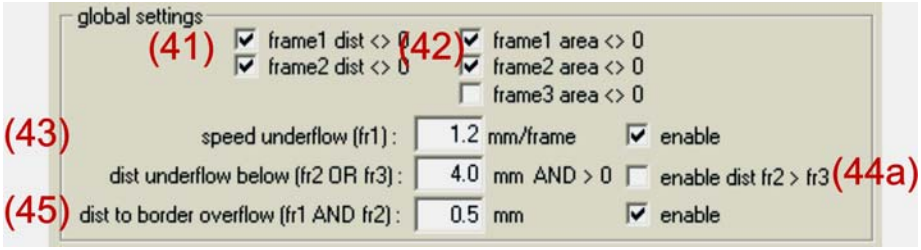


Expression 5 (34) does not describe another form of lunge, but specifies the direction of the fly, i.e. where is the fly's head and where is its abdomen. It is based on the idea that flies hardly walk backwards. Therefore, the head is defined as that end of the object/fly, in which the movement goes. A movement is defined as a shift of the fly's body centre of gravity of more than 3 pixels each (37) in two subsequent frames (38). To exclude other behaviour than walking to have an influence on setting the direction, such as the fly slipping down the wall or lunging (strong backward motions can occur if the attacking fly misses its opponent), the fly's area must be at least 220 pixel^2 big for a frame be to

included in the calculation assuring that all six legs have ground contact (36). The direction will be kept, as long as the fly's change in direction is less than 80° in one frame (38).

This direction is used as a further criterion, since the attacking fly is oriented towards the opponent when starting a lunge. Therefore, the angle between the attacking fly's body axis and the opponent's centre of gravity should not exceed 30° (39) in frame 1 (40):

Global settings concerning the first three frames



(41) during frame 1 and 2 the flies' body centres of gravity must not be fused, as a result of the flies being too close together and the software being not able to distinguish the two dark objects (distance (dist) of the two flies to each other is zero (< > 0)).

(42) both flies have to be detected by the software at frame 1 and frame 2, that is, the flies' area has to be bigger than 0. Actually, if you check (41), you do not need to check (42), because if a fly is not recognized by the software, the flies' distance to each other is zero by definition.

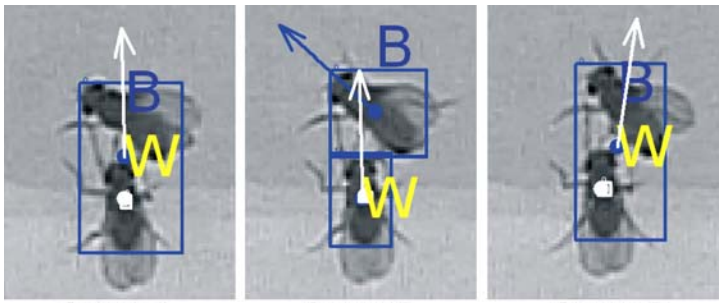
(43) the speed of the attacking fly in frame 1 has to be smaller than 1.2 mm/frame.

(44) The distance of the two flies to each other has to be below 4 mm in frame 2 or in frame 3. IT IS NOT RECOMMENDED TO ENABLE 'DIST FRAME 2 > FRAME 3 (44a), which means for a frame sequence to be counted as a lunge, the distance of the two flies to each other has to be smaller for frame 3 than for frame 2. This is not true for all lunges, since sometimes, the fly misses its opponent and the opponent can escape in time (in frame 3).

(45) the distance of the attacking fly to the wall (border) has to be bigger than 0.5 mm in frame 1 and in frame 2.

These criteria were chosen for the following reasons:

(41a) *frame 1 dist <> 0*: The flies' body centres of gravity must not be fused at frame 1 to avoid false positives such as:

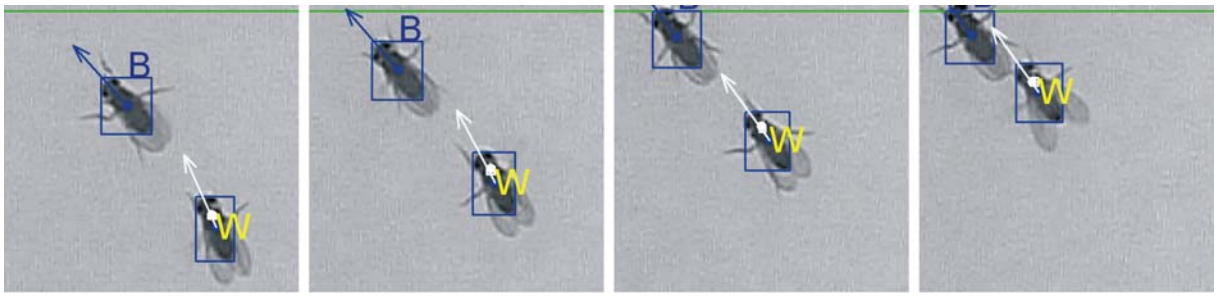


frame 1 area for each fly: 662 pixel^2 | frame 2 dark: 363 pixel^2 bright: 283 pixel^2 | frame 3

If the two flies are too close to each other for the software to distinguish the two objects, each fly is assigned the shared area, which is consequently much bigger than the area of a single fly. If the two flies are then diverging and therefore can be recognized as single objects, to each fly its own area will be assigned, thus, there is a extreme area decrease, which would meet the criteria for expression 2 (or expression 6).

(41b) *frame 2 dist <> 0*: This criterion is a take-over of older version of LungeCount. If the centre of gravity is merged in frame 2 and the flies can be distinguished in the subsequent frame, the shift of the flies' centre of gravity would be artificially high, which might have resulted in false positives for expression 3 (and expression 4). However, as these expressions now also include the criterion that the strong shift in the attacking fly's body centre of gravity has to be in the direction of the opponent, this criterion (41b) is presumably superfluous.

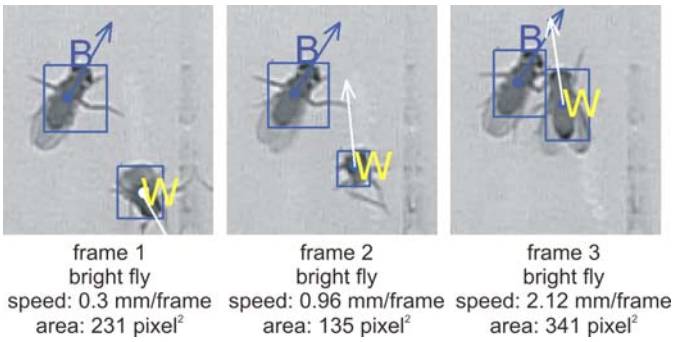
(43) *speed underflow (fr1)*: The speed of the attacking fly must be below 1.2 mm/frame to avoid false positives such as the following chasing sequence:



During chasing, two important criteria for expression 3 are fulfilled: flies reach high velocities and the distance between the two flies converge. Therefore, chasing sequences would frequently be falsely regarded as lunges. Since typically a fly slows down before it starts a lunge to position its legs, in particular its front-legs (see high-speed videos captured from top view), the speed during the first frame must not exceed 1.2 mm/frame.

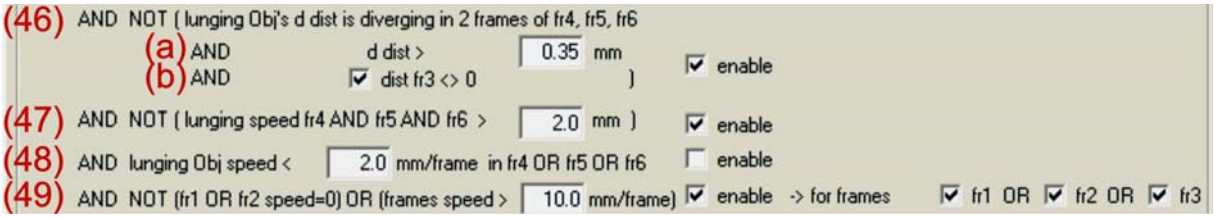
(44) *dist underflow below (fr2 OR fr3):* Since lunges occur only in situation when flies are close to each other, i.e. the flies' body centres of gravity are less than 4 mm apart, this criterion excludes many frames. However, rarely, lunges might be missed, because the flies' centres of gravity are a little bit further away than 4 mm.

(45) *dist to border overflow (fr1 AND fr2):* The distance of the attacking fly to the border/wall has to be bigger than 0.5 mm to avoid false positives such as:

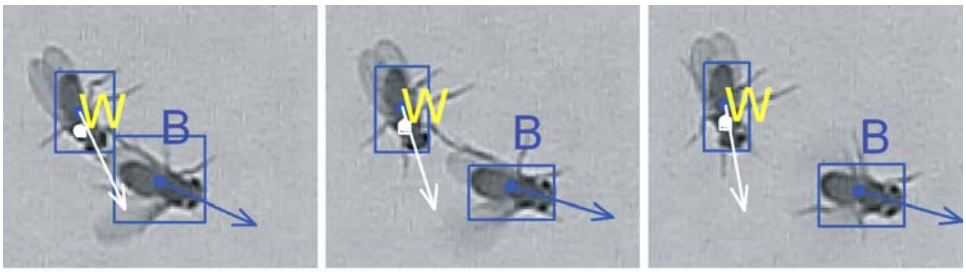


Since the attacked fly wants to escape, it tries to climb up the wall; thereby the fly's area will decrease. If it then slips due to the Fluon, not only a decrease in area, but also an increase in velocity occurs, thereby fulfilling criterion for all lunge expressions.

Global settings concerning mainly the last three frames



(46) *AND NOT lunging Obj's d dist is diverging in 2 frames of fr4,fr5,fr6:* after a lunge, the attacked fly but NOT the attacking fly escapes. For a fly to be defined as 'escaping', in at least two of the three frames following a lunge the distance between the two flies has to become bigger (d dist > 0) and in these two frames the same escaping fly must have the higher velocity concerning its body centre of gravity compared to its opponent. In the example given below, the dark fly escapes:



frame 1	frame 2	frame 2
d dist: + 0.47	d dist: + 0.84	d dist: + 0.72
dark speed: 0.80 pixel/fr	dark speed: 0.87 pixel/fr	dark speed: 0.72 pixel/fr
bright speed: 0.08 pixel/fr	bright speed: 0.15 pixel/fr	bright speed: 0.10 pixel/fr

To be more precise, the threshold for d dist is in fact not > 0 mm/frame, but >0.35 mm/frame (46a).

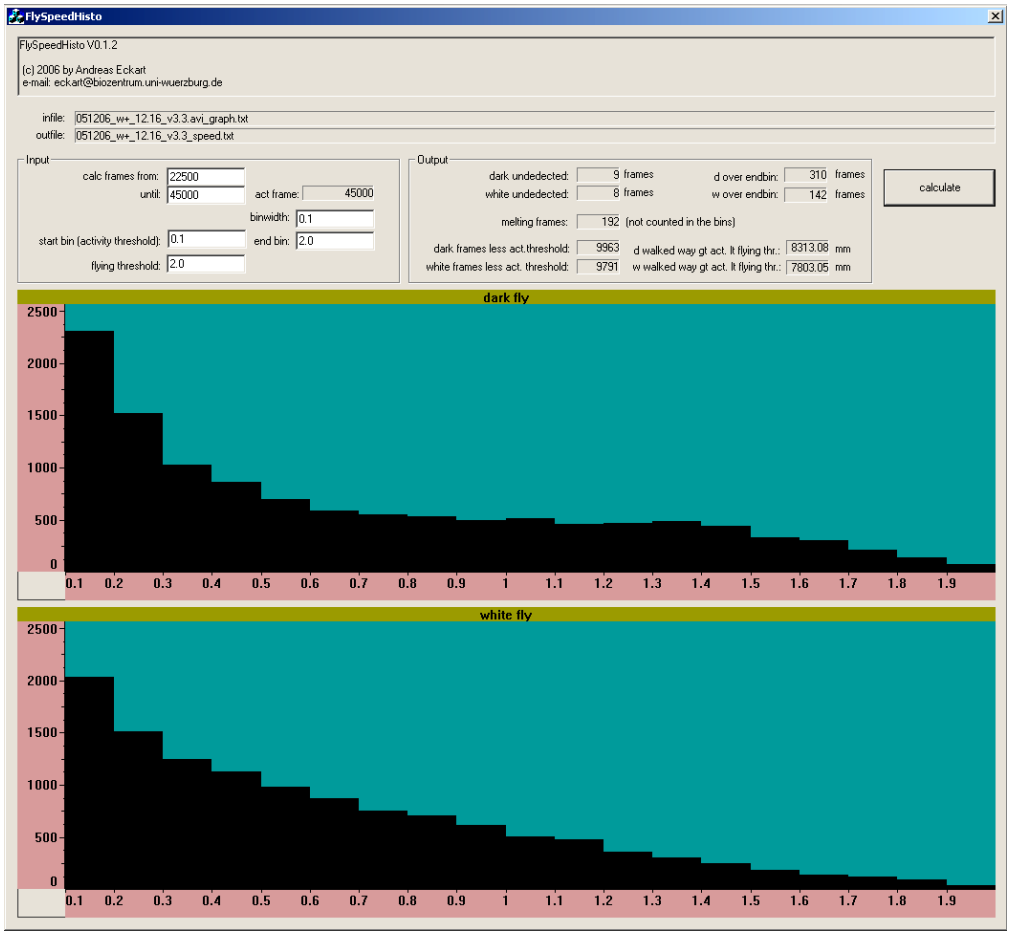
The criterion of escaping is not applied, if in frame 3 the flies' body centres of gravity are merged (46b), because d dist is automatically getting extremely large, as soon as the two flies can be distinguished, therefore it artificially appears, as if one fly escapes.

(47) *AND NOT lunging speed fr4 AND fr5 AND fr6 >:* The speed of the attacking fly must not exceed 2.0 mm in all three frames following the lunge. This criterion excludes flying flies, which sometimes fulfil all the other criteria.

(48) *AND lunging Obj speed < 2.0 mm/frame in fr4 OR fr5 OR fr6:* In one of the three frames following the lunge the speed of the attacking fly must not exceed 2.0 mm/frame. This criterion did not prove to be beneficial; therefore it is not included in our settings.

(49) *AND NOT (fr1 OR fr2 speed=0) OR (frames speed > 10 mm/frame):* This criterion should be better listed in the part 'global settings concerning the first three frames'. The first part of this criterion 'AND NOT (fr1 or fr2 speed = 0)' is actually superfluous, as a speed of zero occurs only, if the fly is not detectable. However, if a fly is not detectable not only the speed but also the area of the corresponding fly and the distance between the two flies would be zero. The latter two settings were already excluded in 'global settings concerning the first three frames' (see (1) and (2)). The second part 'frames speed > 10.0 mm/frame' excludes again flying flies, as a speed of bigger than 10 m/frame is only achieved during flying.

3.2.6 FlySpeedHisto Version 0.1.2

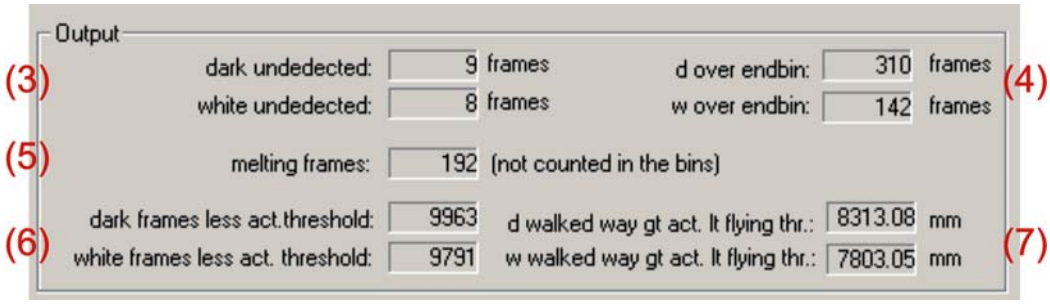


FlySpeedHisto describes each fly's walking activity. It calculates per frame per fly the shift in the centre of gravity of the fly's body, i.e. the distance travelled. Walking is defined as a shift in the centre of gravity of 0.1 – 2.0 mm/frame to avoid an impact of behaviours other than walking, e.g. cleaning and flying.

3.2.6.1 Running an analysis

Open FlySpeedHisto. Click on the text field to the right of 'infile' (1). Select a text file with the suffix avi_graph. Click on the text field to the right of 'outfile' (2) and select where you want to store the output file and under which name. Click 'save' and then 'calculate'. Repeat these steps for each file you would like to be processed.

3.2.6.2 FlySpeedHisto output (file)



(3) *dark/white undetected*: number of frames, in which the respective fly was not on the floor.

(4) *dark/white over endbin*: number of frames, in which the respective fly exceeded the speed specified in flying threshold (11).

(5) *melting frames*: number of frames, in which the flies were so close to each other that the flies' body centre of gravity, were fused. These frames are not included into the summation leading to the total walked distance.

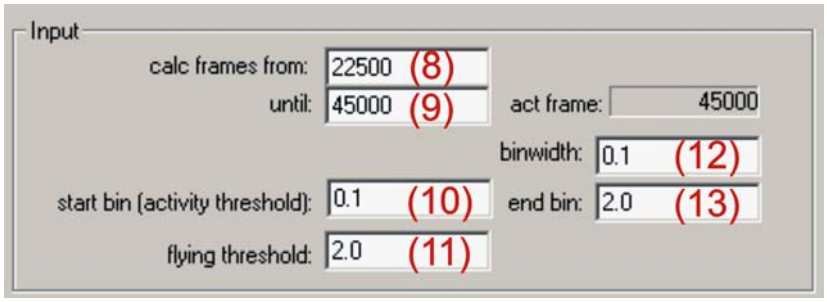
(6) *dark/white frames less action threshold*: number of frames below the activity threshold specified in (10), i.e., the time each fly was idle.

(7) *dark/white walked way*: distances within the activity window defined in the section input (10) and (11), are summed up to give the total walked distance for each fly.

All information is additionally listed in the FlySpeedHisto output text file.

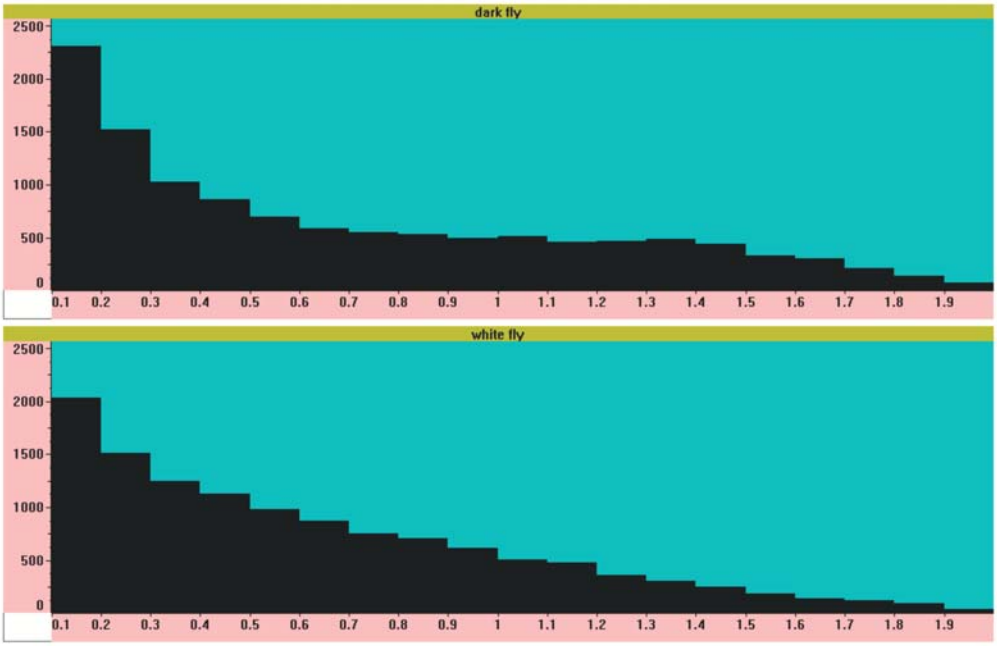
3.2.6.3 Specify the input

Determine from which frame (8) to which frame (9) you would like the file to be analysed. To avoid an impact of behaviours other than walking, define 'walking' by selecting an activity window ((10) and (11)) that excludes behaviours such as cleaning and flying. Attention is invited to the fact that the centre of gravity flickers, even if the fly stands still. Choose a bin width (12) and the final bin (13) for the Histogram being displayed in the lower half of the window.

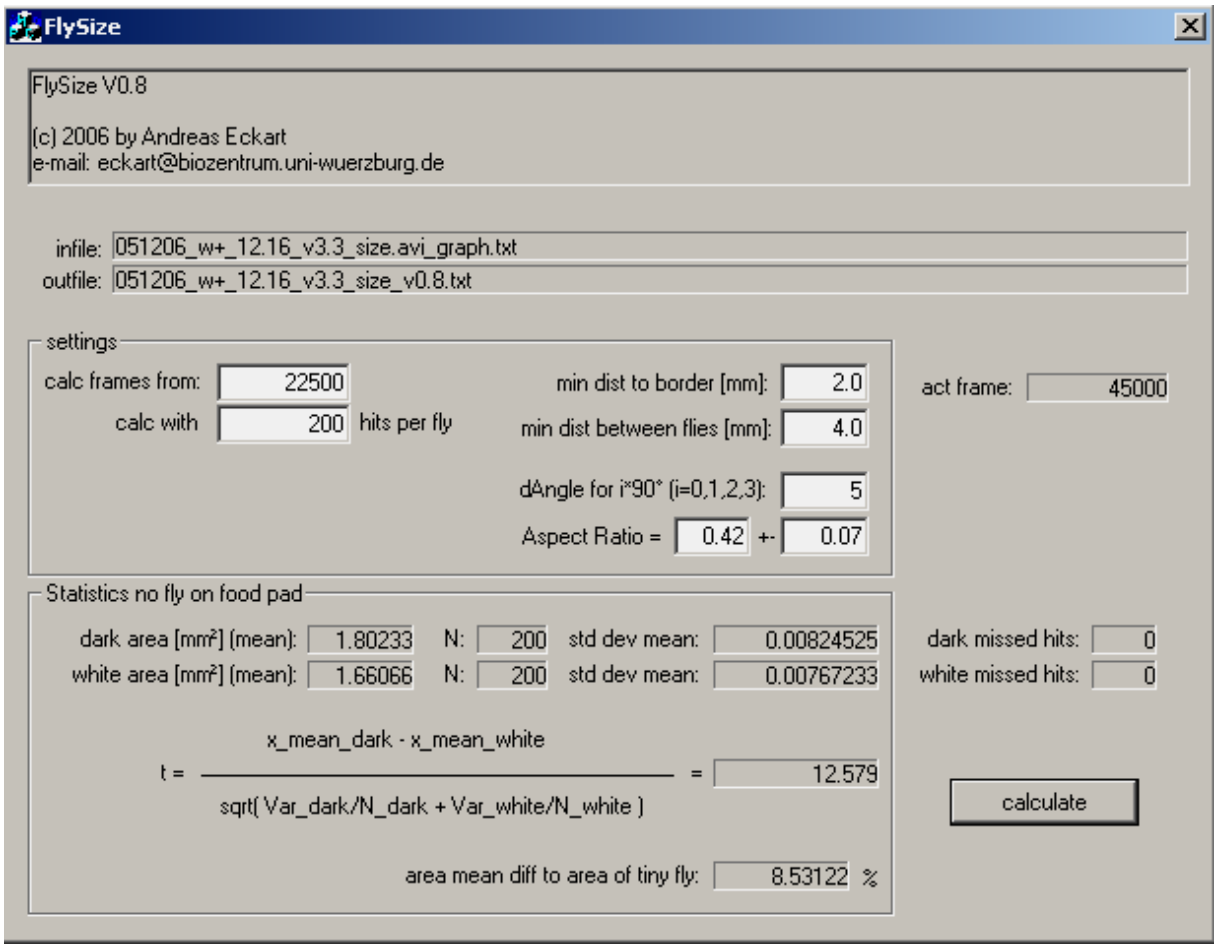


3.2.6.4 Histogram

A Histogram for each fly illustrates in how many frames (y-axis) the respective fly covered a certain distance (x-axis):

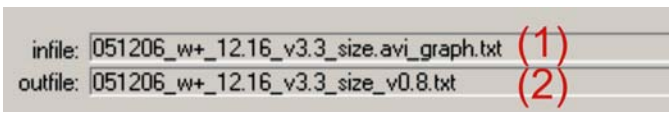


3.2.7 FlySize Version 0.8



FlySize measures the fly's size based on the 2-dimensional area covered by the fly from top view. The calculations are based on an output file generated by LoopRun. This output file differs from the one used in 'LungeCount' and 'FlySpeedHisto'! Be sure that for this output file you have checked '**Fill holes within objects**' for **dark objects**, when setting the properties in **CalcProp**. For further information, refer to CalcProp→ produce a demo-file→ dark settings.

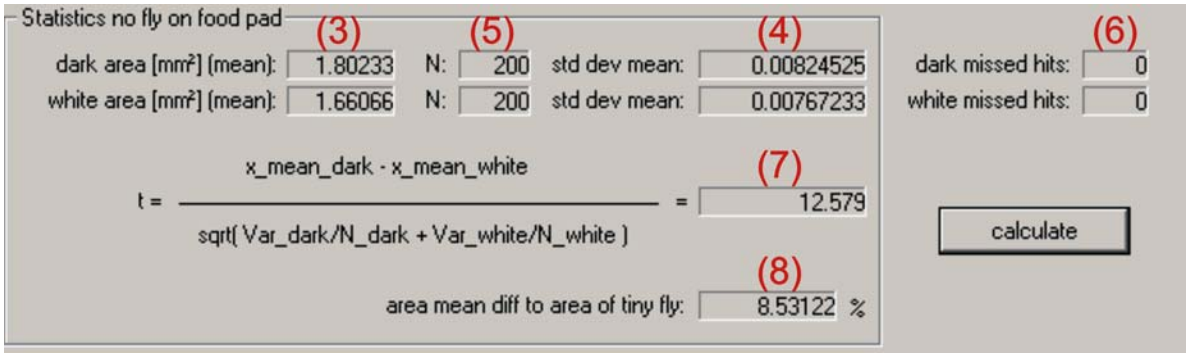
3.2.7.1 Running an analysis



Click on the text field to the right of infile (1). Select a text file with the suffix avi_graph. Click on the text field to the right of outfile (2) and select where and under which

name you would like to save the output file. Click calculate. Repeat these steps for every file you would like to be processed.

3.2.7.2 FlySize output (file)



The software calculates each fly's mean body size (3) and SEM (4) in mm^2 based on optimally 200 frames (5). The number of frames that lack to reach this optimum is given in (6). To compare the size of the two flies, a t-value is calculated (7). Additionally, the software calculates how much bigger the big fly is compared to the small fly (8). All information is additionally listed in the output file.

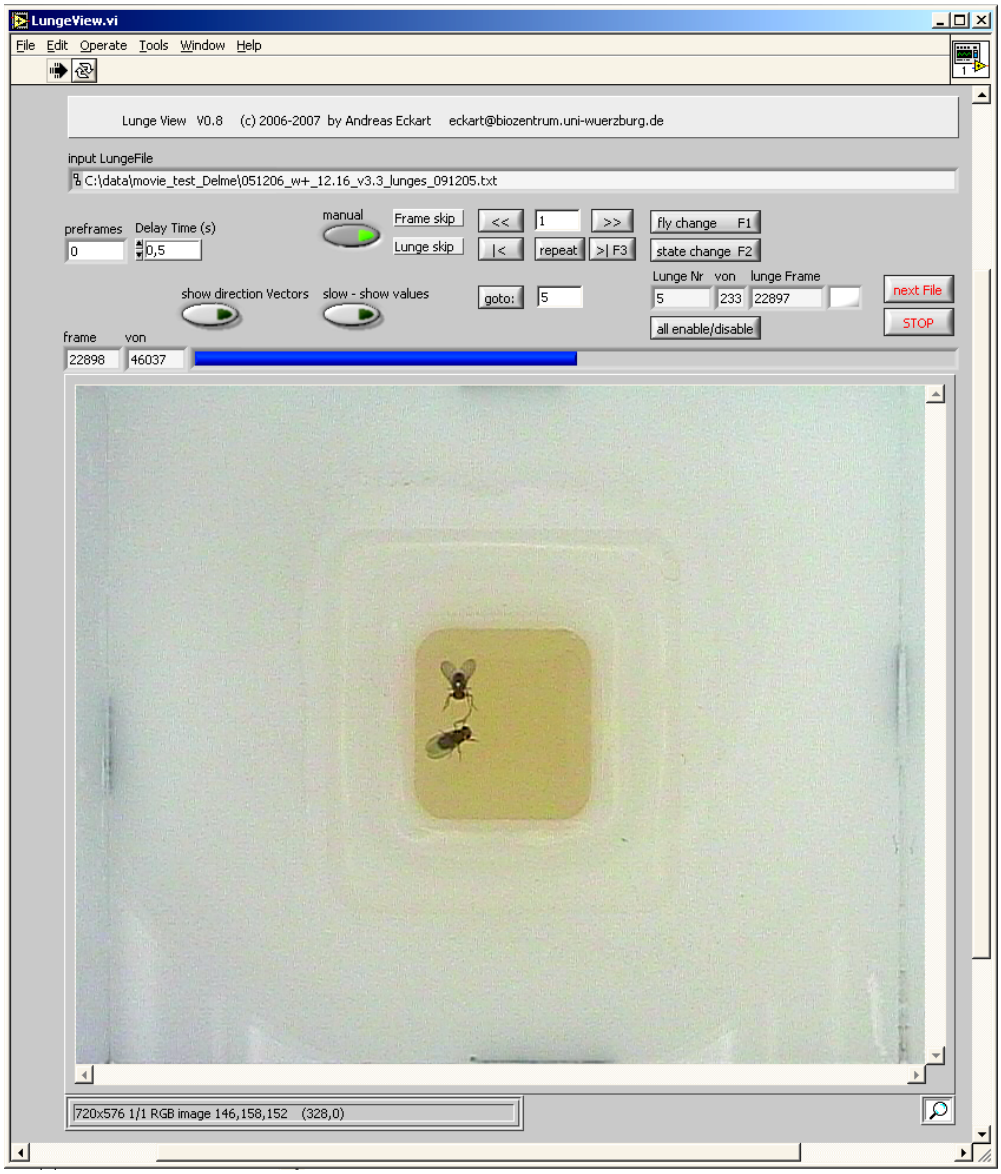
3.2.7.3 Specify the input

Specify from which frame onwards you would like the file to be processed (9) and the number of frames (10), on which the size calculations should be based. Each frame used for calculating the fly's size has to meet four criteria:

1. the fly's distance to the wall must be larger than 2 mm to control for body posture (11).
2. the two flies have to be more than 4 mm apart from each other to exclude interactions and thus to control for body posture (12).
3. the aspect ratio of body width to body length must be 0.42 ± 0.07 (13) and the fly's orientation has to be parallel to either the horizontal or vertical line (14) to control for body posture

4. the fly must not be on the food patch to assure constant contrast.

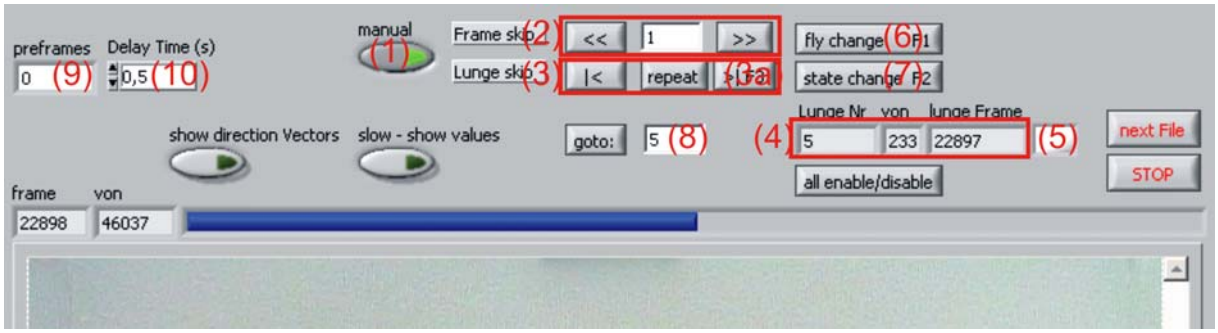
3.2.8 LungeView Version 0.8



LungeView enables the investigator to focus on those frame sequences LungeCount claims to contain lunges. The investigator can then decide whether the selected frame sequences indeed represent lunges, thereby eliminating all false positives. Furthermore, if the investigator would like to know why a lunge was not detected (false negative) or why a frame sequence was wrongly claimed to represent a lunge (false positives), LungeView displays a summary of all information for a selected frame sequence used by LungeCount. Thereby, it is

easy to find out why the error occurred, and consequently the investigator can systematically alter settings in CalcProp.

3.2.8.1 Eliminating false positive lunges

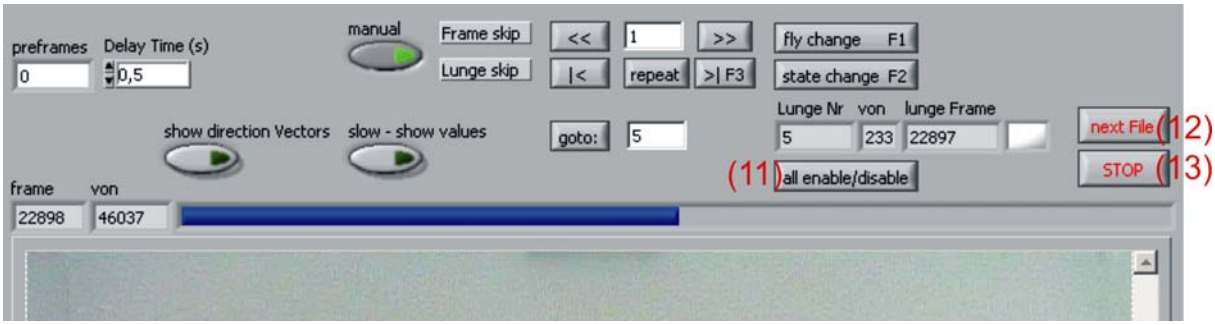


Open LungeView. A dialog appears asking you which file to select. Select the output file created by LungeCount. Click 'ok'. The first frame of the video will be displayed in the window. By default, 'manual' (1) lightens up, which enables you to skip manually either from frame to frame (2; rectangle) or from lunge to lunge (3; rectangle). Skip to the next lunge by pressing (3a) or F3. (4; rectangle) shows which lunge of all detected lunges is displayed in the window and at which frame this particular lunge is taking place. The colour in (5) reflects which fly is performing the lunge: If (5) is white, the bright fly lunges; if it is black, the dark fly lunges; and if it is grey, the investigator has judged this frame sequence as false positive. Imagine, the bright fly is claimed to perform a lunge, as shown in the figure above. There are three possible outcomes:

1. The bright fly lunges indeed. Skip to the next lunge.
2. There is a lunge, but the dark fly lunges. Press fly change (6) or F1. (5) will turn from white to black. Skip to the next lunge.
3. There is no lunge. Press state change (7) or F2. (5) will turn from white to grey. Skip to the next lunge.

With (3; rectangle) you skip either one lunge forward or one lunge backward or you can repeat the same lunge. Alternatively, you can go directly to a lunge of choice by typing in the respective lunge number in (8) and clicking on 'goto'. If you would like to skip not directly to a lunge, but to a discrete number of frames before the lunge, you can type in the respective number in (9). The next time you skip to a lunge, you will have the respective number of frames coming before the lunge. To go from frame to frame, you can click either

(2; rectangle) or you disable 'manual' (1): the software will then play the video frame by frame with a delay time between each frame specified in (10).



In the default state, all frame sequences from LungeCount are claimed to represent lunges. Therefore, if a frame sequence was falsely claimed to contain a lunge, you must press 'state change' to eliminate the false positive. There are videos, where most frame sequences are false positives and only rarely a frame sequence indeed represents a lunge (e.g. fights between blind flies). To save time, you might prefer to claim all frame sequences offered by LungeCount to be false positives and only if there is a lunge, you change the state of the frame sequence to 'correct'. To claim all lunges to be wrong, you have to press 'all enable/disable' (11), which will turn the default state 'all correct' to 'all incorrect'. By pressing 'all enable/disable' (11), you can go back and forth. After you have checked all frame sequences, you can either choose a new file to be presented (12) or you can stop (13). In either case, LungeView will ask you whether you would like to save the corrected version of the file. Press 'save' and give the file a new name.

3.2.8.2 LungeView output file

```
manual corrected lunges:

frame fly
24251 dark   fly
24814 dark   fly

dark   lunges:    2
bright lunges:    0


discarded lunges:

29065 dark   fly
29491 dark   fly
```

(1) (2) (3)

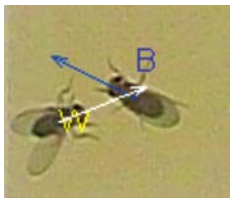
The output file given by LungeView lists

(1) all frames sequences (frame 1) for each fly, which the investigator claimed to contain lunges,

(2) the total number of lunges for each fly and

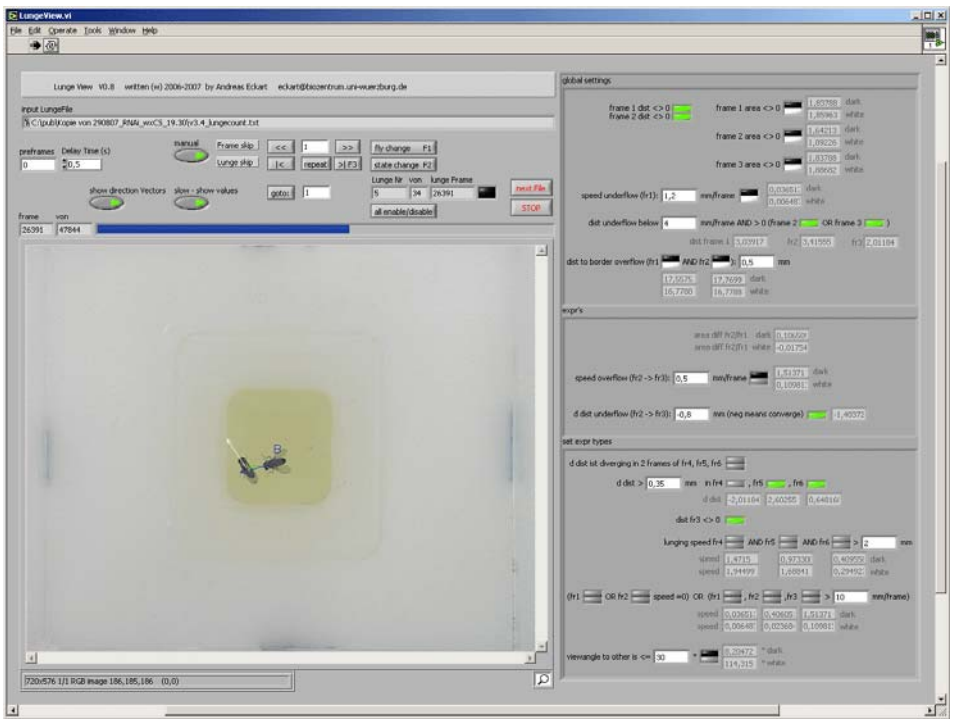
(3) the discarded lunges for each fly.

3.2.8.3 Displaying information used by LungeCount



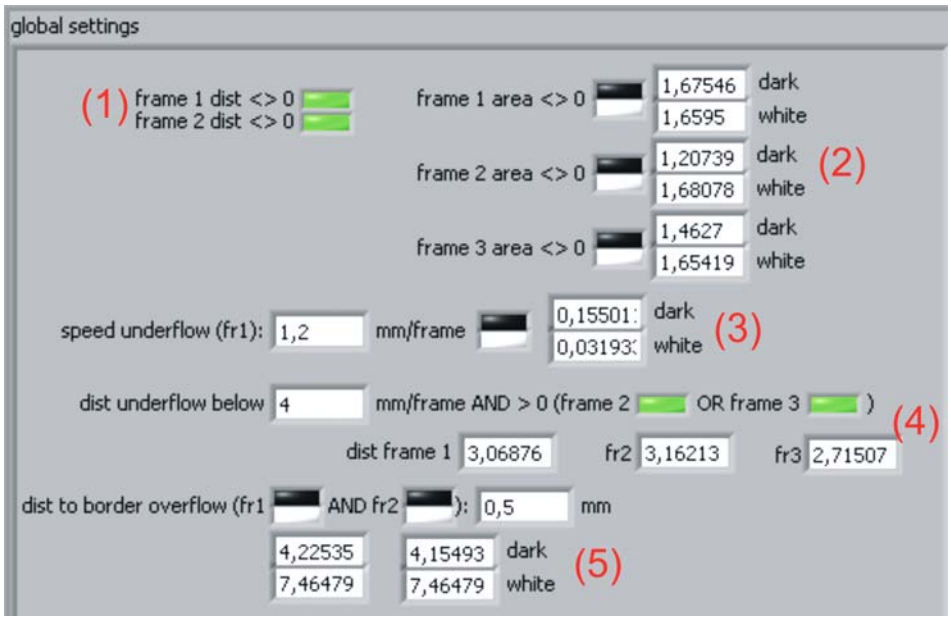
If you would like to see the fly's orientation for each frame as calculated by LungeCount, press 'show direction vectors', click 'next' and choose the same text file again. The inner window will now display the orientation of each fly in each frame.

If you would like to change the settings for CalcProp, it is essential to know the reasons why certain lunges were not detected, whereas other frame sequences were falsely claimed to contain lunges. By pressing 'show values', click 'next' and choose the same text file (it can take a while), LungeView will illustrate all relevant information that is used by LungeCount for the whole frame sequence (frame 1-6) starting with the frame displayed in the window.



Therefore, go to the first frame of the frame sequence of interest (frame 1 according to LungeCount). If the criteria of LungeCount are met, the according fields next to each criterion will lighten up, either green, if the according criterion includes both flies (e.g. distance of the flies < 4mm), or black, if this criterion is related to only the dark fly (e.g. speed), or white, if this criterion is related to only the bright fly. In addition, the according values are shown next to the field. Thus, the investigator quickly overviews, which field does/does not lighten up, which should not/should lighten up.

Global settings concerning the first three frames:



Here, all information is met:

(1) the distance of the two flies is more than 0 mm for frame 1 and frame 2.

(2) Each fly has an area bigger than 0 for frame one to three.

(3) Both flies displayed a speed less than 1.2 mm/frame in the first frame.

(4) The distance of the two flies is less than 4 mm for both frame 2 and frame 3.

(5) And both flies are further than 0.5 mm away from the wall regarding frame 1 and 2.

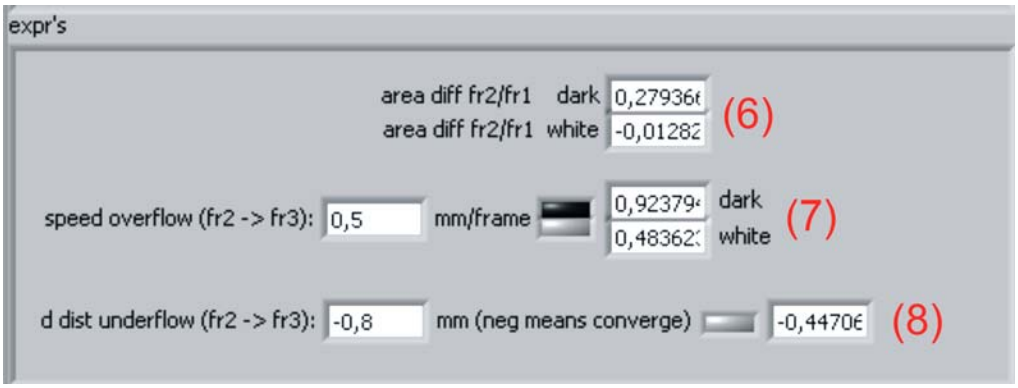
Lunge expressions:

(6) gives the decrease in area from frame 1 to frame 2, which is for the black fly 27.9%

(7) Only the black fly exceeds the criterion of 0.5 mm/frame for frame 3, thereby only the field of the dark fly is black, whereas the field of the white fly stays grey.

(8) The difference in distance between the two flies from frame 2 to frame 3 (do the flies converge?), is - 0.44 mm.

Therefore, only the black fly would meet the criterion for a lunge (expressions 1, 2 and 6).



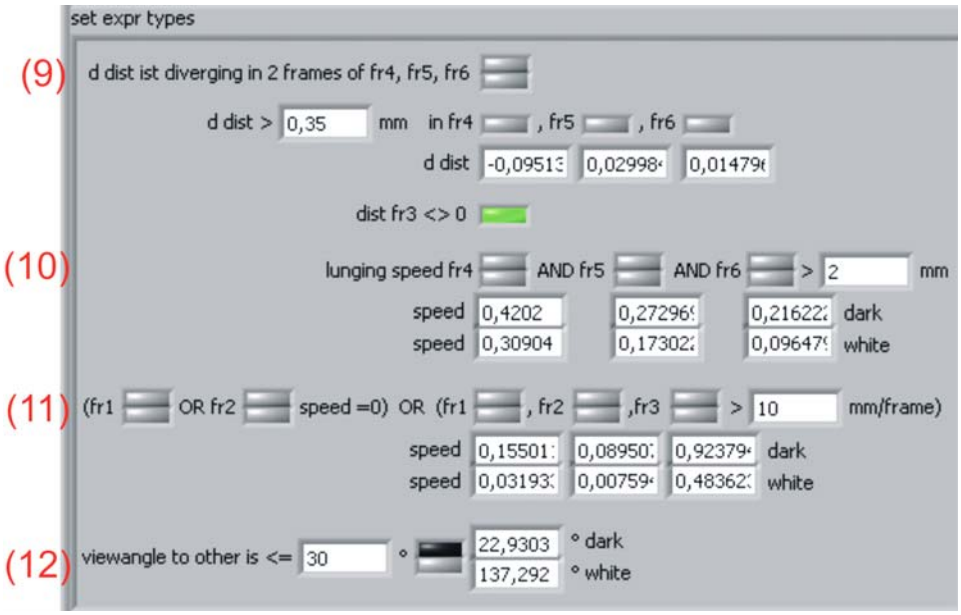
Global settings concerning mainly the last three frames:

(9) None of the flies is escaping after the lunge (both fields grey). The requirements for escaping are not met, since in none of the three frames following the lunge, the difference in the fly's distance to each other from frame to frame was bigger than 0.35.

(10) Neither the dark nor the bright fly flies following the frames after the lunge (all fields grey).

(11) Neither the dark nor the bright fly flies during the first three lunges (all fields grey).

(12) The dark fly is oriented to the bright fly (22.9°), but the bright fly is not oriented to the dark fly.



3.3 EVALUATION OF THE AUTOMATED ANALYSIS

The present software program gives the number of lunges for each fly in a certain time interval. In addition, it provides information such as the distance the fly walked, his size and the time he spent on the food patch and in the periphery. As the lunge has been reported to be the most frequent behaviour by which an opponent is displaced from the food patch (Hoffmann, 1987a), the number of lunges of a male may serve, at least to some extent, as an indicator of his overall aggressiveness.

To evaluate the reliability of the software, the same clips were analysed twice with respect to the number of lunges: once by the software and once 'by hand'. The software is designed to minimize false positive assignments (counting frame sequences wrongly as lunges). This leads to a slightly larger number of false negatives (missing lunges; Figure 6B). The software underestimates the occurrence of lunges by about 11%, as indicated by the slope of the red line in Figure 6A. This value is independent of the lunge frequency (Figure 6B). Importantly, it is also largely independent of genotype (Figure 6C). Only if a genotype results in a high percentage of non-fighting males, the overall error rate differs from that of wild-type, since for non-fighting males the number of lunges can only be overestimated (Figure 6C).

Overestimating lunge frequency for non-fighting males can hide subtle differences between genotypes. Therefore, we added a ‘lunge view’ software program that enables the investigator to focus only on those frame sequences of a clip that contain lunges according to the ‘lunge count’ software. The investigator can then decide whether the selected frame sequences indeed represent lunges, thereby eliminating false positives. Thus, with the help of the ‘lunge count’ and ‘lunge view’ software, aggressive interactions between two male *Drosophila melanogaster* of a genotype of choice can now be analyzed either automatically or if preferred semi-automatically.

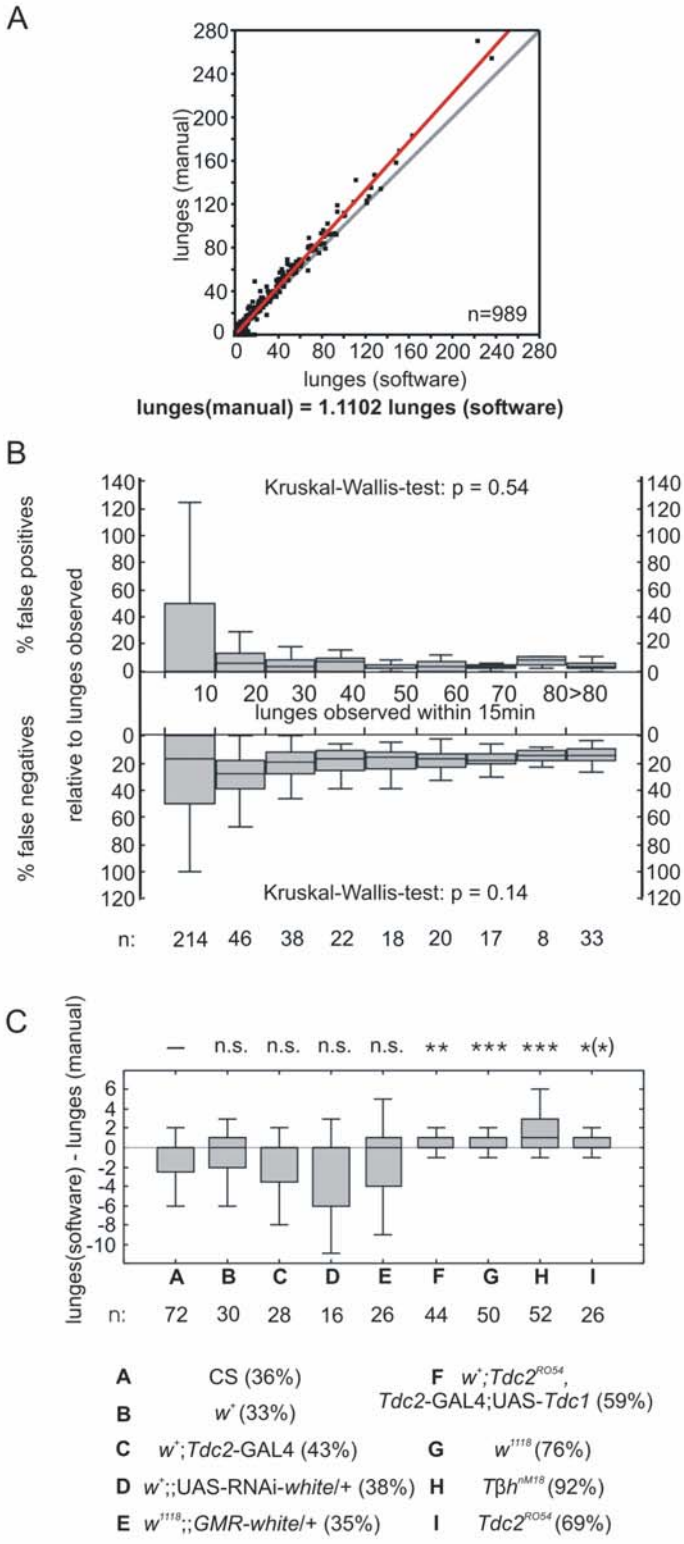


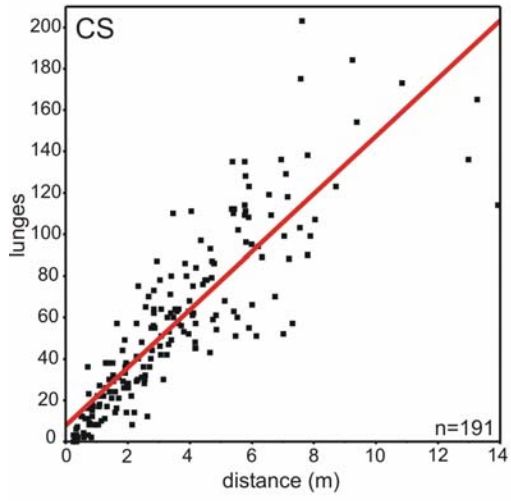
Figure 6:

The software underestimates the amount of lunges by ~11% - independent of both the lunge frequency and the genotype. **(A)** The software underestimates the amount of lunges by ~11%, as indicated by the slope of the red line. The x-axis represents the number of lunges detected by the software, whereas the y-axis gives the number of lunges counted by hand. Each data point represents one male. For comparison, the grey line shows the ideal detection of every single lunge (slope equals 1). **(B)** For each male all lunges were determined by hand in order to check how often the software counted a frame sequence wrongly as a lunge (false positive) and how often it missed a lunge (false negative). The error rate is given in percent relative to the total number of lunges the male performed. Males were pooled into bins depending on how many lunges they performed. The error rate for false positives (upper half of the panel) and false negatives (lower half of the panel) is constant over all lunge frequencies. **(C)** The nine genotypes A to I were screened in this study. The y-axis illustrates how many lunges the software counted compared to a manual evaluation. For each genotype the accuracy in detecting lunges was compared to CS, as the software was developed on CS fights. Only genotypes that result in a high percentage of non-fighting males (percentages given in parentheses) differ from CS, since for non-fighters the number of lunges can only be overestimated.

3.4 WALKING ACTIVITY AND BODY SIZE

To determine baseline aggressive behaviour of wild-type flies in our paradigm, CantonS (CS) males were tested. Independent of the time of day ($p = 0.17$; n per hour = 8 – 32), a pair of five-day old CS males performed 3.85 ± 2.82 lunges/min (mean ± S.D.; n = 191) demonstrating the high variability already observed in other paradigms of *Drosophila* male aggression (Skrzipek et al., 1979; Hoffmann, 1987a; Yurkovic et al., 2006). In the present study aggression was recorded from the 15th to the 30th min constituting a period when flies already had decreased activity and displayed constant aggression at a level indistinguishable from that of the two subsequent 15 min time bins (data not shown).

The total number of lunges performed by a pair of males correlated positively with their overall walked distance, i.e., the more the two flies walked the more lunges they performed. This correlation could be demonstrated for numerous genotypes (Figure 7). We decided to normalize lunges to walking activity, since variance was strongly reduced by this step. A pair of five-day old CS males performed 16.4 ± 6.6 lunges/m (mean ± S.D.; n = 191). Moreover, normalizing for walking activity also compensated for effects of higher temperature on lunge frequency. A pair of CS males performed significantly less lunges when tested at 31.5°C compared to 25°C ($p = 0.006$; n = 21), the lunge frequency being decreased by 70%. However, since walking activity was also strongly reduced ($p = 0.001$), temperature did not affect the number of lunges a pair of CS males performed per meter ($p = 0.31$)



genotype	Rsquare	n	β =0
w^+;*GMR-white*/+	0.93	18	<0.0001
w^+;*Tdc2*-GAL4/+	0.82	39	
w^{1118};*Tdc2*-GAL4/+;*Appl*-GAL4/+	0.82	24	
w^+;;*GMR-white*/+	0.81	10	
w^+;$Tdc2^{RO54}$,*Tdc2*-GAL4/+	0.78	23	
CS	0.74	191	
w^{1118};;*GMR-white*/+	0.74	37	
w^+;;*Appl*-GAL4/+	0.73	18	<0.0001
w^{1118};;*Appl*-GAL4/+	0.72	32	<0.0001
w^+,y^-	0.71	12	0.0006
w^+;*GMR*-GAL4/+	0.63	19	<0.0001
w^+;;UAS-*RNAi-white*/+	0.62	21	<0.0001
w^+;$Tdc2^{RO54}$/+	0.62	17	0.0002
w^{1118};*GMR-white*/+	0.56	21	
w^+;UAS-*Kir2.1*/+	0.50	20	0.0005
w^+;cn^1	0.44	17	0.0038
w^{1118};*GMR-white*/*GMR-white* (III)	0.36	13	0.0315
w^{1118};*GMR-white*/*GMR-white* (II)	0.34	16	

Figure 7: The lunge frequency correlates with the walking activity. The graph depicts the correlation of the total distance a pair of CS flies walked to the total number of performed lunges. Each dot represents a pair of males. The chart lists the R square value of the according correlation for 18 genotypes of this study. Where the requirements for testing were met, the p-value indicates the probability for no correlation between the number of lunges and the walking activity (slope equals zero). Since for all 18 genotypes the total number of lunges performed by a pair of males correlated positively with their overall walked distance, the indicator of aggression in this study is lunges per meter to normalize for the walking activity.

The two males did not lunge equally often within the recording period. We defined animals as winners that performed more than 70% of all lunges. In 91% of all pairs that performed at least 10 lunges (pairs performing 10 lunges or more: n = 172) a winner could be identified by this criterion. Yurkovic et al. (2006) defined winners as flies that lunged three times in a row, while the opponent retreated each time. They reported that winners spend more time on the food patch than losers. This is consistent with our findings, since winners by the 70% criterion nearly always spent more time on the food patch than losers (p = <0.0001; n = 157 pairs). Instead, the latter spent significantly more time in the border zone than the winners (p = <0.0001; n = 157). The border zone was defined as the outer 4 mm of the arena.

The size difference between two males strongly influenced, which one was going to become the dominant animal. Our data show that if the size difference was bigger than 8% (measured as the projection area from above), the bigger fly was likely to lunge more often than the smaller fly (Figure 8). Three different genotypes were pooled for the statistical analysis. They all showed the same trend. Since 8% difference in body size cannot be detected by human eye, fights were only set up between two males of the same genotype throughout this study, in order to avoid a confounding influence of size when investigating the effect of a specific genotype.

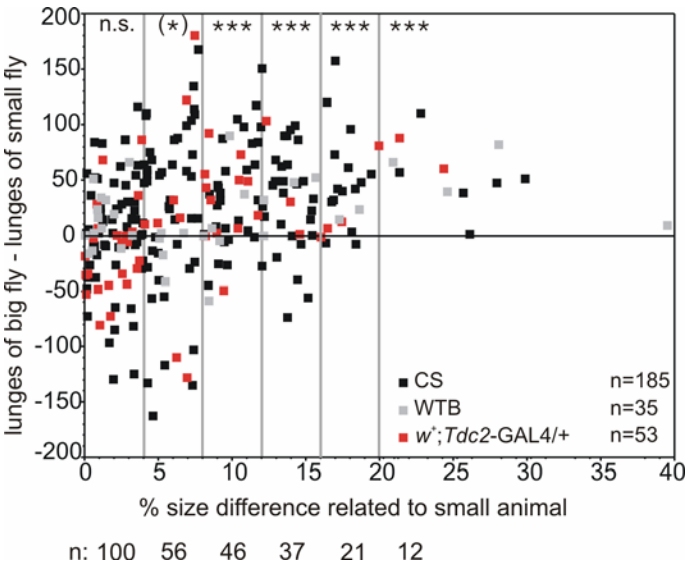


Figure 8: Size differences between two males from 8% up influence the outcome of a fight. That is, if a fly is bigger than its opponent, it is likely to lunge more frequently than the smaller fly. Size was measured from dorsal as the 2-dimensional area of the fly (compare to Fig. 4H). Size differences between two males were calculated with respect to the smaller male (x-axis). The difference in lunges was determined by subtracting the number of lunges of the smaller fly from the number of lunges of the bigger fly (y-axis). Data from three different genotypes were pooled and subdivided into 4% bins. Each bin was tested for a significant deviation from zero using Wilcoxon-Sign-Rank.

3.5 *WHITE*

Many transgenic fly lines are generated and kept in a *white* mutant background. We therefore examined the role of the *white* (*w*) gene in aggressive behaviour. Males mutant for the null allele w^{1118} (Hazelrigg et al., 1984) were strongly impaired in aggression, lunging at a

rate of only 3% of wild-type male levels (Figure 9A). Providing w^{1118} males with a *mini-white*$^{+}$ transgene had differing effects: *Tdc2*-GAL4 heterozygote males performed as many lunges/m as w^{1118} males did, i.e. their aggression was reduced by 98% compared to wild-type. In contrast, *Appl*-GAL4 heterozygous males as well as males heterozygous for both *Tdc2*-GAL4 and *Appl*-GAL4 showed partially restored aggression with 29% and 53% of wild-type level, respectively. In line with this finding, males of the latter two genotypes had more intense eye pigmentation than *Tdc2*-GAL4 heterozygous males (Figure 9A). The absent or incomplete rescue of the aggression phenotype is not due to side-effects of the constructs, since w^{+} males carrying either one of the two transgenes fought indistinguishably from wild-type males (Figure 9A).

Mutant w^{1118} flies lacking the characteristic red pigmentation of the eyes are visually impaired (Hengstenberg and Gotz, 1967; Wehner et al., 1969). Therefore, we tested whether the decrease in aggressive behaviour observed in w^{1118} mutant flies was due to the peripheral visual system. This assumption was supported by the behaviour of blind $norpA^{P24}$ hemizygote (Hotta and Benzer, 1970; Pak et al., 1970) and motion-blind $ninaE^{17}$ homozygote males (Otousa et al., 1985; Zuker et al., 1985; Strauss et al., 2001). Males of both genotypes performed significantly less lunges/m than WT-B males (< 10%; Figure 9B). Next we asked whether to show aggression males needed the *white* gene function in vision for proper pattern contrast in the eye. For a tissue specific knock-down we used the eye specific GMR-GAL4 line (Freeman, 1996) to drive a UAS-RNAi-*white* transgene. These males showed only a light colouring of the adult eye and the aggression phenotype of w^{1118} mutant males could be mimicked, thus aggression was almost completely abolished (Figure 9C). In an inverse experiment we rescued the eye colour phenotype in males carrying a GMR-*white* construct in a w^{1118} mutant background. Interestingly, the aggression was only partially restored independent of whether one or two copies of the GMR-*white* construct were present. This effect could be observed for two different insertion sites of the construct, one located on the 2nd and one on the 3rd chromosome, with flies fighting at 28% - 65% of wild-type level. This suggests that an intact visual system is required for proper aggressive behaviour. Since the flies' eye colours were dark red but still clearly distinguishable from wild-type CS males (Figure 9D), this experiment did not rule out that the lower than WT level of aggression reflected an incomplete restoration of contrast transfer in these eyes.

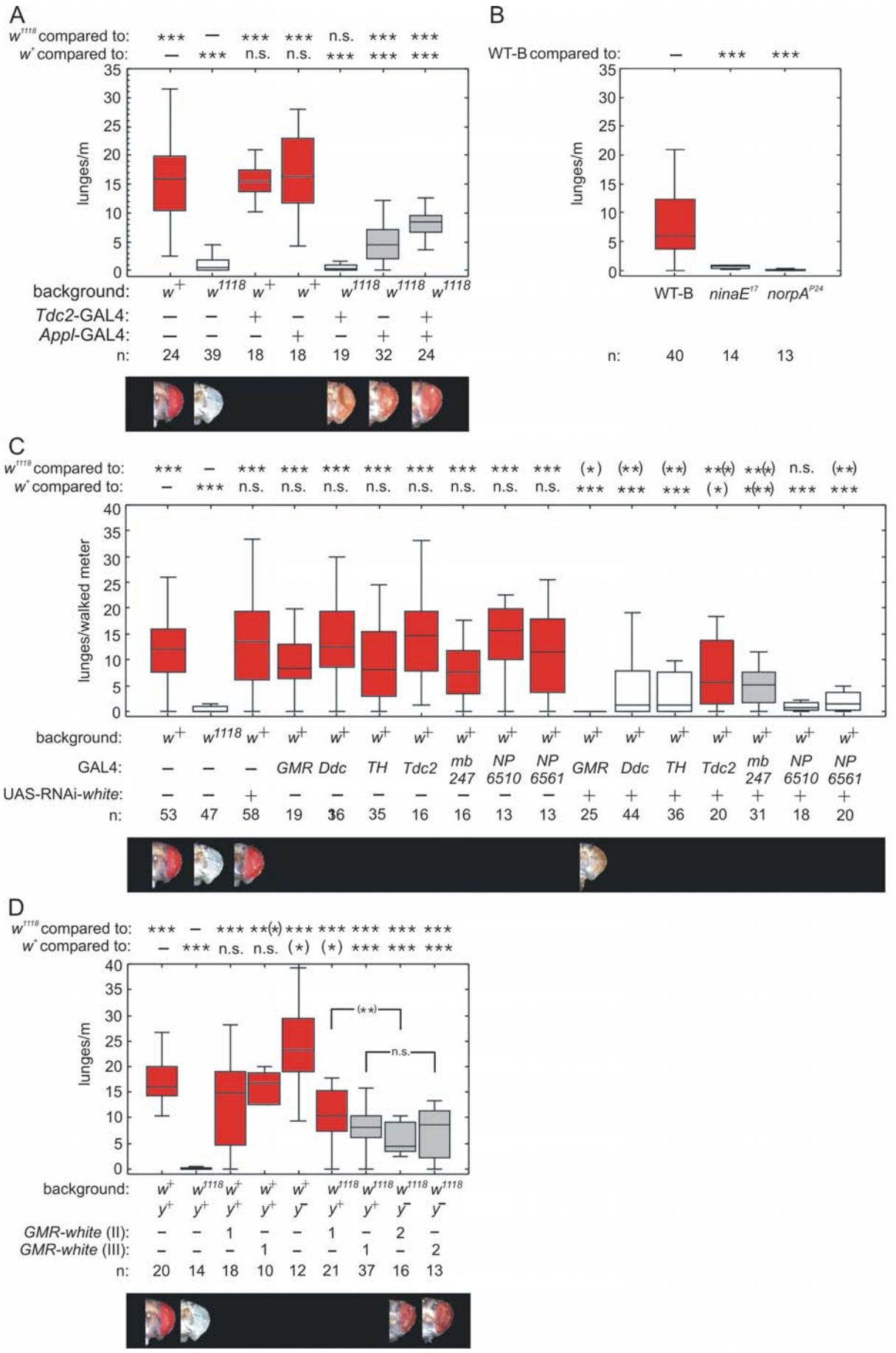

A
w1118 compared to: *** – *** *** n.s. *** ***
w+ compared to: – *** n.s. n.s. *** *** ***
lunges/m
background: w+ w1118 w+ w+ w1118 w1118 w1118
Tdc2-GAL4: – – + – + – +
Appl-GAL4: – – – + – + +
n: 24 39 18 18 19 32 24
B
WT-B compared to: – *** ***
lunges/m
WT-B ninaE17 norpAP24
n: 40 14 13
C
w1118 compared to: *** – *** *** *** *** *** *** *** *** (*) (**) (**) **(*) **(*) n.s. (**)
w+ compared to: – *** n.s. n.s. n.s. n.s. n.s. n.s. n.s. n.s. *** *** *** (*) *(**) *** ***
lunges/walked meter
background: w+ w1118 w+ w+ w+ w+ w+ w+ w+ w+ w+ w+ w+ w+ w+ w+ w+
GAL4: – – – GMR Ddc TH Tdc2 mb 247 NP 6510 NP 6561 GMR Ddc TH Tdc2 mb 247 NP 6510 NP 6561
UAS-RNAi-white: – – + – – – – – – – + + + + + + +
n: 53 47 58 19 36 35 16 16 13 13 25 44 36 20 31 18 20
D
w1118 compared to: *** – *** **(*) *** *** *** *** ***
w+ compared to: – *** n.s. n.s. (*) (*) *** *** ***
lunges/m
(**)
n.s.
background: w+ w1118 w+ w+ w+ w1118 w1118 w1118 w1118
y+ y+ y+ y+ y– y+ y+ y– y–
GMR-white (II): – – 1 – – 1 – 2 –
GMR-white (III): – – – 1 – – 1 – 2
n: 20 14 18 10 12 21 37 16 13

Figure 9: *w*^{1118} null mutants are strongly impaired in aggressive behaviour. This decrement is substantially, but not exclusively, due to the loss of *white* function in vision. **(A)** In *w*^{1118} null mutant males aggression is almost abolished (p = 0). To investigate whether *mini-white*$^{+}$ transgenes rescue the *w*^{1118} phenotype, males carrying either one or both of two different GAL4-constructs were tested for their aggression. By themselves, only one of the two *mini-white*$^{+}$ transgenes partially restored male aggressive behaviour (*w*^{1118}*; Appl*-GAL4; p = 0.00001). In *w*^{1118}*; Tdc2*-GAL4 males aggression was indistinguishable from aggression displayed by *w*^{1118} males (p = 0.44), presumably due to the insufficient eye pigmentation. Combining both transgenes also resulted only in a partial rescue compared to *w*$^{+}$ (p = 0). **(B)** That the decrease in aggressive behaviour observed in *w*^{1118} mutant flies was partially due to the impairment of the peripheral visual system is supported by findings for blind *norpA*^{P24} hemizygote and motion-blind *ninaE*^{17} homozygote males. Males of both genotypes performed significantly less lunges/m than wild-type (WT-B) males (< 10%; for both p < 0.00001). **(C)** Knocking-down *white* expression only in the eye using *GMR*-GAL4/UAS-RNAi-*white* led to a decrease of lunges per walked meter indistinguishable from the level of *w*^{1118} (p = 0.06). However, the *white* mutation seems to also affect neurons outside the eye, since knocking-down *white* in various neurons of the central brain reduced the frequency of lunges to varying degrees. **(D)** Rescuing the *white* mutation exclusively in the eye with two different insertions of the same *GMR-white*$^{+}$ construct, one being located on the 2nd the other on the 3rd chromosome, only partially restored aggressive behaviour independently of whether one or two copies were present (for all p < 0.00005). Genotypes represented in red boxes do not differ from *w*$^{+}$ with respect to their aggressive behaviour, whereas genotypes represented in white boxes are not distinguishable from *w*^{1118}. Grey boxes show genotypes different from both *w*$^{+}$ and *w*^{1118} regarding their aggression. Where informative, the eye colour of a male of a specific genotype is illustrated below the according box of the plot.

On the other hand, *white* gene function might be required in tissues of the fly other than the pigment producing cells in the eye. The latter idea is supported by findings of Campbell and Nash (2001) who detected *white* messenger RNA in *so*^{1} flies by using RT-PCR. Mutant *so*^{1} flies have neither eyes nor ocelli and should therefore lack pigment producing cells. Also, in a place learning paradigm in complete darkness (heat box) *w*^{1118} null mutant flies are impaired (Diegelmann et al., 2006). The *white* mutation, therefore, seems to also affect neurons outside the eye. To further test this hypothesis, we combined various GAL4 drivers (*Ddc*-GAL4; *TH*-GAL4, *Tdc2*-GAL4, MB247-GAL4; NP6510-GAL4, NP6561-GAL4) expressing GAL4 in groups of neurons in the central brain with the UAS-RNAi-*white* transgene. Indeed, diminishing *white* expression in these cells reduced the frequency of lunges to varying degrees ranging from 5% to 48% of wild-type level (Figure 9C). These results suggest that *white* exerts its effect not only in pigment producing cells but also in other parts of the brain, some of which are involved in the control of aggression.

3.6 OCTOPAMINE: *TBH*^{nM18}

Scoring various components of *Drosophila* aggressive behaviour Baier et al. (2002) report severely reduced aggression of *Tβh*^{nM18} males. *Tβh*^{nM18} mutant flies lack tyramine β-hydroxylase (TβH), an enzyme converting tyramine (TA) to octopamine (OA). These flies have no detectable levels of OA, whereas TA levels are about 10-fold elevated (Monastirioti

et al., 1996). These authors, however, had used $T\beta h^{nM18}$ males carrying the additional w^{1118} null mutant allele. The white-eyed $T\beta h^{nM18}$ males were tested with red-eyed control males. As shown above, the w^{1118} null mutation by itself leads to a phenotype similar to the one observed by Baier et al. (2002): a profoundly reduced aggression. Furthermore, even after back-crossing the $T\beta h^{nM18}$ flies to w^{+}, mutant males were still about 8% smaller than wild-type males ($p = 0.0039$) (Figure 10C). Hence, body size difference might have contributed to the decreased aggression as well. To test whether reduced aggression was indeed due to the $T\beta h^{nM18}$ mutation and independent of body size we repeated the experiment using our w^{+} $T\beta h^{nM18}$ flies in pairs with mutant males only and in our automated recording set-up counting only lunges. Aggression was still almost completely abolished (Figure 10A).

Inconsistent with our results, Certel et al. (2007) in a study of $T\beta h^{nM18}$ males did not report a general decrease in aggression compared to wild-type males (S. Certel, personal communication). To exclude genetic background as the cause for this discrepancy, their $T\beta h^{nM18}$ mutant stock being independently crossed into w^{+} background (S. Certel, HMS, Boston) was tested in our paradigm. These males displayed profoundly less lunges per meter compared to wild-type males (Figure 10D), even though the remaining level of 17% of wild-type was slightly more than our mutant stock in which aggression was almost completely abolished.

In our paradigm, walking activity of $T\beta h^{nM18}$ males was reduced compared to wild-type males by ~38% (Figure 10B). Interestingly though, this effect was reversed when looking at the first 15 min of an experiment. During this phase $T\beta h^{nM18}$ males were about 67% more active than wild-type males ($p = 0.008$). Quantifying walking activity of individual $T\beta h^{nM18}$ males, Hardie and colleagues (2007) detected no alterations for $T\beta h^{nM18}$ males compared to controls.

Based on published effects of OA, we tested two hypotheses that might explain the strong decrease in aggression observed for $T\beta h^{nM18}$ males. (1) Zumstein et al. (2004) studied distance of and force production during jumping in *Drosophila*: The performance of $T\beta h^{nM18}$ flies is only ~50-60% of wild-type level. Consequently, $T\beta h^{nM18}$ males might be incapable of executing lunges. However, a quantitative high-speed analysis of lunges measuring twelve parameters did reveal only a single small difference between lunges executed by CS versus $T\beta h^{nM18}$ males: While rising up on their hind-legs, $T\beta h^{nM18}$ males did not elevate their body as much as wild-type males (-26%; $p = 0.005$). In other words, only the frequency but not the execution of lunges seemed to be affected. (2) As mentioned in the Introduction, injection of

the OA agonist chlordimeform into crickets causes normally submissive losers to re-engage in fights faster (Stevenson et al., 2005). Therefore, appropriate levels of OA might be required to motivate former losers to fight again. If *Tβh*^{nM18} males establish a hierarchy within the first 15 min and the loser thereafter avoids re-engaging in further fights, lunges might become a rare event. To test this hypothesis, the first 15 min immediately after pairing the flies were analysed. Right from the beginning *Tβh*^{nM18} males performed hardly any lunge ($p < 0.0001$) indicating a general loss of aggressiveness independent of former experiences.

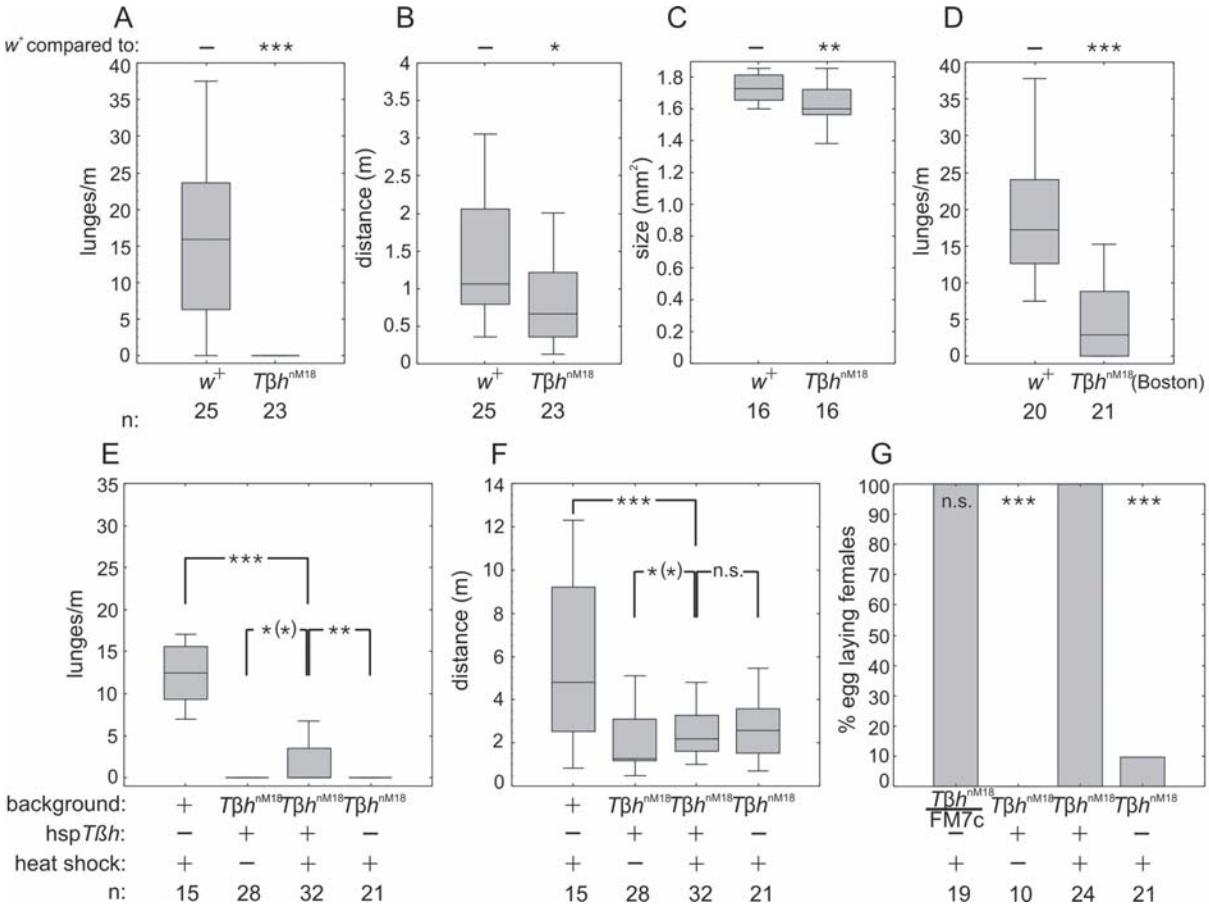


Figure 10: TßhnM18 males display less aggression than wild-type males. (A) *Tßh*^{nM18} males performed fewer lunges per meter compared to wild-type males ($p = 0$). **(B)** *Tßh*^{nM18} males walked slightly less than wild-type males ($p = 0.027$). **(C)** *Tßh*^{nM18} males were smaller than wild-type males ($p = 0.004$). Therefore, in (A) fights were set up with two males of the same genotype. **(D)** The decrease in aggression is not due to genetic background, as *Tßh*^{nM18} males of a second stock also displayed less lunges per meter compared to wild-type males ($p = 0.000002$) (see Results). **(E)** Heat-shock induced expression of *Tßh* in adult *Tßh*^{nM18} mutant males partially restored aggression compared to both males of the same genotype without heat-shock ($p = 0.004$) and to heat-shocked *Tßh*^{nM18} males lacking the hs-*Tßh* construct ($p = 0.003$). **(F)** The walking activity of heat-shocked *Tßh*^{nM18} males with hs-*Tßh* construct did not differ from heat-shocked *Tßh*^{nM18} males without the construct ($p = 0.61$). However, it was different from males of the same genotype without a heat-shock ($p = 0.004$). **(G)** Heat-shock induced expression of *Tßh* in adult *Tßh*^{nM18} females resulted in a full rescue of egg laying behaviour.

We investigated whether restoring OA in *Tβh*^{nM18} males would increase the frequency of lunges. This would strengthen the assumption, that it is indeed the lack of OA that elicits the low aggression phenotype. *Tβh*^{nM18} females are sterile and fecundity can be restored by feeding octopamine (Monastirioti et al., 1996; Monastirioti, 2003). Moreover, feeding OA successfully rescues a memory deficit of *Tβh*^{nM18} flies (Schwaerzel et al., 2003). In that study, OA should have crossed the insect blood-brain barrier, since it was supposed to have its effect in the mushroom body, a structure of the central brain. We provided 5mg/ml OA in normal fly food either throughout the whole life span or only during adult life. Neither treatment restored aggression in *Tβh*^{nM18} males compared to wild-type males (both $p < 0.0001$). The same feeding protocol, however, reverted female sterility independent of the onset of OA supplement ($p = 0.42$ and $p = 0.64$) indicating that OA was ingested and still active in the fly.

Tβh^{nM18} males carrying a wild-type *Tβh* cDNA downstream of the *hsp70* promoter (*hsp-Tβh*) were used to show that the *Tβh* locus is responsible for the behavioural changes measured here. The heat-shock protocol applied had already successfully been used to rescue the above mentioned memory deficit of *Tβh*^{nM18} flies (Schwaerzel et al., 2003). Heat-shock induced expression of *Tβh* in adult *Tβh*^{nM18} males restored aggression to a small, but significant extent compared to both males of the same genotype without heat-shock and to heat-shocked *Tβh*^{nM18} males lacking the *hsp-Tβh* construct (Figure 10E). 47% of all mutant *Tβh*^{nM18} pairs that temporarily expressed TβH in all cells showed at least one lunge, whereas only 14% and 9% of all pairs of the same genotype without heat-shock and of *Tβh*^{nM18} males lacking the *hsp-Tβh* construct showed at least one lunge, respectively. This result substantiates the role of octopamine in modulating *Drosophila* male aggression. Since this partial rescue was hidden in the noise of the software, clips were evaluated manually (see above).

To rescue fecundity in females, a slightly stronger heat-shock protocol was applied. It resulted in a percentage of *Tβh;; hsp-Tβh* egg-laying females indistinguishable from wild-type ($n = 19–24$; due to technical reasons, Fisher's exact test could not be applied; Figure 10G). The walking activity of *Tβh;; hsp-Tβh* males did not differ from *Tβh*^{nM18} males subjected to heat-shocks. However, walking activity differed between *Tβh;; hsp-Tβh* males with and without heat-shock treatment (Figure 10F).

The rather poor performance of *Tβh*^{nM18} males that temporarily expressed TβH in all cells might be due to the short time window in which TβH was expressed. In the light of immunohistochemical data indicating that there are neurons expressing tyramine but not

octopamine (Nagaya et al., 2002) misexpression of TβH, alternatively, might change tyraminergic into octopaminergic neurons, which might have deleterious effects on aggression.

3.7 OCTOPAMINE: *TDC2^{RO54}*

Since OA supplemented food did not rescue aggression in *Tβh^{nM18}* males, we next examined whether the increased TA rather than the lack of OA in *Tβh^{nM18}* males might have caused the aggression phenotype. To address this issue, we used mutants of the *tyrosine decarboxylase 2* (*Tdc2*) gene (*Tdc2^{RO54}*). Tyrosine decarboxylase 2 (TDC2) converts tyrosine to TA in neurons. HPLC measurements reveal no detectable levels of TA and OA in *Tdc2^{RO54}* mutant brains (Cole et al., 2005). We used males homozygous for a mutation in the nearby *cinnabar* gene (*cn^{1}*) as a control, because the *Tdc2^{RO54}* mutant also carried it. *Tdc2^{RO54}cn*^{1} males were strongly reduced in aggression compared to *Tdc2^{RO54}cn*^{1} heterozygote males and to *cn^{1}* males. Their lunge frequency was at about 5% of control levels (Figure 11A) using 'lunge count' software. In other words, *Tdc2^{RO54}* males display an aggression phenotype similar to *Tβh^{nM18}* males. This result strongly suggests that in *Tdc2^{RO54}* and *Tβh^{nM18}* males it is indeed the missing OA that causes the aggression phenotype. TA could only still be held responsible, if too little TA was as deleterious for aggression as too much.

Providing mutant *Tdc2^{RO54}* males with TA/OA supplemented food during adulthood again did not restore aggression (Figure 11A). The same feeding protocol, however, rescued *Tdc2^{RO54}* female sterility (Figure 11C). The applied protocol has been demonstrated to restore brain TA and OA levels of *Tdc2^{RO54}* mutant flies to wild-type levels (Hardie et al., 2007). Interestingly, the authors report that feeding only TA could not restore OA levels, “as if ectopically supplied amines were not transported into the appropriate neurons where the metabolic conversion could take place”.

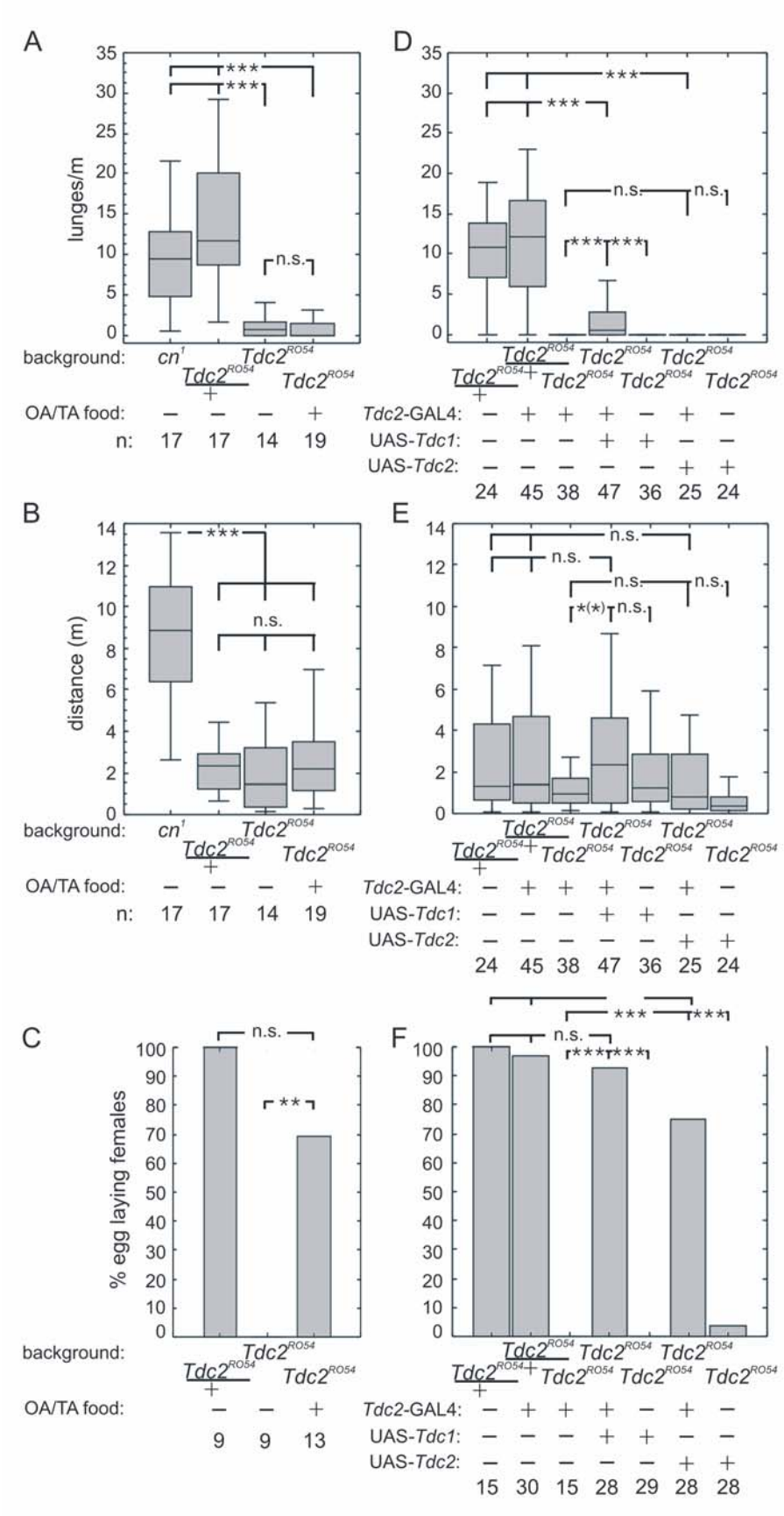


Figure 11: *Tdc2^{RO54}* mutant males lacking tyramine (TA) and octopamine (OA) performed hardly any lunges. This phenotype could be rescued by expressing UAS-*Tdc1* in octopaminergic and tyraminergic neurons. (A) *Tdc2^{RO54} cn^{1}* males lacking TA and OA were strongly reduced in

aggression compared to $Tdc2^{RO54}$ cn^1 heterozygote males and to cn^1 males (for both $p < 0.00003$). Feeding $Tdc2^{RO54}$ cn^1 flies TA/OA during the five days prior testing did not restore male aggression ($p = 0.46$). **(B)** Only cn^1 males differed in walking activity from $TDC2^{RO54}$ cn^1 heterozygote males and $Tdc2^{RO54}$ cn^1 homozygote males with and without OA/TA supplement ($p < 0.00001$ for all three genotypes). **(C)** Feeding $Tdc2^{RO54}$ cn^1 flies TA/OA rescued female sterility, when taking the percentage of egg-laying females as the indicator ($p = 0.12$). **(D)** Expressing UAS-*Tdc1* in octopaminergic and tyraminergic neurons of $Tdc2^{RO54}$ cn^1 males restored aggression partially compared to control flies without GAL4 ($Tdc2^{RO54}$ cn^1; UAS-*Tdc1*) ($p = 0.0002$) or UAS-*Tdc1* ($Tdc2^{RO54}$ cn^1 *Tdc2*-GAL4) ($p = 0.0008$). Expressing UAS-*Tdc2* in the same set of cells did not restore male aggression compared to the two controls ($p = 0.96$ and 0.84, respectively). In wild-type flies, *Tdc1* is expressed non-neuronally, whereas *Tdc2* is expressed neuronally. **(E)** Walking activity differed only for $Tdc2^{RO54}$ cn^1 *Tdc2*-GAL4; UAS-*Tdc1* males compared to $Tdc2^{RO54}$ cn^1 *Tdc2*-GAL4 ($p = 0.006$). **(F)** In $Tdc2^{RO54}$ cn^1 females, expressing UAS-*Tdc1* via *Tdc2*-GAL4 fully rescued female sterility compared to $Tdc2^{RO54}$ cn^1 heterozygotes ($p = 0.42$), whereas expressing UAS-*Tdc2* only partially rescued female sterility ($p = 0.04$).

To ensure restoration of OA and TA levels within neurons, UAS-*Tdc* was expressed in all tyraminergic and octopaminergic neurons using *Tdc2*-GAL4. There are two genes encoding for a TDC in flies: *Tdc1* is expressed non-neuronally, *Tdc2* in neurons only (Cole et al., 2005). Surprisingly, not *Tdc2* expression, but *Tdc1* expression in *Tdc2*-neurons yielded a small, but significant rescue of aggression compared with $Tdc2^{RO54}$ males carrying either only the *Tdc2*-GAL4 transgene or the UAS-*Tdc1* construct (Figure 11D). $Tdc2^{RO54}$, *Tdc2*-GAL4;UAS-*Tdc1* males lunged at a rate of 3% compared to the heterozygote controls, whereas $Tdc2^{RO54}$ males displayed very rarely a lunge. Evaluating lunges automatically, we had measured for $Tdc2^{RO54}$ males 5% of heterozygote control levels (see above) but re-evaluating the experiment semi-automatically using the 'lunge view' software we found 0%.

In general, the aggressive behaviour displayed was highly variable: two separately collected data-sets were pooled for Figure 11D-F: in one of the two experiments $Tdc2^{RO54}$, *Tdc2*-GAL4;UAS-*Tdc1* males were only significantly different to one control. In accordance with previous reports (Cole et al., 2005; Hardie et al., 2007) and with our findings on aggression, *Tdc1* expression seemed to be more potent in rescuing female sterility than *Tdc2* expression, the latter restoring female fecundity only partially (Figure 11F). Strikingly, HPLC analysis revealed that both OA and TA were higher in flies expressing UAS-*Tdc2* than in flies expressing UAS-*Tdc1*. In fact, no TA was detected in brains of $Tdc2^{RO54}$, *Tdc2*-GAL4;UAS-*Tdc1* flies, whereas TA was strongly elevated in brains of $Tdc2^{RO54}$, *Tdc2*-GAL4;UAS-*Tdc2* flies compared to wild-type flies (Cole et al., 2005). It appears as if *Drosophila* male aggression would be highly fine-tuned regarding the OA/TA systems, in other words, small deviations from wild-type levels would severely suppress aggression. Interestingly, the walking activity of $TDC2^{RO54}$ males in our recordings was unaltered compared to

heterozygous males (Figure 11B and E), which is in contrast to a pronounced locomotion defect detected for individual *Tdc2*^{RO54} males in a locomotor assay (Hardie et al., 2007).

3.8 Octopamine: UAS-*Kir 2.1* and UAS-*shi*^{TS1}

The finding that rescuing neuronal OA and TA only partially restored aggression points to OA/TA being required either outside neurons or neuronal OA/TA being required at a specific (1) concentration, (2) time point and (3) place to enable flies to express aggression. To test the importance of tyraminergic and octopaminergic neurons for the control of aggression, these neurons were selectively blocked. Inhibiting action potential generation via UAS-*Kir2.1* expression (Baines et al., 2001) in *Tdc2*-neurons mimicked the *Tβh*^{nM18} mutant phenotype. That is, *Tdc2*-GAL4/UAS-*Kir2.1* males showed a significant decrease in lunges/m compared to males both of the driver and the effector line, lunges occurring at a rate of about 22% of the controls (Figure 12A).

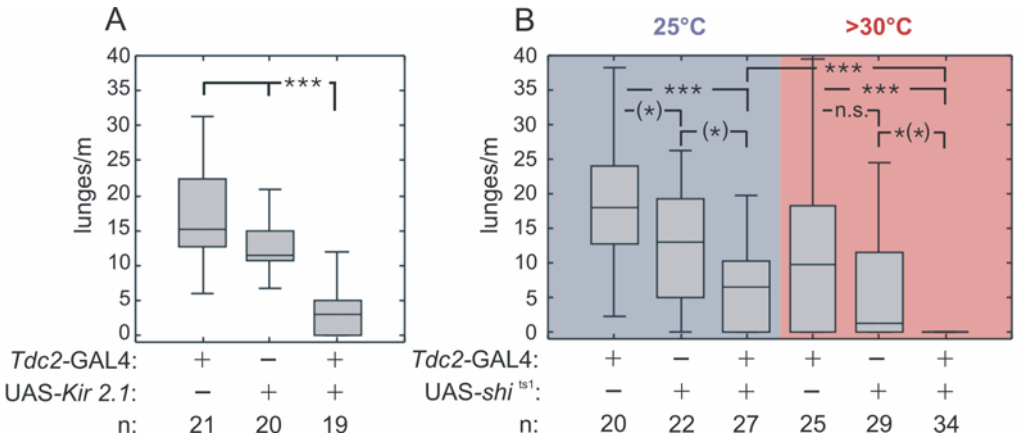


Figure 12: Inhibiting neuronal action potentials or synaptic transmission in both tyraminergic and octopaminergic neurons mimicked the TßhnM18 phenotype. (A) Males carrying both the *Tdc2*-GAL4 construct and the UAS-*Kir2.1* construct performed fewer lunges than males of the driver line (p = 0.000001) and males of the effector line (p = 0). **(B)** Males expressing UAS-*shi*^{ts1} in octopaminergic and tyraminergic neurons perform at the permissive (25°C) and the restrictive (>30°C) temperature fewer lunges than controls carrying only either GAL4 (*w*$^{+}$*; Tdc2*-GAL4) (25°C p = 0.00008; 30°C p = 0.000008) or UAS (*w*$^{+}$*;* UAS-*shi*^{ts1}) (25°C p = 0.032; 30°C p = 0.005). *w*$^{+}$*;Tdc2*-GAL4;UAS-*shi*^{ts1} males perform fewer lunges at the restrictive temperature than at the permissive temperature (p = 0.00001).

To restrict blockage of tyraminergic and octopaminergic neurons to a small time-window, we used the temperature-sensitive UAS-*shibire* transgene (Kitamoto, 2001, 2002) driven by *Tdc2*-GAL4. Blocking synaptic transmission only during the experimental period by raising the temperature to more than 30°C almost abolished aggression in *Tdc2*-GAL4/UAS-*shi*^{ts1} males compared to males of the same genotype fighting at the permissive temperature of 25°C (Figure 12B). However, using the UAS-*shi*^{ts1} transgene for studying

Drosophila aggression proved to be difficult due to a general trend of high temperature to reduce aggression. The general reduction in aggression due to high temperature made it difficult to detect differences between genotypes, especially when comparing UAS-*shi*^{ts1} males with *Tdc2*-GAL4/UAS-*shi*^{ts1} males at the high temperature ($p = 0.005$), which required a manual evaluation. The marginal decrease in aggression found for UAS-*shi*^{ts1} males at 25°C compared to *Tdc2*-GAL4 males ($p = 0.047$) is presumably due to a slightly higher walking activity of UAS-*shi*^{ts1} males, since the pure number of lunges was not affected ($p = 0.66$). Despite the problems with using the UAS-*shi*^{ts1} transgene, the results obtained with both UAS-*Kir2.1* and UAS-*shi*^{ts1} strengthen our hypothesis that octopaminergic neurons and potentially tyraminergic neurons are necessary for aggressive behaviour.

3.9 TEMPERATURE

In *Drosophila*, temporal control over onset/offset of genetic interventions can be achieved by applying temperature-sensitive tools among others. In the original GAL4/UAS system, the gene of choice (the effector) is expressed in a spatiotemporal pattern determined by the driver line, therefore often throughout the animal's whole life. The temperature sensitive GAL80 (GAL80^{ts}) enables the investigator to specify the time window of expression (= TARGET system; temporal and regional gene expression targeting (McGuire et al., 2003)). At 18°C, GAL80^{ts} protein represses GAL4 transcription and thus expression of the effector. At 30°C, however, GAL80 protein becomes inactive; consequently, the effector becomes expressed. An alternative method to restrict the time window of expression is the temperature-sensitive *shibire*-transgene. *Shibire* encodes a protein that is essential for endocytosis and recycling of synaptic vesicles. As a dominant negative mutant allele of the gene, *shi*^{ts} reversibly inhibits vesicle recycling at the non-permissive temperatures (34°C) and thus, suppresses synaptic transmission. At the permissive temperature of 25°C, it leaves synaptic transmission undisturbed. Both tools, however, require the behaviour under investigation to be fairly insensitive to alterations in temperature.

To test how elevated temperature affects *Drosophila* male aggression, CS fights were set up at 25°C and 31°C. Even though higher temperature did not affect aggression measured as lunges/m (Figure 13A), the behavioural pattern changed dramatically. At 31.5°C CS males performed only 30% of lunges performed by males of the same genotype at 25°C (Figure 13B). Simultaneously, walking activity was pronouncedly decreased by about 82% (Figure 13C).

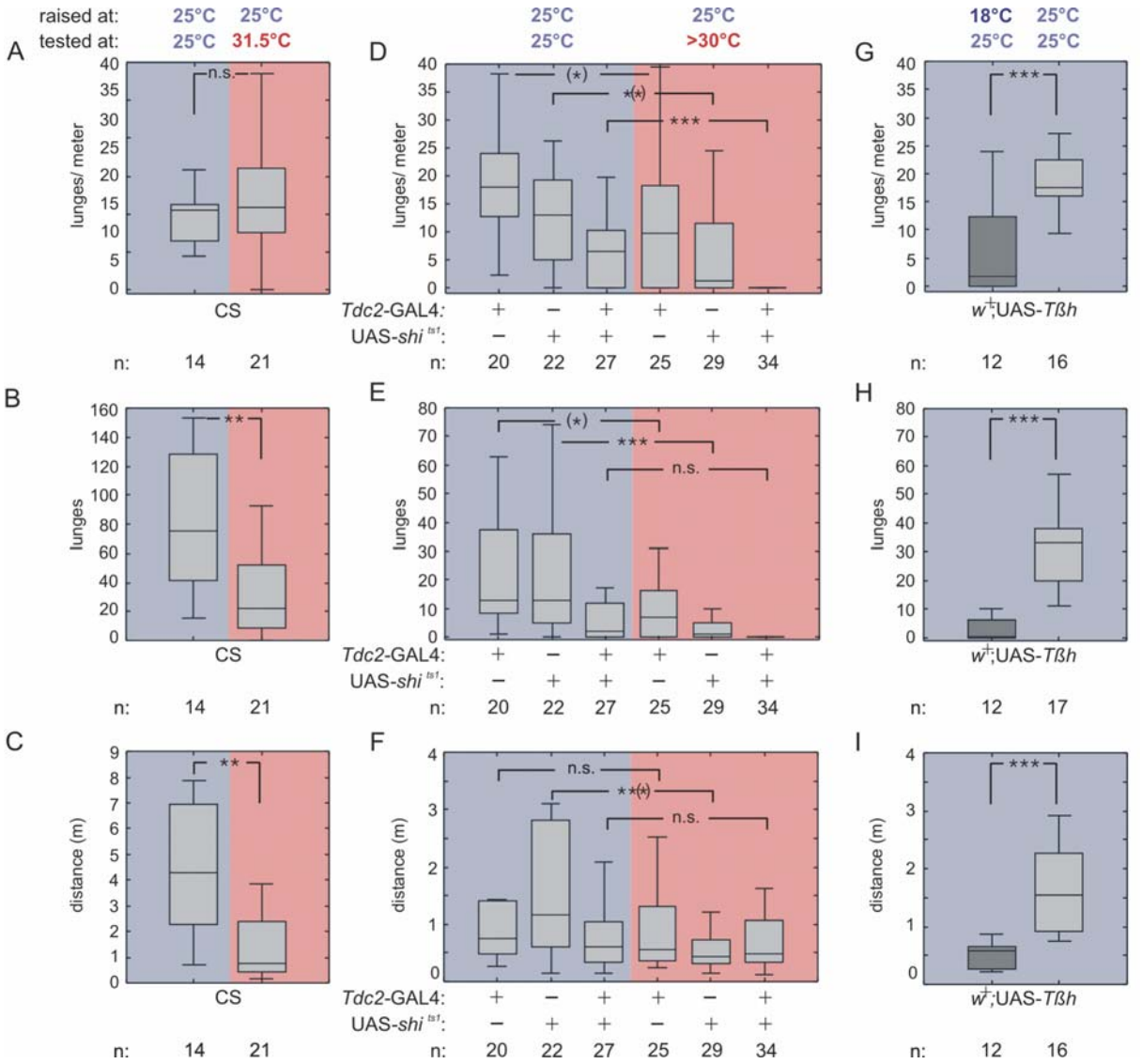


Figure 13: Male aggression is highly sensitive to the temperature during fights and to the temperature males were raised at. (A) Aggression of CS males was not affected by higher temperature when measured as lunges/m (p = 0.31) **(B)** However, lunge frequency was strongly decreased at 31.5°C compared to 25°C (p = 0.0063). **(C)** Simultaneously, walking activity was pronouncedly reduced (p = 0.0012). **(D)** Control males carrying either only the *Tdc2*-GAL4 construct or the UAS-*shi* ^{ts1} transgene performed less aggression at the high temperature when measured as lunges/m (p = 0.044 and p = 0.006). Please refer to Figure 12 for further information regarding the experimental group. **(E)** Similarly, lunge frequency was reduced in control males (w^+; *Tdc2*-GAL4 males: p = 0.016 and w^+; UAS-*shi* ^{ts1} males: p = 0.00016). This strong reduction observed for w^+; UAS-*shi* ^{ts1} males might be due to UAS-promoter leakiness. **(F)** Only in w^+; UAS-*shi* ^{ts1} males walking activity was decreased (p = 0.0007). **(G)** w^+; UAS-*Tβh* males perform significantly less lunges/m when raised at 18°C rather than at 25°C (p = 0.00047). **(H)** The same holds true for the lunge frequency (p = 0.0063). **(I)** Walking activity is also reduced (p = 0.0012).

In contrast to CS males, high temperature affected aggression measured as lunges/m in w^+; *Tdc2*-GAL4 and w^+; UAS-*shi*^{ts1} control males when testing for effects of blocking synaptic transmission in tyraminergic and octopaminergic neurons by expressing *shibire*^{ts1} (Figure 13D). Each genotype was tested at 25°C and at 33°C, 31.5°C, and 30.5°C. Temperature above 30°C affected aggression to the same extend, therefore data were pooled.

Even control males carrying only the *Tdc2*-GAL4 construct performed with 54% less aggression ($p = 0.044$) at the high temperature. This effect was more pronounced for w^+; UAS-*shi*^{ts} males ($p = 0.008$), which lunged only at a rate of 10% at the high temperature. This effect might be due to UAS-promoter leakiness, i.e. the expression of the effector even though a driver-line is lacking, a problem, which can be visualized when looking at reporter-lines, such as UAS-GFP (Ito et al., 2003). As shibire^{ts} functions optimally at temperatures above 29°C (Kitamoto, 2002), the paradigm for eliciting *Drosophila* male aggression needs further refinement, for example by reducing the floor size, for triggering aggression at a sufficiently high rate at higher temperatures.

Temperature can diminish the problem of UAS-promoter leakiness. Since the GAL4-system, and thus leakiness, is temperature-dependent (Duffy, 2002), a breeding temperature of 18°C might be favoured over 25°C. To test how reduced breeding temperature affects *Drosophila* male aggression, males carrying only the UAS-TβH transgene were raised at 18°C, but tested for their aggressiveness at 25°C. These males lunged profoundly less with 2% compared to males of the same genotype raised at 25°C (Figure 13H), an effect that was still apparent when looking at the lunges/m (9%) (Figure 13G). Thus it appears as if *Drosophila* male aggression is highly sensitive to both the temperature during fights and to the temperature that males were raised at. More studies are required to find the adequate temperature and maintenance conditions under which *Drosophila* males still reliably display aggression, but enable the investigator to apply temperature-sensitive tools and circumvent problems such as UAS-promoter leakiness.

3.10 MUSHROOM BODY

The mushroom bodies (MBs) are among the most striking neuropil structures in the insect brain, consisting of thousands of small and mostly parallel intrinsic neurons. The MBs are involved in olfactory discrimination learning, context generalization, and control of walking activity (reviewed in Heisenberg, 2003). Baier et al. (2002) report that males with blocked mushroom body output caused by spatially restricted expression of tetanus neurotoxin (TNT) display strongly reduced aggression compared to control males expressing an impotent form of TNT. TNT expression prevents evoked synaptic vesicle release by cleaving synaptobrevin (Sweeney et al., 1995). The interpretation of the result is hampered by the finding that males expressing the impotent form of TNT show elevated levels of aggression compared to wild-type males. Baier and colleagues suggest that this phenotype is a

side-effect of the P-element insertion site, since expressing the inactive form of TNT should have no effect on synaptic transmission.

In this study, MBs were deleted by feeding hydroxyurea (HU), having the advantage that the genetic background is almost identical between control and experimental group (both wild-type CantonS). Feeding HU to newly hatched larvae in a small time window selectively deletes those neuroblasts which give rise to the MBs, thus, resulting in an almost complete MB ablation with an exception of 200 Kenyon cells remaining (Debelle and Heisenberg, 1994). The almost complete ablation was individually assayed at the level of the light microscope by observing autofluorescence. In accordance with previous reports (Debelle and Heisenberg, 1994), size was not affected by HU treatment (Figure 14A). Therefore, fights were set-up between MB-ablated males and wild-type males. Males lacking the MB performed significantly less aggression than wild-type flies (Figure 14B and C), whereas walking activity was not affected (Figure 14D) as already shown in Buridan's paradigm (Debelle and Heisenberg, 1994). This is a further indication, that MBs play a role in the control of *Drosophila* male aggression.

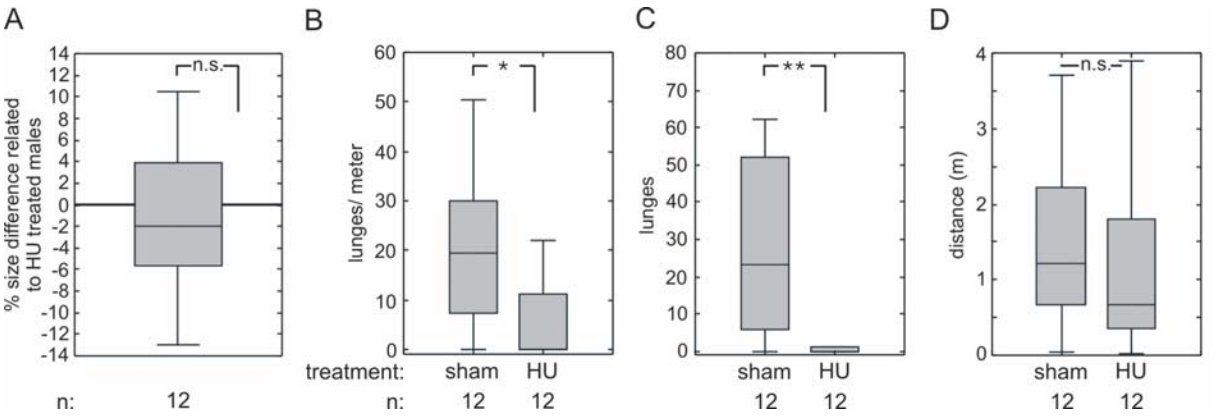


Figure 14: Males with impaired mushroom bodies display significantly less aggression than wild-type males when fighting against the latter. (A) Feeding HU to newly hatched larvae does not affect size of adult males (p = 0.52). **(B)** When fighting against wild-type males, males with only ~200 Kenyon cells perform significantly less lunges/m than sham-treated wild-type males (p = 0.027). For sham-treatment larvae were treated as for HU-treatment, however no HU was added to the yeast. **(C)** Also the lunge frequency is strongly reduced (p = 0.0058). **(D)** HU-treatment does not affect walking activity (p = 0.46).

4 DISCUSSION

4.1 OCTOPAMINE

As in other arthropod species, also in *Drosophila* octopamine (OA) is involved in modulating aggression. In fact, aggression is almost abolished in flies lacking OA as a result of blocking two enzymes (TβH and TDC2) required for OA synthesis one at a time. Restoring enzymatic activity in the $T\beta h^{nM18}$ mutant by expressing *Tβh* in all cells during adulthood or in the $Tdc2^{RO54}$ mutant by expressing *Tdc1* particularly in octopaminergic and tyraminergic neurons throughout the mutant's life, restored aggression partially. Consistently, blocking octopaminergic and tyraminergic neurons by inhibiting action potential formation or synaptic transmission strongly reduced aggression. Thus, several independent approaches point to OA playing an important role in modulating aggression.

The first indication that OA is involved in aggression control came from a study conducted by Baier et al. (2002). The authors report an overall strong reduction of various aggressive behavioural components for $T\beta h^{nM18}$ males compared to wild-type males when fighting against each other. Wild-type males, however, are on average 8% bigger than $T\beta h^{nM18}$ males, a body size difference sufficient to influence which fly is going to perform more lunges (Figure 8). That is, body size advantage of wild-type males over $T\beta h^{nM18}$ males could account for the differences in displayed aggression. Of even greater importance is the fact, that the $T\beta h^{nM18}$ males tested by Baier et al. (2002) carried in addition the w^{1118} mutation. The white-eyed mutant flies were tested against red-eyed control flies. I found that w^{1118} on its own almost completely abolishes *Drosophila* male aggression (Figure 9) – thereby being a phenocopy of the octopamine mutant. Thus, Baier and co-workers arrived at the right conclusion but had no proper controls.

In contrast to our findings, Certel et al. (2007) report no general reduction in lunge frequency displayed by $T\beta h^{nM18}$ males when fighting against each other (S. Certel, personal communication). This difference is not due to genetic background, as in our paradigm their $T\beta h^{nM18}$ males displayed aggression at a rate of only 17% of wild-type. Thus, the discrepancy in results is presumably due to either the set-up and/or data analysis. Whereas in my analysis almost every pair of flies is included, flies are only included in their analysis if entering the food patch, which is exclusively monitored by the camera. Under these conditions, in their set-up wild-type CS flies perform about 1 lunge/min (Y.-B. Chan, personal communication),

whereas in my set-up about 3.8 lunges/min are performed, presumably because escape attempts by the submissive animal are impeded due to the Fluon on the walls. To scale up overall displayed aggression might simplify detecting decreases in aggression. Certel et al. (2007) detected differences only in a well-defined, rare situation, in which one male approaches another male by vibrating its wing(s). Here, $T\beta h^{nM18}$ males transitioned less often to aggressive behaviours than wild-type males, instead they switched more frequently to courtship components. Whether $T\beta h^{nM18}$ males also perform increased courtship in my set-up needs further investigation, as courtship was not quantified. Preliminary observation confirmed that in some pairs of $T\beta h^{nM18}$ males courtship was remarkably frequent.

As in $T\beta h^{nM18}$ flies not only OA levels, but also tyramine (TA) levels are affected – the latter being about 10fold elevated (Monastirioti et al., 1996) – , the decreased aggression displayed by $T\beta h^{nM18}$ males could be due to higher TA levels. As discussed in the Introduction, TA is not merely the precursor of octopamine, but also a signalling molecule. However, $Tdc2^{RO54}$ males lacking OA and TA showed a significant decrease in aggression, thereby strongly suggesting that the lack of OA leads to reduced aggression. Otherwise, a dose-dependent biphasic effect of TA had to be postulated, that is, too little ($Tdc2^{RO54}$) and too much ($T\beta h^{nM18}$) of TA had to reduce aggression. This hypothesis is highly unlikely, in particular, if the site of action is considered. A dose-dependent biphasic effect of TA should be located in neurons using tyramine as the signalling molecule, therefore being presumably devoid of octopamine (Nagaya et al., 2002). However, the elevated TA levels detected in $T\beta h^{nM18}$ flies should be the result of higher TA levels in octopaminergic neurons, not in tyraminergic neurons, as TA should accumulate exclusively in neurons lacking TβH.

The question arises how OA exerts its action on aggression, so that lack of octopaminergic signalling reduces aggressive display in such a dramatic way. Is it an essential neurotransmitter required in the innumerable neuronal processes taking place before the investigator can observe the according motor output, the lunge? Is it involved in modulating specific neuronal circuits, e.g. input from sensory systems? Or does it influence aggressiveness, i.e. the underlying motivation to become aggressive - leading to the question, what motivation is. There are many models of motivation. Motivation can be thought of as being triggered by the organism's internal state departing from an optimum. Internal changes, e.g. lack of oxygen, interact with external stimuli thereby potentially leading to compensatory behaviour (overview of models of motivation given in Barnard, 2004). Biogenic amines are suggested to promote the occurrence of compensatory behaviour rather than being involved in its production and execution (Huber, 2005). Unfortunately, it proves to be extremely difficult

to detangle whether octopamine promotes the occurrence of aggressive behaviours or whether it produces the behaviour *per se*. A first hint might give the location where OA exerts its action on aggression.

So far it can only be excluded that OA alters male cuticular hydrocarbons, particularly 7-tricosene (Certel et al., 2007), which suggests that a male still elicits pheromonal signals informing the opponent truthfully about its gender. Furthermore, as demonstrated in this study, lunge execution, i.e. the motor output, is only minimally affected by lack of octopamine.

Rescue experiments can help to unravel where OA is influencing aggressive display. Aggression was partially restored by expressing either *Tβh* in all cells of adult $T\beta h^{nM18}$ males or *Tdc1* in octopaminergic and tyraminergic neurons of $Tdc2^{RO54}$ mutant flies (Figures 10E and 11D). Hence, these two rescues confirm that the decrease in aggression observed for $T\beta h^{nM18}$ males and $Tdc2^{RO54}$ males is not due to a second-site mutation, but to the lack of OA. Concerning the site(s) where OA exerts its action on aggression, the experiments suggest that it is located within the ~140 neurons targeted by *Tdc2*-GAL4 (S. Busch, personal communication). As aggression was only partially rescued and rescued mutants performed still poorly even though the wild-type enzyme was expressed in a large set of neurons/in all cells, I refrained from pursuing rescue experiments by refining the set of neurons expressing the wild-type enzyme. It appears as if aggression is highly fine-tuned to alterations in octopaminergic signalling, since the applied tools are fully functional as proven by successfully rescuing other deficits triggered by lack of OA or TA, such as deficits in egg laying, memory and cocaine sensitivity (Monastirioti, 2003; Schwaerzel et al., 2003; Cole et al., 2005; Hardie et al., 2007). One could imagine that OA modifies neural functions in various processes. If each process requires a distinct range of OA concentration to support aggressive display, it is easy to imagine that the presumably rather crude increase of OA levels due to expressing transgenes rather globally (throughout development) led only to a partial rescue of lunge performance.

Suppressing activity of refined sets of neurons should be therefore the easier approach to locate the octopaminergic cells mediating the strong reduction in aggression. Indeed, inhibiting action potential formation or synaptic transmission in octopaminergic and tyraminergic neurons strongly reduced aggression (Figure 12). Unfortunately, there is so far no driver-line available specific for octopaminergic neurons, not to mention a driver-line targeting only specific subsets of them.

Not only the region of action but also its timing can indicate how OA exerts its action on aggression. Does it have an organizational effect during development that later enables the adult fly to express aggression? Or is there an activational effect of OA in the adult, as required if OA were to modulate underlying aggressiveness of a fly? With the data at hand, this question can not be fully answered. Expressing TβH in $T\beta h^{nM18}$ males shortly before the fight partially restored aggression (Figure 10E). This hints to OA having an activational effect on *Drosophila* male aggression. Pointing in the same direction, inhibiting synaptic transmission in octopaminergic and tyraminergic neurons exclusively during the fight appears to suppress aggression. It would be of great value for future studies to gain a better control over the dynamics of the effector gene to distinguish between potentially organizational and activational effects on *Drosophila* male aggression. Studies using temperature-sensitive tools to gain temporal control are hampered by the pronounced decrease on lunge frequency and general activity observed at temperatures departing from the optimum of 25°C.

To sum up, data collected in this thesis demonstrate that OA is involved in modulating *Drosophila* aggression, potentially having an activational effect on it. Presumably due to aggression being extremely sensitive to deviations from the wild-type OA pattern – with respect to space, time and concentration – attempts to localize the specific site and timing of OA's action on aggressive display have been hampered.

4.2 AUTOMATED RECORDING OF LUNGES

All experiments conducted in this thesis underlie the assumption that lunges reflect the overall aggressiveness of *Drosophila* males. The lunge was chosen as the single indicator of aggression for three reasons: (1) lunges are unique to aggression; (2) they are the most frequent behaviour by which an opponent is displaced from the food patch (Hoffmann, 1987a); and (3) their distinct characteristics lend themselves to an automated registration. However, focusing on only a single indicator of aggression regards aggression as a unitary phenomenon, where different components are modulated by identical mechanisms and can be therefore fully represented by one behavioural component. This is unlikely to be true. For example, low-intensity aggressive components, such as fencing, might be controlled separately. To answer this question it would be worthwhile to know the neuronal underpinnings of at least one component, e.g. the lunge, from which one can then ask whether other components are regulated the same way.

I did not try to bring the lunge detection to perfection. The detection could have been refined by increasing resolution of time and space or by adding a 2nd camera including the lateral view. However, my aim was to develop a paradigm, which is easy (and rather cheap) to install in a lab and which can be used without much prior experience. Also, the paradigm had to enable the investigator to collect huge data sets, since every fight was recorded for 30 min. Therefore, I accepted an error rate of 11%, which is mainly due to undetected lunges (tight exclusion criteria). This error rate turned out to be problematic, when false positives confounded detection of subtle mutant rescue effects. Therefore, the 'lunge view' software was added. It enables the investigator to first loosen the criteria for detecting lunges and to then eliminate all false positives. The other situation, where the 'lunge view' software might be helpful to increase accuracy of lunge detection, is tussling, a mixture of boxing and lunging. Here, lunges were less precisely detected. This problem could be ignored in this study, as all genotypes under investigation tussled very rarely during the observation time.

The main advantage of an automated analysis of lunges is the potential to deal with huge data-sets. To fully analyse a clip, i.e. regarding the number of lunges, walking activity, the fly's body size etc., the investigator needs to spend only 2-3 min. Except for the very low end of the scale, the error rate is independent of lunge frequency. In addition, it is largely independent of the genotypes used in this study.

4.3 WALKING ACTIVITY

Regarding a pair of *Drosophila* males, there is a strong correlation of their lunge frequency and their walking activity, which is intuitively plausible, as flies have to first encounter each other before displaying aggressive behaviour. The encounter frequency is naturally dependent on the walking activity. The question arises which comes first: does increased walking activity lead to higher lunge frequency or the other way around, does increased aggression lead to higher walking activity, potentially by altering arousal. Also, is the higher walking activity due to behaviour of the attacking fly or due to behaviour of the attacked fly? If escape is denied, rats 'freeze' if they are faced with a threatening stimulus, such as a predator or a territory holder (Blanchard and Blanchard, 1989). Likewise, preliminary observations suggest that in *Drosophila* standing still could help the subordinate male to avoid further harassment by the dominant male, as moving seems to trigger aggression. Obviously, this hypothesis needs to be tested.

I normalized lunge frequency by walking activity for two reasons: (1) to reduce the pronounced variance in displayed aggression and (2) to prevent that in mutant studies alterations in lunge frequency are wrongly interpreted as changes in aggressiveness. (e.g. alterations in lunge frequency might actually be side-effects of differences in walking activity in cn^1 flies.)

4.4 BODY SIZE

In *Drosophila*, as in many other species, larger (heavier) males win more aggressive encounters than their smaller (lighter) opponent as observed in the field (Partridge et al., 1987; Shelly, 1987; Bell and Kipp, 1994) and under laboratory conditions (Partridge and Farquhar, 1983; Hoffmann, 1987b; Hoffmann, 1987a). The effect was most obvious when the weight differences between the opponents were pronounced (50%) due to maintenance conditions (Hoffmann, 1987b). In this thesis, I show that body size differences of 8% and higher influence which fly is going to perform more lunges and as a consequence, which fly is excluded from the resource (the food patch) (Figure 8). This finding has strong implications for studies on *Drosophila* aggression, as 8% size differences are not detectable by the human eye. Therefore, in order to avoid the effects of size, one should observe fights between two males of identical genotype/treatment, if feasible. For example, Yurkovic et al. (2006) report that former losers did not win second fights against former winners interpreting the outcome as indication of a loser effect in *Drosophila* males. One may hypothesize that during the first fights bigger flies and smaller flies become winners and losers, respectively. If then a former winner is paired with a former loser, there is an increased likelihood that the former winner is bigger than the former loser, therefore the latter is prone to lose the fight again. Interestingly, if two former losers are paired, they establish a winner/loser hierarchy rather than ‘drawing’. Thus, to explain this experiment reported by Yurkovic and colleagues (2006), it would not be necessary to postulate a loser effect, a pronounced body size effect as demonstrated in this study could be sufficient. By further tightening the criteria for an animal to be defined as a winner, the same authors show that fewer loser/loser pairs formed hierarchical relationships than winner/winner pairs. As in this experiment two animals of identical ‘treatment’ were paired, the outcome should be unaffected by size differences.

How is the size difference perceived? Do flies have an internal representation of how big they are and compare this mirror image to their opponent’s? More likely, do they perceive

the opponent's size (weight) relative to their own by pushing each other as observed during fencing? How *Drosophila* males detect size differences is yet unknown.

In territorial insects, body size strongly influences fighting ability, the so-called resource holding power (RHP) (Baker, 1983). Game theory predicts that if animals are able to assess each other's RHP, fights should only escalate if disparity in RHP is small. Otherwise, the animal with the smaller RHP should retreat before escalation (Parker, 1974; Smith and Parker, 1976). Focusing on the 15th to the 30th minute of a fight, one of the two males involved in a contest performed less than five lunges in 75% of all CS fights (n = 191). I defined all fights, in which both males performed at least five lunges as 'escalated', without knowing in which context these lunges occurred (tussling or 'pure' lunges). In accordance with the predictions by game theory, 'escalated' fights occurred significantly more often in pairs with size differences of less than 4% (33 out of 99) than in pairs with size differences of more than 8% (18 out of 115) ($p = 0.0036$; three different genotypes pooled). When interpreting this result one has to keep in mind that at the time of measurement the experiment was already ongoing for 15 min. *Drosophila* males establish hierarchical relationships within the first minutes after encountering each other (Nilsen et al., 2004). Hence, it is likely, that already during the first 15 minutes the majority of mutual lunges occurred. In addition, after 15 min flies presumably differed not only in body size but also in being the resource holder or not, which is predicted to also shape the course of a fight (Parker, 1974). Interestingly, it appears that even though a hierarchical relationship is formed, subordinate *Drosophila* males tend to come frequently back to the food patch. As *Drosophila* presumably cannot directly hurt each other, the main costs of a fight may be time and energy expenditures.

4.5 Locations involved in modulating aggression

As pointed out earlier, neurons included in the *Tdc2*-GAL4 driver line are highly likely to mediate OA's effect on aggression, even though their location can be so far not further specified. Moreover, studies investigating the effect of white on *Drosophila* male aggression demonstrate that a functional peripheral visual system is required for wild-type aggression. The white null mutation w^{1118} , which impairs contrast perception (Hengstenberg and Gotz, 1967; Wehner et al., 1969), almost abolished aggression, an effect that could be mimicked by specifically knocking-down *white* in the eye.

Notably, knocking-down *white* using UAS-RNAi-*white* in cells within the central nervous system by using the following six driver-lines decreased aggression to varying

degrees: (1) *Tdc2*-GAL4 (tyraminergic and octopaminergic neurons), (2) *Ddc*-GAL4 (serotonergic and dopaminergic neurons), (3) *TH*-GAL4 (dopaminergic neurons), (4) NP 6510-GAL4 (cells of fan-shaped body), (5) NP 6561-GAL4 (cells of fan-shaped body), and (6) MB247-GAL4 (cells of mushroom bodies). Consistently, males, in which the *white* gene was restored exclusively in the eye, showed only a partial rescue of aggression. Also, w^{1118} null mutant males tested in complete darkness have altered memory compared to wild-type flies in the heat box learning paradigm (Diegelmann et al., 2006). Strikingly, there is almost no evidence of *white* being located in the central nervous system. Immunostainings using White antibody detected White only in pigment cells of the retina and subretinal glia, but not in the central brain (I. Meinertzhagen, personal communication). Possibly, compared to the strong expression in the visual system the faint staining in the central brain might appear as mere background.

The presumably rather faint expression of *white* in the central nervous system stands in contrast to the strong effects caused by knocking-down *white* in small subsets of central brain neurons (e.g. NP6510-GAL4 and NP6561-GAL4). The question arises, whether there are off-target effects of the UAS-RNAi-*white* construct. White is one of the 15 ABCG-transporters in *Drosophila* (Dean et al., 2001). As ABC transporters are required for transporting various molecules across membranes, off-target effects interfering with other ABC transporters might be deleterious for the respective neurons and thus for aggressive display. Independent of the specific mechanism of action, the observed alterations in aggressive display due to UAS-RNAi-*white* expression mentioned above indicate that all of the targeted cell types might indeed modulate aggressive behaviour.

It is surprising that expressing UAS-RNAi-*white* in only a few neurons located in the horizontal stratum near the lower margin of the fan-shaped body (NP 6510-GAL4 and NP6561-GAL4) (Liu et al., 2006) almost completely abolished aggression (Figure 9). The fan-shaped body is part of the central complex, which is an unpaired, highly structured neuropil region residing in the centre of the protocerebrum. The central complex is involved in the regulation of various aspects of locomotor behaviour, such as locomotor activity, step length and compensations of site-asymmetries interfering with normal walking (Strauss, 2002). It could therefore be involved in modulating aggression indirectly by altering walking activity. However, in none of the males expressing UAS-RNAi-*white* walking activity differed from wild-type males ($p = 0.39$). The few neurons targeted by NP6510-GAL4 and NP6561-GAL4 were shown to store the memory trace for ‘contour orientation’ (Liu et al., 2006), a skill, where flies learn to differentiate between two patterns, such as / and \. In

addition to its role in 'contour orientation' memory, these neurons might have other roles which are of importance for *Drosophila* aggression. Alternatively, the expression pattern of the two GAL4-lines might be more widespread during development and the decrease in aggression is in fact due to defects acquired during the development and not during adulthood.

This might also be the case for MB247-GAL4. MB247-GAL4 is reported to be expressed in the alpha/beta and gamma lobes of the mushroom bodies (MBs) (Thum et al., 2006) and is, according to Ito and colleagues (2003), the most specific MB driver strain identified so far. However, MB247-GAL4/UAS-RNAi-white males have an eye colour distinct from wild-type males. This change in eye pigmentation indicates that MB247-GAL4 also targets cells outside the mushroom body during development, thereby affecting eye pigmentation; an effect which lasts throughout the animal's life, even though the GAL4 expression pattern becomes more refined after eclosion. Thus, one cannot fully exclude that the decreased aggression observed for MB247-GAL4/UAS-RNAi-*white* males is rather mediated by changes in eye pigmentation, than by affecting processes in the mushroom body. However, there is strong indication that MBs are involved in aggression control: Males with ablated MBs due to chemical intervention performed significantly less lunges than wild-type males.

These findings suggest that various neurons within the central brain are involved in aggression control. This is not surprising, as complex behaviours such as aggression rely on the functionality of many neural circuits, among them being all sensory systems involved in perceiving the opponent and neural assemblies executing the behaviour *per se*. It becomes apparent that more refined tools concerning dose and dynamics are needed to shed further light on the specific contribution of certain neurons and neuromodulators to the control of such a fascinating behaviour as *Drosophila* aggression.

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6 SUMMARY

Aggression is a strikingly multi-faceted phenomenon occurring in vertebrates as well as in invertebrates. Despite its omnipresence, the neuronal basis of aggressive behaviours is yet barely understood. Many studies however, imply a role for biogenic amines in aggression. This PhD project aimed at contributing to the understanding of the neuronal correlates of aggression, with a main focus on the biogenic amine octopamine, using *Drosophila melanogaster* as the model system.

In *Drosophila*, agonistic encounters of males and females are composed of a variety of both offensive and defensive components, some of which are displayed more often in one sex than in the other. To simplify analysis and to standardize evaluation, I chose to focus on a single indicator of aggression: the lunge, a striking feature unique to *Drosophila* male aggression. By evaluating the lunge I developed in cooperation with Andreas Eckart for the first time an automated, video-based analysis of *Drosophila* male aggression. The present software program gives the number of lunges for each fly in a certain time interval. In addition, it provides information such as the distance the fly walked and his size among others. In combination with a second software program that we developed, aggressive interactions between two male *Drosophila melanogaster* of a genotype of choice can now be registered either completely automatically or if preferred semi-automatically.

Using these softwares, I demonstrate that (1) body size differences of 8% and higher influence the outcome of a fight in favour of the larger male; (2) walking activity alters lunge frequency with more lunges performed by more active pairs of males; (3) flies mutant for the *white* gene, one member of the ABC transporter family in *Drosophila*, are profoundly impaired in aggression, an effect that is partially due to reduced visual performance. (4) Either knocking-down *white* in various brain regions or chemically ablating the mushroom body located in the central brain by deleting its neuroblast precursors diminishes aggression, indicating that integrity of various neural circuits/brain regions is required for wild-type aggression to occur. Furthermore, I show that (5) flies lacking octopamine signalling but having altered tyramine signalling display hardly any lunge. A quantitative high-speed analysis revealed that lunge execution is almost indistinguishable from wild-type males. The results from the experiments in which octopamine levels and/or tyramine levels were restored suggest that an elaborate pattern of octopamine levels in time and space is required to enable flies to express wild-type aggressive behaviour.

7 ZUSAMMENFASSUNG

Aggression ist ein facettenreiches Phänomen, das sowohl in Vertebraten als auch in Invertebraten auftritt. Trotz der weiten Verbreitung dieses Verhaltens sind die neuronalen Netzwerke, die der Aggression zugrunde liegen, noch kaum bekannt. Zahlreiche Studien weisen den biogenen Aminen eine prominente Rolle in der Modulation von Aggression zu. Das Ziel dieser Doktorarbeit war mit Hilfe des Modellorganismus *Drosophila melanogaster* zu der Aufschlüsselung der neuronalen Korrelate von Aggression beizutragen, insbesondere im Hinblick auf das biogene Amin Oktopamin.

In *Drosophila* sind aggressive Interaktionen aus einer Vielzahl von offensiven und defensiven Verhaltensweisen zusammengesetzt, von denen einige bezüglich der Häufigkeit ihres Auftretens geschlechtsspezifisch sind. Um die Auswertung dieser vielseitigen Verhaltensweisen zu vereinfachen, wurde die Analyse auf einen einzigen Indikator für Aggression beschränkt: den „lunge“. Diese bemerkenswerte Verhaltensweise tritt nur im Kontext der Aggression auf und ist charakteristisch für Männchen. In Kooperation mit Andreas Eckart habe ich ein Computerprogramm entwickelt, das eine automatische Auszählung der lunges in einem vom Forscher gewählten Zeitraum durchführt. Zusätzlich erhält man u.a. Informationen über die Laufstrecke der einzelnen Tiere wie auch über ihre Größe. Dank eines weiteren von uns entwickelten Programms ist es möglich, Kämpfe zweier *Drosophila* Männchen unabhängig von deren Genotyp wahlweise automatisch oder halb-automatisch auszuwerten.

Mit Hilfe dieser Programme wurde gezeigt, dass (1) die gemeinsame Laufaktivität der beiden Männchen mit der Anzahl aller aufgetretenen lunges korreliert und, dass (2) ein Größenunterschied von 8% ausreichend ist, um zu beeinflussen, welches Tier mehr lunges durchführt. Ebenfalls konnte festgestellt werden, dass (3) eine Nullmutation im ‚*white*' Gen, welches einen ABC-Transporter kodiert, aggressives Verhalten fast vollständig unterdrückt, was teilweise auf eine visuelle Beeinträchtigung zurückzuführen ist. Außerdem führt (4) das Absenken des White-Levels in verschiedenen Bereichen des Zentralgehirns zu reduzierter Aggression; ein Effekt, der auch durch die chemische Entfernung der Pilzkörper, einer Struktur des zentralen Gehirns, hervorgerufen werden kann. Dies weist darauf hin, dass die Integrität verschiedener neuronaler Netzwerke/Gehirnbereiche erforderlich ist, um wildtypische Aggression zu ermöglichen. Zusätzlich konnte (5) anhand von Mutationen in zwei Genen der Oktopaminsynthese, die beide die Oktopamin-Konzentration zwar

erniedrigen, die Tyramin-Konzentration jedoch heben bzw. senken, demonstriert werden, dass Oktopaminmangel Aggression fast vollständig zum Erliegen bringt. Wird ein lunge durchgeführt, so ist dessen Ausführung fast wildtypisch. Rettungsversuche, in denen Oktopamin- und/oder Tyramin-Konzentrationen wiederhergestellt werden, legen nahe, dass ein sehr spezifisches Muster von Oktopamin räumlich und zeitlich gewährleistet sein muss, um ein so komplexes und faszinierendes Verhalten wie die Aggression in *Drosophila* hervorzurufen.

8 APPENDIX

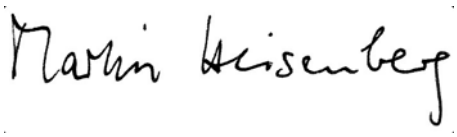
8.1 ERKLÄRUNG

Erklärung gemäß § 4 Absatz 3 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universtität zu Würzburg vom 15. März 1999:

Hiermit erkläre ich, die vorgelegte Dissertation selbständig angefertigt zu haben und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Die mit meiner Publikation wortgleichen oder nahezu wortgleichen Textpassagen habe ich selbst verfasst. Alle aus der Literatur entnommenen Stellen sind als solche kenntlich gemacht.

Des Weiteren erkläre ich, dass die vorliegende Arbeit weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat. Ich habe zuvor keinen Versuch unternommen, den akademischen Grad eines Doktors der Naturwissenschaften zu erwerben.

Würzburg, den 20. Oktober 2007



Susanne Hoyer Prof. Martin Heisenberg

8.2 CURRICULUM VITAE

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PERSONAL DETAILS

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SCIENTIFIC EDUCATION

1997 – 1999	Undergraduate studies in biology; University of Muenster, Germany
09/1999	Intermediate examination in biology (Vordiplom); University of Muenster
1999 – 2003	Graduate studies in biology; University of Wuerzburg, Germany
08/2000 – 05/2001	Studies in biology; Northern Arizona University, Flagstaff, AZ
05/2002 – 05/2003	Diploma thesis: 'Brain plasticity, biogenic amines, and aggression in the ponerine ant *Harpegnathos saltator*'
05/2003	Final examination in biology (Diplom); University of Wuerzburg
since 09/2003	Doctoral student in the group of Prof. Dr. Martin Heisenberg, Department of Neurobiology and Genetics, University of Wuerzburg. Thesis topic: Neuronal correlates of aggression in *Drosophila melanogaster*

SCHOOL EDUCATION

1988 - 1997	Immanuel-Kant-Gymnasium Muenster-Hiltrup; Abitur

Additional Courses

2003	Cognitive neurosciences – models and mechanisms of higher brain functions (summer academy of the German National Merit Foundation)
2004	Neurobiology of *Drosophila* (Cold Spring Harbor Laboratory course)
2006	New aspects of stem cell biology and therapy (summer academy of the German National Merit Foundation)

Collaborative studies Abroad

03/1999 – 04/1999	Internship at the ‚Iguana research and breeding station' (supported by the ‚Zoologische Gesellschaft Frankfurt' and the ‚Senckenbergische Naturforschenden Gesellschaft')
08/2000 – 05/2001	Internship at the Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ, USA
06/2001 – 09/2001	Internship at the Arizona Research Laboratories Division of Neurobiology, University of Arizona, Tucson, AZ, USA
09/2003 – 01/2004	Visiting graduate student in the lab of Prof. Dr. E.A. Kravitz , Department of Neurobiology, Harvard Medical School, Boston

Scholarship / Fellowship

02/2000 – 03/2003	Scholarship of the German National Merit Foundation
08/2000 – 05/2001	Scholarship of the ‚Federation of German-American Clubs e.V.' for studies at the Northern Arizona University, Flagstaff, AZ
08/2000 – 09/2001	Fulbright travel grant
11/2001 – present	e-fellows.net
09/2003 – 12/2003	DAAD ‚Kurzstipendium' for the stay abroad at the Harvard Medical School
01/2004 – 08/2006	Scholarship of the Boehringer Ingelheim Fonds
03/2004 – 03/2007	Scholarship of the German National Merit Foundation

Wuerzburg, 20.10.2007

8.3 PUBLICATIONS AND TALKS

8.3.1 Publications

1. **S.C. Hoyer**, A. Eckart, A. Herrel, T. Zars, S. Fischer, M. Heisenberg. Octopamine in Male Aggression of *Drosophila* (submitted).
2. **S.C. Hoyer**, J. Liebig, W. Rössler, 2005. Biogenic amines in the ponerine ant *Harpegnathos saltator*: serotonin and dopamine immunoreactivity in the brain. Arthropod Structure & Development 34, 429 – 440

8.3.2 Talks

1. **S.C. Hoyer**, A. Eckart, A. Herrel, M. Heisenberg, 2007. The neuronal underpinnings of aggression: ABC-transporters and octopamine. Neurobiology of *Drosophila* Conference, Cold Spring Harbor, USA.
2. **S.C. Hoyer**, A. Eckart, A. Herrel, M. Heisenberg, 2006. Automated analysis of aggressive behaviour in *Drosophila melanogaster*. 11th European *Drosophila* Neurobiology Conference, Leuven, Belgium.

8.4 DANKSAGUNG

Ich möchte mich bei Herrn Prof. Martin Heisenberg für seine langjährige Unterstützung und Förderung bedanken, die bereits mein Studium begleitet hat. Während meiner Doktorarbeit hat mir Herr Heisenberg jederzeit Unterstützung angeboten und gleichzeitig eine enorme Freiheit gelassen; eine Kombination, die mir sehr dabei geholfen hat, viele Facetten der Wissenschaft kennen zu lernen.

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Meinen Freundinnen möchte ich danken, dass sie akzeptiert haben, dass die Frequenz der Telefonanrufe während meiner Doktorarbeit signifikant abgenommen hat und ich trotzdem ständig Gehör bei ihnen gefunden habe.

Ohne die finanzielle Unterstützung des Boehringer Ingelheim Fonds wäre diese Doktorarbeit nicht möglich gewesen. Auch die Menschen, die ich dank des Boehringer Ingelheim Fonds kennen gelernt habe, haben mein Leben stark beeinflusst. Der Studienstiftung des deutschen Volkes danke ich für ihre ideelle Unterstützung.

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Meine Eltern und Geschwister waren immer für mich da. Danke.