Characterization of memories and *ignorant (S6KII)* mutants in operant conditioning in the heat-box

Dissertation zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

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aus Wertheim

Würzburg 2002

Eingereicht am: ....16. August 2002

Mitglieder der Promotionskommission:

Vorsitzender: ....Prof. Dr. R. Hedrich

Gutachter: Prof. Dr. Martin Heisenberg

Gutachter: Prof. Dr. Georg Krohne

Tag des Promotionskolloquiums: ....18. Dezember 2002

Doktorurkunde ausgehändigt am: ....

Dankeschön!

Allen voran danke ich **Prof. Dr. Martin Heisenberg** für meinen Arbeitsplatz, den monatlichen Geldsegen und die Chance aus fremden Gefilden in die Genetik eintauchen zu dürfen. Ich bin sehr froh in die ‚Heisenberg´sche Schule' gegangen zu sein und einen guten Ratgeber an der Seite gehabt zu haben. Daneben gibt es einige weitere Menschen, die mir mit Rat und Tat zur Seite standen...

...Sören, der mich in die Welt der Naßbiologie eingeführt hat und mit mir durch Walzer und Tango geht. ...**Martin und Sebastian**, die eine einmalige Atmosphäre im Naßlabor geschaffen haben und keinerlei Respekt davor hatten, einer Frau Jahre ihres Lebens zu rauben. ...**Troy Zars**, ein weiterer heat-box Anhänger, der mich bei der Planung meiner Versuche unterstützt hat und dem ich für die Korrektur meiner Arbeit danke. ...**Björn**, ein ehrlicher, einmaliger und erstklassiger Freund, der noch nächtelange Diskussionen jeglicher Art zu schätzen weiß, und den ich niemals eintauschen möchte. ...**Bertram Gerber**, der mein Manuskript mit emsiger Mühe und scharfem Verstand unter die Lupe genommen hat. ...**Eva Dierichs-Schmitt** mit der ich neue molekulare Techniken erobert habe und ohne die ich heute noch vor dem rohen Spargel stünde. ...**Sonja Flurschütz-Twardzik**, die die lohnenswerten Mühen des Screens mitgetragen hat. ... **Reinhard Wolf, Konrad Öchsner, und Hans Kaderschabek**, ohne die es die Hitzekammer erst gar nicht gäbe. ...**Reinhard** rettete mich aus mancher technischen Notlage und war jederzeit für mich da. ...**Willi**, für die schönen und anregenden Pausen am Mittag ...**Andy**, den man einfach nehmen muß, wie er ist oder nicht ist und der im Ernstfall zur Stelle ist. ...**Eike Kibler** für die Bereitschaft mir einige Gehirne zu präparieren. ...**Ulrich Schäfer**, ohne dessen Fliegenstämme nur die halbe Doktorarbeit möglich gewesen wäre. ...**meinen Eltern**, die mir Möglichkeiten gaben, die sie nicht hatten, und die Zeit diese Möglichkeiten zu nutzen. ... und **all meinen Freunden,** die mir das wichtigste Standbein waren!!! **Eva**, die da war **DANKE!** als es am Nötigsten war...und meinem Schwesterherz **Eve**.

Eva	Erbach	Eva
Anne	Valaas	Anne
Björn	Brembs	Björn
Willi	Spatz	Willi
Gabi	Endres	Gabi
Toni	Uecker	Toni
Dirk	Schleif	Dirk
Inge	Klinger	Inge
Jojo	Huth	Jojo
Martin	Heil	Martin
Marlies	Höfer	Marlies
Susanne	Klühspieß	Susanne
Sören	Diegelmann	Sören

Hier setze ich den Schlußpunkt, damit meiner *Drosi* die Beine nicht bis in den Himmel wachsen!

Meinem Zwilling
Eve

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1 INTRODUCTION

Behavioral plasticity is a key to the study of central brain function. It provides access not only to various forms of memory but also to cognitive functions such as attention, context generalisation, and configural learning (Liu et al., 1999; Menzel and Giurfa, 2001).

Learning has often been divided into two principle classes, associative and non-associative learning (Rescorla, 1988). While associative learning requires close temporal contiguity of stimuli, non-associative learning does not require a pairing of events (Lukowiak et al., 1996). Well known forms of associative learning are classical and operant conditioning: in classical conditioning a contingency is established between a stimulus and reinforcement, whereas in operant conditioning, a contingency is established between a response and a reinforcer (Cook and Carew, 1986). Hence, the animal modifies its behavior in response to a comparison between its own behavioral activity and its experiences. The following chapter gives an historical overview of studies focussing on operant conditioning.

1.1 Operant conditioning

Since operant conditioning was introduced by Thorndike (1911), studies to enlighten its underlying principles have been performed in many vertebrates, like rats, bats, monkeys, and man (Skinner, 1950; Beecher, 1971; Berger, 1968; Jaeger et al., 1987; Wolpaw, 1987; Feng-Chen and Wolpaw, 1996), but also in two molluscs, *Aplysia* and *Lymnea stagnalis* (Cook and Carew, 1986, 1989a, 1989b, 1989c; Hawkins et al., 1985; Nargeot et al., 1995, 1996, 1997, 1999a, 1999b; Lukowiak et al., 1996; Spencer et al., 1999). With regard to arthropods, Horridge (1962) discovered an example of 'simple' insect learning where a single cockroach can be trained to avoid certain leg postures by electric shock. The physiological basis of this operant behavior has been studied by Hoyle in locusts (Hoyle, 1979).

In the fly *Drosophila melanogaster*, several paradigms of associative learning and memory have been developed in which different behaviors are modified (Wolf et al., 1998). In 1981, Booker and Quinn adapted the Horridge leg paradigm to *Drosophila*, showing that the flies can be trained to lift their legs to avoid electric shock, even when they are decapitated (Booker and Quinn, 1981). Subsequently, Mariath invented an operant conditioning paradigm where single tethered flies learn to control a heat source by moving a platform with the legs in a certain direction (Mariath, 1985). Operant learning of *Drosophila* in the torque meter was reported for the first time in 1991 (Wolf and Heisenberg, 1991). In the following years, the flight simulator was extensively used to investigate pattern recognition (Dill et al., 1993, 1995a; Heisenberg, 1995; Ernst and Heisenberg, 1999), structure function relationships in the brain (Weidtmann, 1993; Wolf et al., 1998; Liu et al., 1999) and to behaviorally analyse learning / memory processes (Eyding, 1993; Dill et al., 1995b; Guo et al., 1996; Guo and Götz, 1997; Wolf and Heisenberg, 1997; Xia et al., 1997a, 1997b, 1999).

Since Rescorla´s statement, that „...one is unlikely to achieve a stimulus that bears purely Pavlovian or purely instrumental relation to an outcome....“, studies focussed precisely on separating mechanisms of classical and operant conditioning (Rescorla, 1994). With *Drosophila melanogaster* at the flight simulator, this aim was achieved, enabling a direct comparison of operant and classical learning with very similar stimulus situations (Brembs, 1996; Brembs and Heisenberg, 2000).

Here I investigate heat-box learning (Wustmann et al., 1996) which was developed for large scale mutant screening and is one of the simplest and most efficient operant learning paradigms.

1.2 Heat-box

Conditioning in the heat-box is an operant process in which flies develop a spatial preference for one side of an experimental chamber. Single flies walking freely back and forth in a narrow alley in complete darkness,

are conditioned to avoid one half of the length of the alley by being heated instantaneously upon entering that half. The temporal scheme of heating and cooling simulates for the fly a spatial temperature gradient in the chamber. The training is followed by a test period without temperature change. During the whole experiment, the position of the fly in the chamber is monitored and the fraction of time the flies spent on the 'unpunished' side is calculated. Besides temperature, the fly can use only tactile information and path integration for orientation (ideothetic orientation, i.e. the accumulation of the internal representations of the fly's turns and steps; Wustmann and Heisenberg, 1997).

One of the advantages of this paradigm is that the procedure is fast and robust, making it suitable for large-scale mutant screening. Additionally, learning scores are obtained automatically without the interference of an experimenter. As flies are freely walking in the apparatus and are not damaged during the experiment, they can be used afterwards in further behavioral, histological or genetic investigations.

A critical step in developing the heat-box paradigm had been to show that performance in the test period indeed demonstrates memory. One problem arises from the fact that the test directly follows the conditioning process. At the end of the training period, most flies avoid being heated and are therefore found on the unpunished side. With the test starting directly after the training, all these flies contribute positively to the memory score. However, it is not possible to distinguish between an after-effect of simple heat avoidance and a conditioned preference for the previously unpunished side of the chamber. To avoid this problem, Wustmann et al. (1996) started the evaluation of the position traces for the memory test after the first midline crossing of the fly. This evaluation, however, underestimates the memory score.
A further problem addressed in previous studies were potential odor marks: While being heated, flies might deposit odorants and later during the test period avoid these. To investigate this possibility, flies were transferred from one chamber to another between training and test. As flies turned out to lose track of the unpunished side during the transfer, a 10-sec reminder training was

introduced to re-establish after the transfer the polarity of the new chamber with respect to hot and cold. From these experiments it was concluded that flies indeed learned a spatial preference for the unpunished side (Wustmann and Heisenberg, 1997). However, an alternative explanation still remains. Instead of remembering from the first training that its position in the chamber can influence the chamber temperature, the fly might be conditioned by the experimental situation of the training period (darkness, isolation, etc.) to learn faster during the short reminder training.

In the heat-box, well known memory mutants like *dunce* and *rutabaga* (*rut*) show reduced performance in the test (Wustmann et al., 1996). Flies mutant for *rut* were used by Zars et al. (2000a) to map the structures in the central nervous system requiring normal *rut* adenylate cyclase for heat-box learning. Candidate structures in the antennal lobes, median bundle and ventral ganglion were identified. Neither the mushroom bodies nor the central complex require normal *rut* expression. Mushroom body-less flies perform as well in heat-box learning as normal ones (Wolf et al., 1998).

Many questions about learning and memory processes in the heat-box are still open. One way to learn more about genes and signaling pathways involved in this paradigm is to behaviorally screen for candidate genes and characterize them. *p90 ribosomal S6 kinase* (*S6KII*) is such a candidate gene which was discovered in a P-element mutant screen and which might play a role in heat-box conditioning. In a detailed analysis, I investigated the effect of several mutations of that *Drosophila* gene. The next chapter is an introduction to the structure and functions of p90 ribosomal S6 kinases (RSKs). Readers familiar with RSKs may skip chapter 1.3.

1.3 p90 ribosomal S6 kinases

1.3.1 Structure of RSKs

The RSKs are a family of cellular serine-threonine kinases that are also known as p90 [rsk] or mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1). Initially, the p90 ribosomal S6 kinase was discovered in

Xenopus laevis oocytes and was shown to mediate the phosphorylation of S6, a 31-kDa protein, which is an integral component of the ribosomal 40S subunit (Erikson et al., 1985, 1987; Nebreda and Gavin, 1999). The phosphorylation of this protein is believed to promote the translation of selected mRNAs important for cell growth (Jefferies et al., 1997). Two ribosomal S6 protein kinases (S6KI and S6KII) with molecular weight of 90 kDa and about 90 % identity were identified by fractionation of cell extracts (Jones et al., 1988). Subsequently, homologues of S6KI and S6KII (renamed p90rsk or RSK) were cloned from mouse, chicken, rat, and *Caenorhabditis elegans* (Alcorta et al., 1989; Grove et al., 1993). Mammals have at least three RSK isoforms: RSK-1, RSK-2, and RSK-3 (De Cesare et al., 1998). In 1994, the *Drosophila melanogaster* gene for p90 ribosomal S6 kinase II (*S6KII*), that encodes a serine/threonine kinase of 910 aa, was isolated from an eye-antennal imaginal disk library and sequenced (Wassarman et al., 1994). In the following text, RSK refers to the broad group of p90 ribosomal S6 kinases in vertebrates and invertebrates, whereas S6KII refers exclusively to the *Drosophila* p90 ribosomal S6 kinase.

The family of RSKs is characterized by two kinase domains, an amino-terminal domain which is related to protein kinase C and the catalytic subunit of cAMP- and cGMP-dependent kinases (40-45 %) and a carboxy-terminal domain that bears 30-35 % homology to phosphorylase b kinase (PhK) and calcium/calmodulin kinases (Jones et al., 1988; Hanks et al., 1988; reviewed by Erikson, 1991). Molecular cloning of RSK isoforms from chicken, mouse, rat, and man have demonstrated a conservation of the two kinase domains along with an overall homology of 75-85 % between different 724-752 amino acid isoforms and between species (Alcorta et al., 1989; Grove et al., 1993; Moller et al., 1994; Bjorbaek et al., 1995a).

In *Drosophila melanogaster* the N-terminal kinase domain extends from aa 195 to 460 and the C-terminal kinase domain from aa 560 to 840. Sequence comparison of a predicted 100-kDa protein revealed identity values of 60, 60, and 63 % and similarity values of 74, 75, and 77 % with mouse, chicken, and Xenopus S6KII proteins (Wassarmann et al., 1994).

The N-terminal kinase domain is responsible for phosphorylating substrates of RSK such as the cAMP response element-binding protein (CREB), c-fos, and Myt1 and recognizes the basophilic consensus motif Arg/Lys-X-Arg-X-X-Ser/Thr or Arg-Arg-X-Ser/Thr (Leighton et al., 1995). The C-terminal kinase domain is able to phosphorylate the linker sequence between the two kinase domains and thereby regulates the activity of the amino terminus (Bjorbaeck et al., 1995b; Nebreda and Gavin, 1999).

The activation of RSK follows binding to and phosphorylation by ERK/MAP kinases. The C-terminal kinase domain contains a short docking motif which is responsible for the specific association of RSK with ERK/MAP kinases (Zhao et al., 1996; Smith et al., 1999). Besides that, Gavin and colleagues identified an additional ERK/MAP kinase docking site at the carboxyl terminus which is required for the efficient phosphorylation and activation of RSKs *in vitro* and *in vivo* and is necessary and sufficient for a stable and specific association with MAP kinase (Gavin and Nebreda, 1999).

Two consensus Thr phosphorylation sites were found within the sequence TPCYTA in subdomain VIII of the C-terminal kinase domain which are conserved in all known RSK isoforms (Alcorta et al., 1989; Lavoinne et al., 1991; Sutherland et al., 1993; Moller et al., 1994). Since the first Thr (Thr[731] in *Drosophila*) residue is followed by Pro, they suggested that this was the major site of phosphorylation by MAPKs which are known to be proline-directed. Furthermore, MAPK phosphorylated only the first Thr in a peptide containing both residues (Davis, 1993; Sutherland et al., 1993). Besides phosphorylation by MAPKs, complete RSK activation requires phosphorylation of the amino-terminal domain by phosphoinositide-dependent kinase 1 (PDK1; Jensen et al., 1999; Richards et al., 1999). RSK activation, therefore, integrates regulatory inputs from both the MAPK- and PDK1-dependent signaling pathways (Nebreda and Gavin, 1999). RSKs contain an α helix downstream of the carboxyl-terminal kinase domain, which results in a permanently activated protein kinase upon deletion or mutation (Poteet-Smith et al., 1999). Additionally, an inhibitory role for the amino-terminal 43 amino acids of RSKs is discussed, which suggests another

mechanism of RSK regulation (in *Xenopus*, Gross et al., 1999).

Besides the family of RSKs there is another major class of S6 kinases, the p70 S6 kinases or pp65-70, which is also involved in phosphorylation of S6 in 40S ribosomal subunits. *In vivo*, S6 activation is mediated by p70 S6 kinases, while, *in vitro*, S6 is also a substrate for p90 ribosomal S6 kinases. p70 S6 kinases differ from p90 ribosomal S6 kinases in that they have only the N-terminal kinase domain, but no C-terminal kinase domain. The presence of two catalytic domains in the RSK gene products suggests that they have the ability to phosphorylate other substrates besides S6 (reviewd by Erikson, 1991).

In mice, two distantly related genes coding for RSKs with decidedly different areas of expression were found. Whereas one is expressed in the intestine, a tissue characterized by rapid cell proliferation, and at low levels in the brain and heart, the second gene has the complementary expression pattern (Alcorta et al., 1989). As brain and heart do not undergo rapid cell proliferation, these data either suggest that the gene product has other substrates or that S6 phosphorylation has a role unrelated to cell proliferation in these tissues (reviewed by Erikson, 1991). In the next chapter roles of RSKs are discussed in more detail.

1.3.2 Functions of RSKs

Role of RSKs in transcriptional regulation
p90 ribosomal S6 kinases are intermediates connecting MAPK activity with the transcriptional activation of regulatory genes. These effects are mediated by the direct association of RSKs with transcriptional regulators including c-Fos, estrogen receptor, NFkappaB/IkappaB alpha, cAMP-response element-binding protein (CREB) and CREB-binding protein, which they phosphorylate and activate (Frödin, 1999; Nebreda and Gavin, 1999). RSK has been shown to phosphorylate CREB at Ser133 (Xing et al., 1996) and is, therefore, proposed to be involved in synaptic plasticity and memory formation (Bito et al., 1997; Dufresne et al., 2001; Impey et al., 1999; Roberson et al., 1999; Swank & Sweatt, 2001). A more general role for RSKs in transcriptional regulation was postulated by

Sassone-Corsi with the observation that some RSKs can phosphorylate histone H3 and, thus, might play a role in chromatin remodeling (Sassone-Corsi et al., 1999).

RSKs and their role in regulation of the cell cycle
RSKs may also be involved in regulation of the cell cycle. They phosphorylate and inactivate the $p34^{cdc2}$ inhibitory kinase Myt1 in oocytes from *Xenopus laevis*, which results in a progression of oocytes through the G_2/M phase of meiosis (Palmer et al., 1998). Wright and colleagues found that such a down-regulation of $p34^{cdc2}$ inhibitory kinase by RSKs might also be important for progression of mammalian somatic cells through the G_2/M phase of mitosis (Wright et al., 1999). Besides the role in cell cycle progression, RSKs have different ways of influencing cell survival. Mammalian RSK-2 can phosphorylate and, thus, suppress the effects of BAD, a protein that promotes apoptosis (reviewed by Ballif and Blenis, 2001). Additionally, RSK-2 is involved in the transcriptional up-regulation of the pro-survival gene Bcl-2 by phosphorylation and activation of the transcription factor CREB (Bonni et al., 1999). Activation of RSK is also necessary and sufficient to cause the MAPK-mediated arrest of eggs at metaphase II of meiosis before fertilisation, which is called the cytostatic factor arrest (CSF; Gross et al., 1999; Bhatt and Ferrell, 1999).

Additional functions of RSKs
Further roles of p90 ribsomal S6 kinases include the feedback inhibition of the Ras-ERK pathway by phosphorylation of the Ras GTP/GDP-exchange factor Sos and the regulation of protein synthesis by phosphorylation of polyribosomal proteins and glycogen synthase kinase-3 (Douville and Downward, 1997; Angenstein et al., 1998). One report even suggests a role of RSK in neurite outgrowth mediated by the cell adhesion molecule L1 (Wong et al., 1996).

1.3.3 MAPK signaling cascade

The MAPK cascade is an evolutionarily conserved signaling cassette that plays a critical role in cell growth and survival in yeast, plants, and vertebrates. In vertebrates, seven MAPK cascades with specialized physiological roles have been identified. The ERK/MAPK signaling cascade is the focus of our interest, as it has p90 ribosomal S6 kinase as a target (Fig. 1; reviewed by Impey et al., 1999). It is distinguished by a characteristic core cascade of three kinases (Chang and Karin, 2001): MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAP kinase (MAPK).

Raf-1 and B-Raf, which belong to the MAPKKKs, activate MEK, a MAPKK, by serine/ threonine phosphorylation. Subsequently, MEK activates p44 MAPK and p42 MAPK by phosphorylating a threonine and a tyrosine residue (reviewed by: Blenis, 1993; Johnson and Vaillancourt, 1994; Treisman, 1996; Grewal et al., 1999; Sweatt, 2001). Besides interacting with other substrates, MAP kinases can then activate RSKs via phosphorylation of serine/threonine residues (Chen et al., 1992; comment by Roberts, 1992). The activated cytoplasmic 'CREBkinase' RSK is translocated to the nucleus (Chen et al., 1992) where it in turn phosphorylates CREB on Ser[133], which then induces target gene expression (Xing et al., 1996; Xing et al., 1998; Impey et al., 1998; Muthusamy and Leiden, 1998; reviewed by De Cesare et al., 1999 and by Walton and Dragunow, 2000).
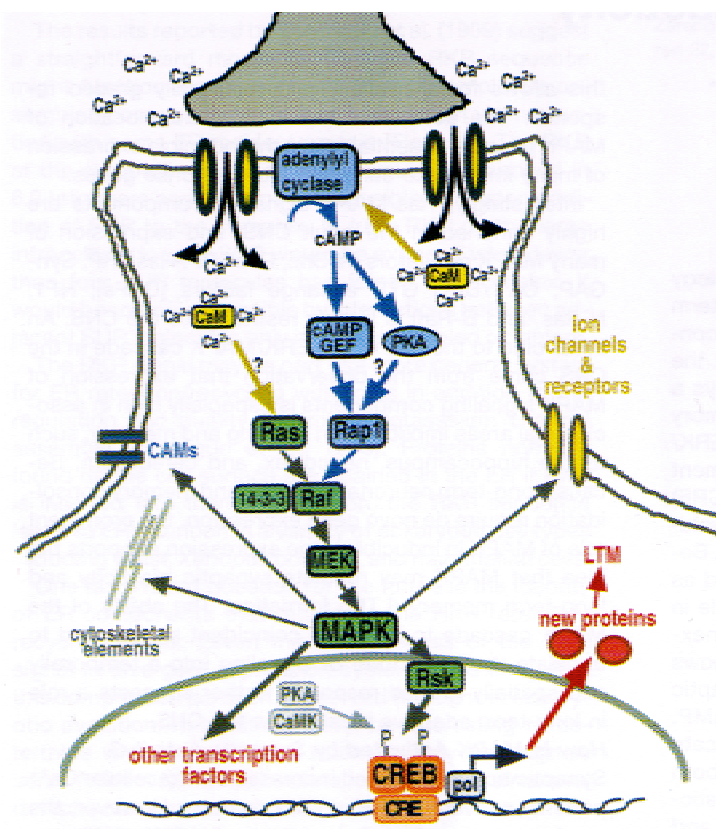
Figure 1: Model for the activation of the ERK/MAPK cascade via synaptic activity and potential regulatory targets. The release of an excitatory neurotransmitter onto a bouton depolarizes a neuron resulting in Ca^{2+} influx and activation of the Ras family G proteins. Following activation of Raf leads to the sequential phosphorylation and activation of MEK and MAPK. MAPK, then, can activate CREB via RSKs and, thus, induce long-term adaptive changes in neurons. Besides RSKs, MAPK has other targets, like cell adhesion molecules (CAM), cytosceletal elements, and ion channels. Figure from Impey and colleagues (Impey et al., 1999).

1.3.4 Role of the ERK/MAPK cascade in neuronal plasticity and memory formation

The expression of many MAPK regulators like N-Shc, RasGFR, RasGRP, SynGAP, Ca^{2+}/DAG GTP exchange factors, NF1, N-Ras and B-Raf is restricted to the CNS. A first clue to the role of the ERK/MAPK cascade in the CNS stems from the observation that MAPK signaling components are higly expressed in the hippocampus, neocortex, and cerebellum, areas which are known to be implicated in learning and memory. The fact that the MAPK cascade plays an important role in inducible expression of immediate-early and late-response genes also supports the idea that it might regulate neuronal plasticity and memory consolidation. Furthermore, the ability of the MAPK cascade to integrate coincident signals and to translate the magnitude of signaling into a temporally and spatially graded response suggest a role in long-term adaptive plasticity in the CNS. In the next chapter, evidence that the activation of the ERK/MAPK cascade plays an important role in neuronal plasticity and memory formation is discussed (reviewed by: Impey et al., 1998; Orban et al., 1999; Sgambato et al., 1998).

1.3.4.1 Role of the MAPK signaling cascade in LTF and LTP

In 1997 studies of long-term facilitation (LTF) in Aplysia for the first time proved a role for the MAPK cascade in invertebrate neuronal plasticity. LTF, a model for long-term sensitisation of the gill withdrawal reflex, can be mimicked by exposing sensory-motor neuron synapses to multiple spaced pulses of serotonin. Martin and colleagues could show that MAPK is activated and translocated to the nucleus of the presynaptic cell during 5-HT-induced long-term facilitation. When MAPK was selectively inhibited in sensory neurons by injection of an inactivating antibody, LTF was attenuated, but STF was not. Pharmacological inhibition of the MAPKK MEK also prevented the aquisition of LTF, without affecting STF (Martin et al., 1997).

Long-term potentiation (LTP) is an activity-dependent strengthening of synaptic efficacy that is the proposed candidate for a cellular mechanism underlying vertebrate memory formation. *In vitro* studies on hippocampal slices showed that stimuli which induce NMDA receptor-dependent LTP in area CA1 also potently activate MAPK and RSKs (English and Sweatt, 1996; Impey et al., 1998), whereas pharmacological inhibition of MEK partially blocks LTP formation in area CA1 of the hippocampus (English and Sweatt, 1997). Application of the MEK inhibitor PD98059 completely blocks the gene expression-dependent late phase of long term potential (L-LTP; Impey et al., 1998). Besides its significance in NMDA receptor-dependent LTP, MAPK signaling is also necessary for NMDA receptor-independent LTP and LTP *in vivo* (Coogan et al., 1999; Mc Gahon et al., 1999; Davis et al., 2000; Rosenblum et al., 2000). Thus, MAPK signaling plays an important role in the induction of LTF as well as LTP.

1.3.4.2. Role of MAPK activation in learning and memory processes in invertebrates

Besides the indication that MAPK signaling plays an important role in the induction of LTP, there are also strong suggestions for a role of the MAPK cascade in memory formation (reviewed by: Sweatt, 2001; Impey et al., 1999). An example is the *Drosophila* mutant *leonardo*. *leonardo* codes for a 14-3-3 family protein that is highly expressed in mushroom body neurons and binds directly to Raf (Fig. 1). This association is critical for the activation of Raf by Ras. Decreased levels of Leonardo expression result in impaired Ras/MAPK signaling and defects in short-term and long-term associative olfactory memory formation (Skoulakis and Davis, 1996).

MAPK is also activated with one-trial *in vitro* Pavlovian conditioning in the sea slug *Hermissenda crassicornis*. The conditioning procedure consisted of light (CS) paired with the application of 5-HT (Crow et al., 1998). After pretreatment with the MEK inhibitor PD98059, the increased phosphorylation of ERK after one-trial conditioning was blocked, suggesting a significant role for an activation of the ERK-MAPK signaling pathway in Pavlovian conditioning in molluscs. However, most studies investigating the significance of

MAPK signaling in memory formation are performed in vertebrates.

1.3.4.3 Role of MAPK activation in learning and memory processes in vertebrates

Role of MAPK in fear conditioning
A role for the MAPK pathway in vertebrate LTM formation was found in mice deficient for the Ras activator RasGFR (Brambilla et al., 1997). Abnormal LTP was found in the basolateral amygdala, a structure that is involved in associative fear conditioning. Fear conditioning is a robust form of classical conditioning which includes two different forms. Cued conditioning pairs a normally innocuous tone with aversive foot shock, whereas context-dependet conditioning pairs a novel context or spatial environment with foot shock (LeDoux, 1995). Conditioned animals show an increased immobility (‚freezing') reaction compared to untrained ones. While cued conditioning is amygdala- but not hippocampus-dependent, contextual fear conditioning needs both, the amygdala and the hippocampus. RasGFR mutant mice showed a markedly compromised LTM in cued fear conditioning, while learning and short-term memory were intact (Brambilla et al., 1997). A possible role of Ras/MAPK activation in fear conditioning is the integration of associative inputs from the thalamus as well as the auditory cortex and the induction of an LTP-like increase in synaptic efficacy.

An alternative approach, avoiding the influence of developmental defects, used selective pharmacological inhibitors of the MAPKK MEK. Atkins and colleagues showed that contextual fear conditioning leads also to a marked activation of MAPK in the hippocampus of rats (Atkins et al., 1998). Injection of the MEK inhibitor SL327 at a dose that blocks conditioning-associated MAPK activation attenuated LTM formation and blocked hippocampal LTP. As injection of the drug also blocked cued fear conditioning in rats, the study supports the idea that MAPK signaling is an essential step in hippocampus-dependent as well as in amygdala-dependent LTM consolidation. In the mentioned study, SL327 was administered intraperitoneally which lead to an inhibition of MEK throughout the animal. Schafe and colleagues infused the MEK inhibitor PD98059 intraventricularly and confirmed that MEK activation is required for fear conditioning. The pharmacological inhibition of MAPK activity blocked LTP and interfered with memory consolidation for fear conditioning (Schafe et al., 1999, 2000; reviewed by Schafe et al., 2001). Comparable approaches in mice also provided evidence that MAPK signaling is necessary for this form of classical conditioning (Selcher et al., 1999).

Role of MAPK in spatial learning:
Additional evidence that the Ras/MAPK cascade is implicated in vertebrate memory consolidation was obtained from a study of Silva and colleagues on neurofibromatosis type 1 (NF1) mutant mice (Silva et al., 1997). NF1 is a neuronal Ras GTPase-activating protein and Ras inhibitor. Heterozygous NF1 mutant mice have been tested in the Morris water maze (Morris, 1981) and revealed partial deficits in hippocampus-dependent spatial memory. The data implied that increased Ras activity perturbed memory formation and suggested that a dynamic balance of Ras activation is essential for memory formation in mice.

Blum and colleagues showed that training of rats in the Morris water maze spatial learning paradigm increased MAPK phosphorylation specifically in the dorsal hippocampus, while infusion of a MEK inhibitor reduced MAPK activity and attenuated the expression of LTM (Blum et al., 1999). A similar study in mice revealed that inhibition of MEK by SL327 does not only lead to an impaired expression of a learned behavior in the Morris water maze task, but already results in an impaired escape latency during training, representing a learning deficit (Selcher et al., 1999). The finding is consistent with Blums observation that training in the Morris water maze increased MAPK activation. An effect of MEK inhibition, however, was only observed 48 hr after training (Blum et al., 1999). Several reasons, like the route of drug administration and differences in training or test paradigms, are discussed to account for this deviation.

Role of MAPK in conditioned taste aversion (CTA):
It was investigated whether the ERK/MAPK cascade is also involved in conditioned taste

aversion which depends on the insular cortex. The conditioning procedure includes the pairing of a novel taste with a noxious stimulus. As this type of learning is critical to the animals survival, it is subserved by an extremely robust learning mechanism. Even after a single experience with novel food that subsequently caused sickness, the animal will exhibit a long-lived aversion to that particular food. Berman and colleagues discovered that MAPK activation is necessary for the formation of stable CTA, as injection of the MEK inhibitor PD98059 into the insular cortex of rats resulted in impaired LTM (Berman et al., 1998).

While Berman and colleagues investigated neurotransmitters involved in the up-stream regulation of MAP kinase by novel taste, Swank and Sweatt concentrated on mitogen-activated protein kinase (MAPK)-dependent events downstream of this pathway (Swank and Sweatt, 2001). They found that the entire ERK/MAPK cascade was activated in the insular cortex by novel taste learning, including activation of Raf, MEK and RSK. Thus, MAPK activation in the insular cortex is necessary for the formation of conditioned taste aversion.

Role of MAPK in step-down inhibitory avoidance task

A method for evaluating passive avoidance- and escape-learning responses had been developed for the study of learning and memory in mice (Kameyama et al., 1986). In the step-down inhibitory avoidance task, animals are trained to avoid stepping down from a platform onto a metal grid floor via punishment with electric shocks. The task depends on the integrated activity of a neural circuit, including the hippocampal area CA1, the entorhinal cortex, and the posterior parietal cortex (Ardenghi et al., 1997; Izquierdo et al., 1997).

Vianna and colleagues evaluated the contribution of the MAPK signaling pathway to step-down inhibitory avoidance learning by using MEK inhibitor PD98059. When the inhibitor was administered immediately after training STM was absent, while LTM was sensitive to PD98059 only when the inhibitor was administered three hours later (Vianna et al., 2000). These results confirm earlier

findings that the induction of STM depends on MEK activation immediately following the training, whereas LTM depends on the activation of hippocampal MEK during the late period (3-6 h) of memory consolidation (Walz et al., 1999, 2000a, 2000b). Walz and colleagues suggest this time-dependent involvement of the MAPK cascade in the post training memory processing of inhibitory avoidance, as infusion of MEK inhibitor PD98059 into the hippocampus at 0 min, but not at later time points after training, impaired STM.

1.3.4.4 CREB is activated by MAPK via RSKs

Recent studies indicate that CREB is the major target of MAPK during neuronal plasticity induction. However, CREB is not directly phosphorylated by MAPK, but via the MAPK-activated RSK family of protein kinases (Xing et al., 1996; Impey et al., 1998).

In humans, there are three isoforms of the p90 ribosomal S6 kinase (p90^{rsk}) family, RSK-1, RSK-2 and RSK-3. Mutations in the *RSK-2* gene are associated with the Coffin-Lowry syndrome (CLS; Lowry et al., 1971), an X-linked disorder characterized by severe psychomotor retardation, digit and facial dysmorphisms, and progressive skeletal deformations (Young, 1988; Trivier et al., 1996; Abidi et al., 1999; see next page Fig. 2). Analysis of the *RSK-2* gene in CLS patients revealed intragenic deletions, nonsense, missense, and splice-site mutations which resulted in absent or truncated non-functional proteins (Trivier et al., 1996; Merienne et al., 1998; Jacquot et al., 1998). In CLS fibroblasts, a drastic attenuation in the induced Ser-133 phosphorylation of transcription factor CREB was detected in response to epidermal growth factor stimulation (De Cesare et al., 1998). A recent study of Harum and colleagues suggests a correlation between human cognitive performance and the cellular capacity to activate *RSK-2* (Harum et al., 2001).
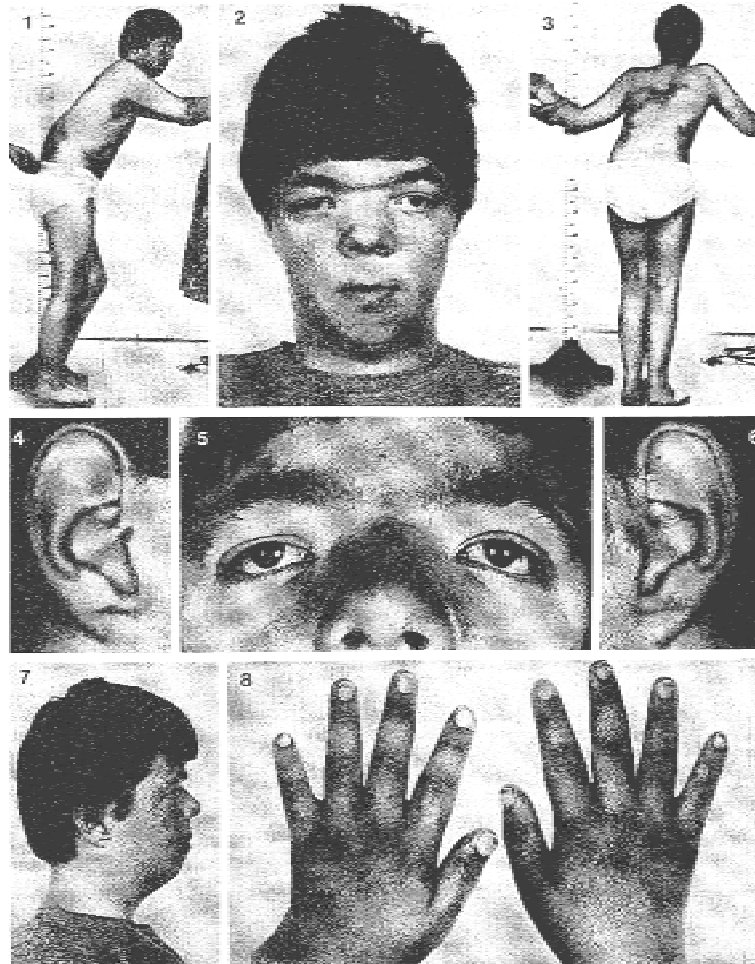
Figure 2: Patient with Coffin-Lowry-Syndrome. Patients suffer from mental retardation, skeletal deformations (1,3), facial and digit dismorphisms (2, 4-8). Figure from Wiedemann and Kunz (1995).

Further evidence that activation of the MAPK signaling cascade is necessary for CREB phosphorylation stems from a study of Roberson and colleagues, who showed that hippocampal MAPK activation is regulated by the PKA and PKC system (Roberson et al., 1999). PKA activation by application of forskolin to hippocampal slices as well as PKC activation by phorbol diacetate (PDA) application resulted in activation of MAPK and increased CREB phosphorylation. Inhibition of MAPKK (MEK) blocked CREB phosphorylation in both preparations (Roberson et al., 1999; Lu et al., 1999).

Moreover, Impey and colleagues demonstrated CREB phosphorylation in the hippocampus due to LTP-inducing stimulation and an attenuation of L-LTP and L-LTP-associated CREB-dependent gene expression by perfusion of hippocampal slices with the MEK inhibitor PD98059 (Impey et al., 1996, 1998). They also confirmed a role for p90 ribosomal S6 kinase in CREB phosphorylation.

Further studies demonstrated that, besides its significance in the hippocampus, MAPK activation is also important for the regulation of CREB activation in the dentate gyrus and in striatal neurons (Davis et al., 2000; Vanhoutte et al., 1999; Perkinton et al., 1999).

In *Drosophila*, it still has to be investigated whether S6KII is involved in the MAPK signaling cascade by phosphorylating CREB or not. Mentioned results of mutant *leonardo* are a first hint that the MAPK cascade plays a role in memory formation also in the flies.

1.4 Aim of this work

Several issues concerning operant learning and memory in the heat-box were addressed in this work. In the first study my aim was to explore learning and memory processes in the heat-box in more detail. The study started with an analysis of the influence of rearing conditions on the performance of flies in heat-box conditioning. I then compared various training procedures and concentrated on the separation of the two components of the memory score. I could show that the increase in memory with more training is not due to an associative memory component but due to the fraction of flies that stay on the unpunished side after the last encounter with heat (*stay-where-you-are* effect). My next goal was to measure how long the memory persists. Thus, transfer experiments were performed with varying memory retention intervals. To avoid the effect of extinction between training and memory test, the flies were transferred to a different environment (a food vial) for that period. The procedure reveals a third memory component which represents conditions of the training other than the heat/position contingency (called exposure effect). The exposure effect is investigated. In addition, I also show that even after transfer and reminder training, heat-box memory is independent of the mushroom bodies.

The second half of my work addressed genes and signaling cascades involved in heat-box conditioning. I studied the behavior of *Drosophila* mutants *amnesiac, dunce, rutabaga* and *radish*, in this paradigm. A similar analysis of *dunce* and *rutabaga* mutants was done by Gerold Wustman who showed that mutants defective in classical conditioning also revealed a defect in heat-box conditioning (Wustmann et al., 1996). Using different mutant strains and a modified version of the heat-box, I tested whether the behavioral phenotypes were consistent. Another way to address the question of which genes and signal transduction cascades might be necessary for operant conditioning is to behaviorally screen collections of *Drosophila* mutants for defects in operant conditioning and to subsequently identify the genetic defect of behaviorally mutant flies. I, thus, performed a large scale *Drosophila* P-element mutant screen together with S. Kramer. Mutants of the candidate gene *ignorant (S6KII)* were isolated and characterized in detail.

2 MATERIALS & METHODS

2.1 Behavioral measurements

2.1.1 Experimental setup

The conditioning apparatus was built in the workshops of the Biocenter. Either the original version of the heat-box described by Wustmann and colleagues (Wustmann et al., 1996) or a modified and improved version was used. Both machines consist of an array of 15 chambers operated in parallel each with peltier elements on top and bottom, which allow for fast heating and cooling (Fig. 3). The peltier elements cover the whole length of the chamber. Chamber size varied between the heat-box versions, with 40 mm length, 2.5 mm height, 4 mm width for the old version and 26 mm length, 2 mm height, 4 mm width for the modified version.

A control circuit and a thermosensor keep the chamber at a defined temperature. Glass side walls enable transmission and detection of an infrared LED source (which is invisible to the flies). While that light is detected by a directionally selective light gate in the original heat-box version, a bar code reader on the opposite side of the chamber detects it in the modified apparatus. The fly casts a shadow on a bar code reader (light gate array in Fig. 3) on the opposite side of the chamber. The position signal of the bar code reader is sent to the computer with a frequency of 10 Hz.

Experiments were performed in complete darkness. Chambers were cleaned with a pipe cleaner every day before experiments. Measurements were performed on at least three days to minimize effects of daily variability. The different groups in one graph were measured strictly in parallel. If not mentioned differently, experiments were performed with the modified heat-box version.

2.1.2 Standard experiment

The standard experiment consists of three phases: pretest, training and test. One half of the chamber is defined as the 'punished' and the other as the 'unpunished' side. These
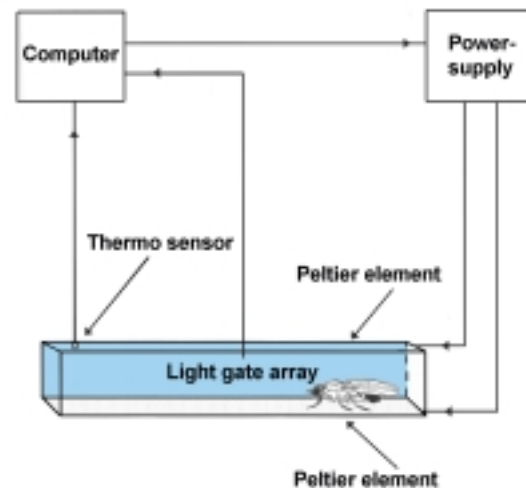
Figure 3: Schematic diagram of one of the 15 modified heat-boxes operated in parallel. For details see text.

designations are altered for every experiment to reduce systematic effects of side use and of potential asymmetries of the apparatus. During the 30-sec pretest, the fly can explore the chamber at a constant temperature of 20 °C; this provides a measure of experience-independent spatial preference. During the subsequent 4-min training period, the whole chamber is heated to 40 °C whenever the fly enters the punished side and is cooled down to 20 °C when it enters the unpunished side.

For analysis, the training and test phases are binned into 1- or 2-min blocks and a Performance Index (PI) is calculated for each block as detailed below. During training, this index provides a combined indicator of heat avoidance and learning. In the following 3-min test period, the chamber is constantly at 20 °C. The PI is calculated as the difference between the time the fly spent in the unpunished versus punished half of the chamber divided by the total time. Thus, the PI can range from −1 to 1, with a PI of 0 indicating no side preference. To yield a measure of general activity, the sum of position changes per period is calculated.

2.1.3 Modifications of the standard experiment – the transfer

The temporal sequence of events in the transfer experiments is explained in Fig. 4.
Direct transfer (experimental design: Fig. 4a): During the training period flies were subjected to four cycles of 4 min training and 1 min test. Afterwards they were removed from the chamber by gently aspirating them into a pipette tip and immediately transferring them into another chamber where they were again trained for 30 sec (reminder training).

During the reminder training the same side was defined as punished side as in the first training period. Subsequently, animals underwent a 6-min memory test. [This procedure differs from that of Wustmann et al. (1996). They trained the animals for 3 min. After the transfer they applied a reminder training of 10 sec and tested memory for only 1 min.]
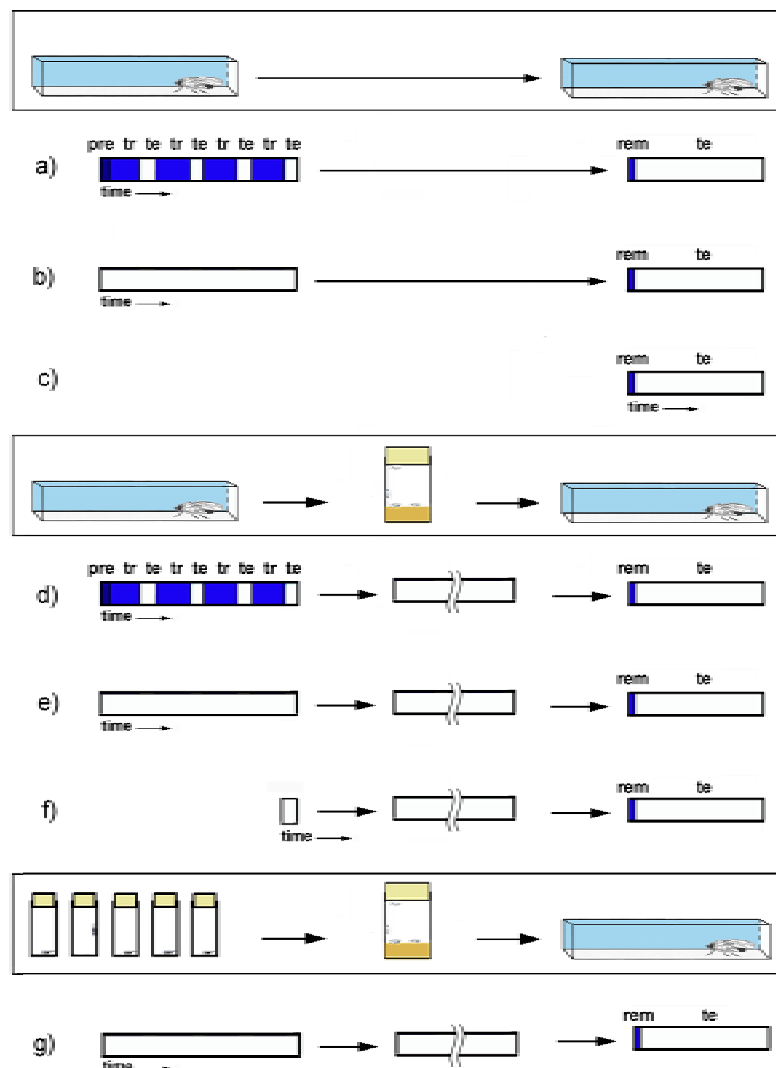
Figure 4: Experimental schedules. (a-c) direct transfer; a) experimental group; b) exposed group, no training; c) naïve control; (d-g) indirect transfer with retention period in food vial; d) experimental group; e) exposed group; no training; f) handling control; flies have only short chamber experience (1-2 sec), no training; g) transfer experiment with single flies in small plastic vials. Figure indicates pretest (pre), training (tr), test (te) and reminder training (rem).

The control conditions for the transfer experiment are outlined in Fig. 4b-c. Flies of the control groups were either exposed to the chamber for 20 min without any heat exposure before the transfer (exposed group) or were taken directly from the food vial before reminder training (naïve group). Both groups underwent a 6 min memory test after the reminder training.

Indirect transfer (experimental design: Fig. 4d-g): Flies were removed from the chambers after training and transferred into a regular food vial (experimental design: Fig. 4d; φ 36.0 x 83 mm). All flies of a given experiment were stored together in a vial until they were, one by one, transferred back into the chambers. After returning flies into the chamber all steps were identical to the direct transfer. Control groups were flies that either had been exposed to the chamber for 20 min without any heat before the indirect transfer (exposed group, Fig. 4e) or naïve flies which had neither received training nor exposure (naïve group). Both control groups were then trained for 30 sec and tested for 6 min.

2.1.4 Thermosensitivity assay

The thermosensitivity assay uses a chamber with peltier elements that can be independently controlled in the front and back half of the chamber (Zars, 2001). A reference temperature of 24 °C is always kept in one half of the chamber, while the other half is stepped to 27 °C, 30 °C, 33 °C, 37 °C, 41 °C, or 45 °C. The side of the chamber set to the reference temperature changes after 60 sec, thus forcing flies to make decisions about their preferred temperature. All points in the chamber reach their final temperature within 2-6 sec. The Performance Index is calculated as described in the learning experiment.

2.1.5 Analysis of data

To exclude animals which do not show substantial motor activity or do not experience punishment, the following criteria were established: flies had to walk at least one chamber length and get at least two heat exposures. For transfer experiments, the following additional criteria applied: After the transfer, flies had to walk one chamber length and had to experience at least one heat period to be included in the data set. As tests for normal distribution of Performance Indices yield varying results, non-paramentrical tests are used for statistical evaluation. Two independent groups were compared by Mann-Whitney U-tests. For comparison of three and more groups Kruskal-Wallis Anova tests were used. Wilcoxon tests were applied to compare single Performance Indices to zero. Repeated measurements were evaluated with a repeated measures Anova. Error bars in the figures are SEMs; n indicates number of flies. Statistically significant differences are shown in the graphs or mentioned in the text; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.2 *Drosophila* techniques

2.2.1 Fly rearing conditions

The *Drosophila melanogaster* CantonS (CS) wild-type strain was used in all experiments. Flies were reared on standard cornmeal / molasses medium (recipe see Guo et al., 1996) in a 16-hr light / 8-hr dark cycle at 60 % humidity and 25 °C. Adults of both sexes were studied (~50 %) at 2-7 days after eclosion. For behavioral experiments the egg laying period of parental flies was restricted to 24 hr.

2.2.2 *Drosophila* crosses

2.2.2.1 Outcrossing P-element lines against control line w^{1118}*Berlin*

To generate a uniform genetic background, several P-element lines (P) were repeatedly outcrossed to a selected w^{1118}*Berlin* stock (next page Fig. 5). Therefore, the w^{1118}*Berlin* stock was used as 'wild-type' comparison in behavioral experiments.

$$\frac{P}{P} \quad \text{x} \quad \frac{w^{1118}Berlin}{y}$$

$$\downarrow$$

$$\frac{P}{w^{1118}Berlin} \quad \text{x} \quad \frac{w^{1118}Berlin}{y}$$

$$\downarrow$$

$$\frac{P}{w^{1118}Berlin} \quad \text{x} \quad \frac{w^{1118}Berlin}{y}$$

↻ further 5 times

$$\frac{P}{w^{1118}Berlin} \quad \text{x} \quad \frac{P}{y}$$

$$\downarrow$$

establish homozygous stock of P-element line
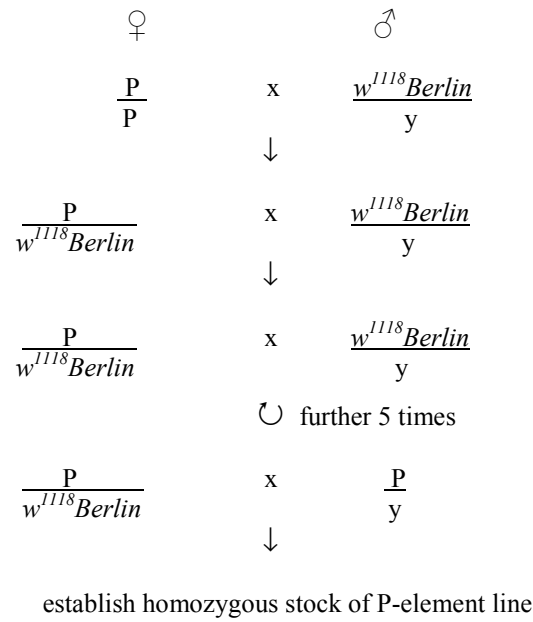
Figure 5: Scheme for the outcrossing procedure of P-element lines (P) to $w^{1118}Berlin$ flies.

2.2.2.2 Generation of jumpout lines

Precise and imprecise excision lines (P(ex)) of P-element line ign^{P1} (P) were established by remobilisation of p[lacW] in females (Fig. 6), but also males (Fig. 7).

♀   ♂

$$\frac{FM7a}{FM7a} \quad \text{x} \quad \frac{w}{y}; \frac{Sb\Delta2\text{-}3}{TM3Ser}$$

$$\downarrow$$

$$\frac{FM7a}{w}; \frac{TM3Ser}{+} \quad \text{x} \quad \frac{P}{y}$$

$$\downarrow$$

$$\frac{P}{FM7a}; \frac{TM3Ser}{+} \quad \text{x} \quad \frac{FM7a}{y}; \frac{Sb\Delta2\text{-}3}{+}$$

$$\downarrow$$

$$\frac{P}{FM7a}; \frac{Sb\Delta2\text{-}3}{TM3Ser} \quad \text{x} \quad \frac{FM6w}{y}$$

$$\downarrow$$

$$\frac{P(ex)}{FM6w}; \frac{TM3Ser}{+} \quad \text{x} \quad \frac{FM6w}{y}$$

$$\downarrow$$

$$\frac{P(ex)}{FM6w} \quad \text{x} \quad \frac{P(ex)}{y}$$

$$\downarrow$$

$$\frac{P(ex)}{P(ex)} \quad \text{x} \quad \frac{P(ex)}{y}$$
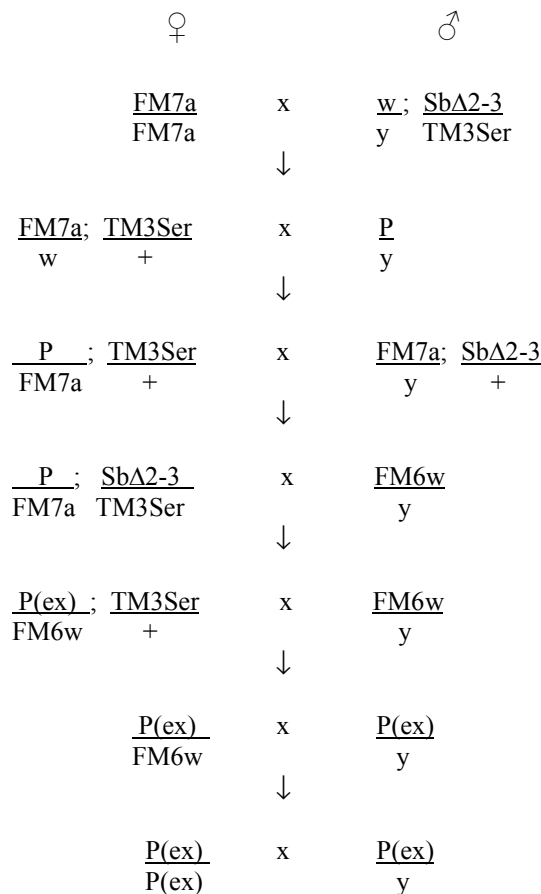
Figure 6: Crossing scheme for the remobilisation of p[lacW] in *Drosophila* females.

$$\female \qquad\qquad \male$$

$$\frac{P}{P} \qquad x \qquad \frac{w\;;\;\;Sb\Delta2\text{-}3}{y\;\;\;\;TM3Ser}$$

$$\downarrow$$

$$\frac{FM6w;\;\;+}{+\qquad+} \qquad x \qquad \frac{P\;;\;\;Sb\Delta2\text{-}3}{y\qquad+}$$

$$\downarrow \qquad 50\ \text{vials (3 } \female + 3\ \male \text{ per vial)}$$

$$\frac{P(ex)\;;\;\;+}{FM6w\qquad+} \qquad x \qquad \frac{FM6w}{y}$$

$$\downarrow \qquad \text{single crosses}$$

$$\frac{P(ex)\;;\;\;+}{FM6w\qquad+} \qquad x \qquad \frac{P(ex)}{y}$$

$$\downarrow$$

$$\frac{P(ex)\;;\;\;+}{P(ex)\qquad+} \qquad x \qquad \frac{P(ex)}{y}$$
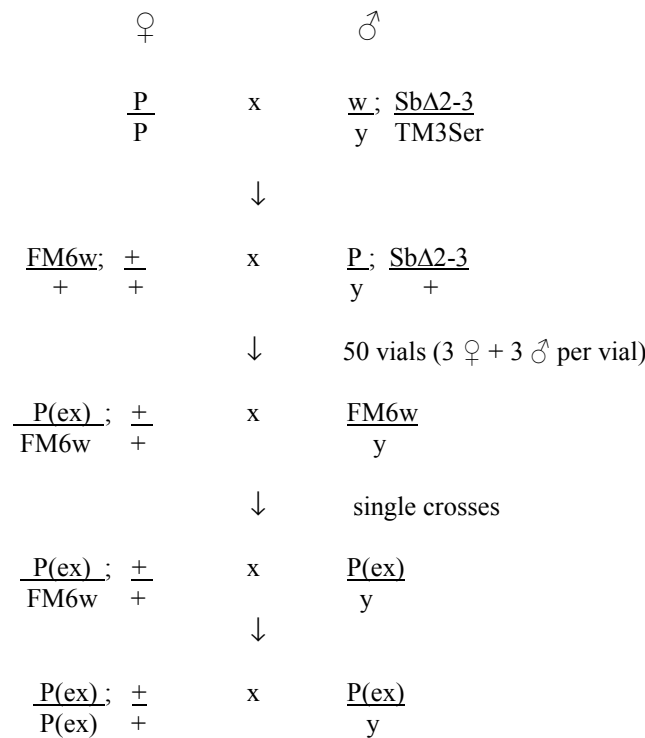
Figure 7: Crossing scheme for the remobilisation of p[lacW] in *Drosophila* males.

2.2.2.3 Cantonisation of P-element line *ign^{P1}* and imprecise jumpouts

The w^+ gene was recombined onto the X-chromosome of P-element line *ign^{P1}* and imprecise jumpout lines *Df(1)ign^{Δ24/3}*, *Df(1)ign^{Δ30/2}*, *Df(1)ign^{Δ58/1}*, and *Df(1)ign^{Δ67/1}*. Selection for recombination events was done by PCR. In parallel, balancer strain FM7a was outcrossed against wild-type CantonS flies for six generations. The recombined lines were afterwards outcrossed to the cantonised FM7a strain (Fig. 8).

$$\female \qquad\qquad \male$$

$$\frac{WT\;\;;\;\;WT\;;\;\;WT}{WT\qquad WT\qquad WT} \qquad x \qquad \frac{w^-,P\;;\;\;+\;;\;\;+}{y\qquad+\qquad+}$$

$$\downarrow$$

$$\frac{w^-,P;\;\;WT;\;\;WT}{w^+\qquad+\qquad+} \qquad x \qquad \frac{WT;\;\;WT;\;\;WT}{y\qquad WT\qquad WT}$$

$$\downarrow \quad \text{single crosses}$$

$$\frac{FM7a\;;\;\;+\;;\;\;+}{FM7a\qquad+\qquad+} \qquad x \qquad \frac{w^+,P;\;\;WT;\;\;WT}{y\qquad+\qquad+}$$

$$\downarrow \quad \text{PCR}$$

$$\frac{w^+, P}{FM7a} ; \frac{+}{WT} ; \frac{+}{WT} \qquad x \qquad \frac{FM7a \text{ (cantonised)}}{y}$$

$$\circlearrowright \quad \text{further 5 times}$$

$$\frac{w^+, P}{FM7a} ; \frac{+}{+} ; \frac{+}{+} \qquad x \qquad \frac{w^+, P}{y} ; \frac{+}{+} ; \frac{+}{+}$$

$$\downarrow$$

establish homozygous stock of outcrossed lines
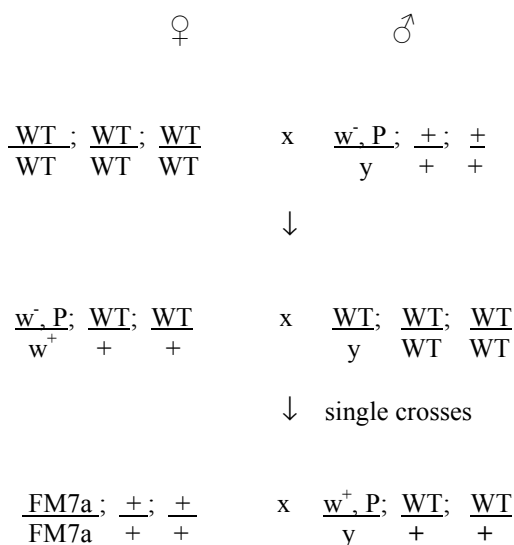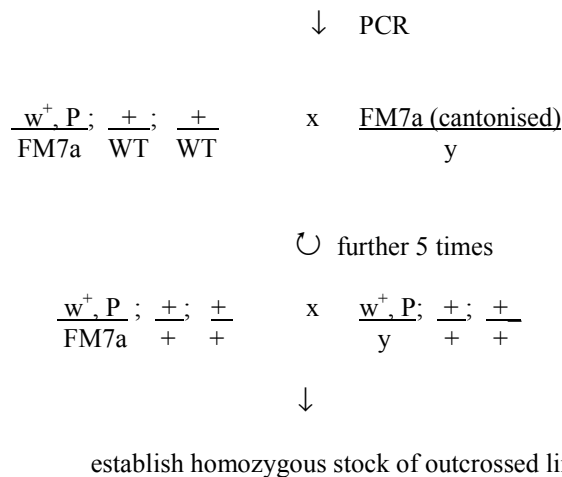
Figure 8: Protocol for the outcrossing procedure of P-element line ign^{P1} and imprecise jumpouts to wild-type CantonS

2.2.2.4 Cantonisation of precise jumpouts

In precise jumpout lines $ign^{\Delta 1P1}$ and $ign^{\Delta 2P1}$ (P(ex)), the w^+ gene was recombined onto the X-chromosome. Selection for recombination events was done by PCR. In parallel, balancer strain FM7a was outcrossed against wild-type CantonS flies for six generations and the recombined lines afterwards outcrossed to the cantonised FM7a strain (Fig. 9).

$$\text{♀} \qquad\qquad\qquad \text{♂}$$

$$\frac{w^-, P(ex)}{w^-, P(ex)} ; \frac{+}{+} ; \frac{+}{+} \qquad x \qquad \frac{w^+, P}{y} ; \frac{+}{+} ; \frac{+}{+}$$

$$\downarrow$$

$$\frac{w^+, P}{w^-, P(ex)} ; \frac{+}{+} ; \frac{+}{+} \qquad x \qquad \frac{FM7a}{y}$$

$$\downarrow$$

$$\frac{FM7a}{FM7a} \qquad x \qquad \frac{w^+, P(ex)}{y} ; \frac{+}{+} ; \frac{+}{+}$$

$$\downarrow \quad \text{PCR, single crosses}$$

$$\frac{w^+, P(ex)}{FM7a} ; \frac{+}{+} ; \frac{+}{+} \qquad x \qquad \frac{FM7a \text{ (cantonised)}}{y}$$

$$\circlearrowright \quad \text{further 5 times}$$

$$\frac{w^+, P(ex)}{FM7a} ; \frac{+}{+} ; \frac{+}{+} \qquad x \qquad \frac{w^+, P(ex)}{y} ; \frac{+}{+} ; \frac{+}{+}$$

$$\downarrow$$

establish homozygous stock of outcrossed precise jumpout lines
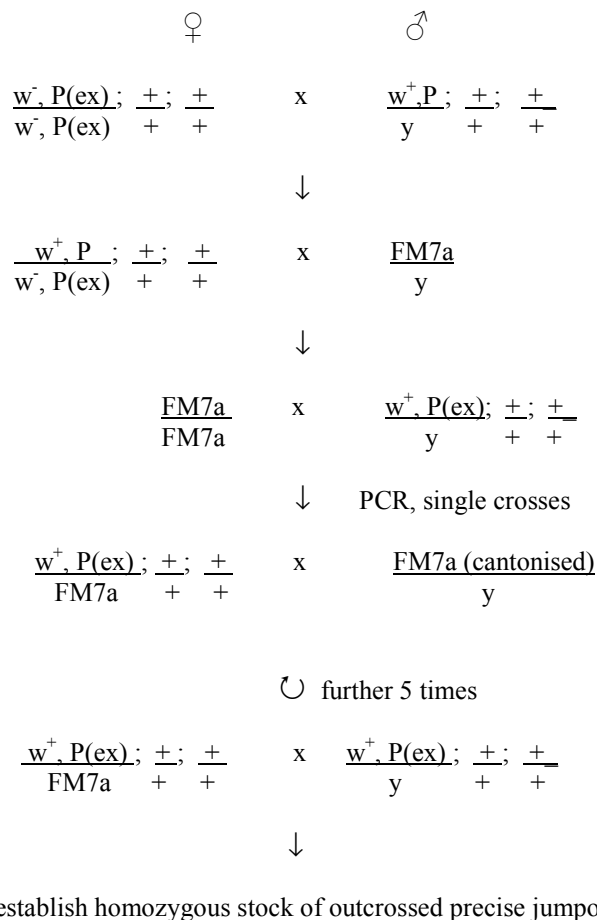
Figure 9: Scheme for the outcrossing procedure of precise jumpouts to wild-type CantonS

2.2.3 Fly strains

Table 1: *Drosophila* stocks used for behavioral and molecular experiments

Stock N°	Description	obtained from
CantonS	wild-type strain	stock collection
Berlin	wild-type strain	stock collection
dunce	allele *dnc^{ML}*	stock collection
amnesiac	allele *amn^1*	stock collection
rutabaga	allele *rut^{2080}*	stock collection
radish	allele *rad*	stock collection
w^{1118}Berlin	berlinised *w^{1118}* strain	stock collection
w^{G8}	berlinised *w^{1118}* strain, selected for good performance in heat-box	G. Putz
w^{G11}	berlinised *w^{1118}* strain, selected for good performance in heat-box	G. Putz
FM6w	balancer strain	stock collection
FM7a	balancer strain berlinised / cantonised	stock collection
w; SbΔ2-3 / TM3Ser	transposase source	stock collection
9885	placW insertion in 18D7-18D9	U. Schaefer
9690	placW insertion in 3B-3C	U. Schaefer
9530	placW insertion in 6E4-6E7	U. Schaefer
8657	placW insertion in 14B	U. Schaefer
8522 = *ign^{P1}*	placW insertion in 20A1	U. Schaefer
8631	placW insertion in 13D2-13D4	U. Schaefer
8570	placW insertion in 19A1	U. Schaefer
8466	placW insertion in 12F	U. Schaefer
6139	placW insertion in 10D5-10D6	U. Schaefer
5054	placW insertion in 19A1	U. Schaefer
further 1211 mutant *Drosophila* stocks	placW insertin on X chromosome	U. Schaefer
Df(1)ign^{Δ76/3}	excision line of 8522 (~ 1 kb deleted)	G. Putz
Df(1)ign^{Δ71/3}	excision line of 8522 (~ 1 kb deleted)	G. Putz
Df(1)ign^{Δ67/1}	excision line of 8522 (11366 bp deleted)	G. Putz
Df(1)ign^{Δ66/3}	excision line of 8522 (~ 1 kb deleted)	G. Putz
Df(1)ign^{Δ58/1}	excision line of 8522 (4762 bp deleted)	G. Putz
Df(1)ign^{Δ53/1}	excision line of 8522 (~ 2 kb deleted)	G. Putz
Df(1)ign^{Δ44/4}	excision line of 8522 (~ 1 kb deleted)	G. Putz
Df(1)ign^{Δ37/1}	excision line of 8522 (1216 bp deleted)	G. Putz
Df(1)ign^{Δ30/2}	excision line of 8522 (2197 bp deleted)	G. Putz
Df(1)ign^{Δ24/3}	excision line of 8522 (1322 bp deleted)	G. Putz
Df(1)ign^{Δ21/5}	excision line of 8522 (~ 1 kb deleted)	G. Putz
Df(1)ign^{Δ9/1}	excision line of 8522 (~ 1 kb deleted)	G. Putz
Df(1)ign^{Δ4/1}	excision line of 8522 (1323 bp deleted)	G. Putz
2/11 = B = *ign^{Δ2P1}*	precise jumpout line of 8522	G. Putz
2/1 = A = *ign^{Δ1P1}*	precise jumpout line of 8522	G. Putz

2.2.4 Histology

Paraffin brain sections were generated following the method of Heisenberg (Heisenberg and Boehl, 1979) and Jager (Jager and Fischbach, 1987).

2.2.5 Wing length and surface area of wings

As an indicator of fly size, length and surface area of wings were determined. Wing length and wing area were automatically calculated from circumference measurements with a custom computer program (Wolf, R.).

2.2.6 HU treatment

To create animals lacking mushroom bodies, first instar larvae were treated with the cytostatic drug hydroxyurea (HU) which leads to the ablation of the mushroom body neuroblasts and hence to adult flies lacking mushroom bodies (de Belle and Heisenberg, 1994). Subsequent to behavioral experiments, a sample of 101 out of 518 HU treated flies were controlled for loss of mushroom bodies by paraffin sectioning of brains. Among the 101 flies, 98 completely lacked the mushroom bodies, while 3 had lost one calyx with one tiny calyx left.

2.3 Chemicals

Frequently used chemicals were ordered from the following companies: Amersham, Appligene, BioRad, Boehringer Mannheim, DuPont, Ferak, Fluka, Gibco-BRL, Invitrogen, Life Technologies, MBI Fermentas (MBI), Merck, New England Biolabs (NEB), Pharmacia, Roth, Schleicher & Schuell, Serva, Sigma, Stratagene, United States Biochemicals (USB), Vector.

2.3.1 Solutions, media and buffers

All used solutions, media and buffers were prepared as described in Sambrock (Sambrock et al., 1989).

2.3.1.1 Media for bacteria

LB-Medium (LB)
>   10 g Bacto / Trypton
>   5 g Bacto-Yeast Extract
>   10 g NaCl
>   add H_2O to final volume of 1 l

YT (2x)
>   16 g Bacto / Trypton
>   10 g Bacto-Yeast Extract
>   5 g NaCl
>   add H_2O to final volume of 1 l

2.3.1.2 Solutions for Mini / Midi preparation

Acetate solution
>   37.5 ml 8 M KoAc
>   11.5 ml acetic acid
>   add 28.5 ml H_2O

Alkaline SDS solution
>   500 µl 5 N NaOH
>   9.1 ml H_2O
>   400 µl 20 % SDS

GTE-buffer
>   20 ml 0.5 M Glucose
>   10 ml 0.2 M EDTA
>   5 ml 1M Tris-Cl (pH 8.0)
>   add H_2O to 200 ml

2.3.1.3 Solutions for *in situ* hybridisation

Blocking buffer (for dot blot)
  0.1 M Tris (pH 7.5)
  0.1 M NaCl
  1 % SDS
  1 % NP40

Detek Hrp complex dilution buffer
  1 ml 0.2 M sodium phosphate (pH 7.2)
  600 µl 5 M NaCl
  200 µl 0.5 M EDTA
  100 µl 10 % TX100
    add H_2O to final volume of 20 ml

Hybridisation buffer
  100 µl H_2O
  250 µl 200 mM Phospate buffer (pH 6.8)
  500 µl 10 % Dextransulfate
  120 µl 5 M NaCl
   20 µl 50x Denhardt
   10 µl 500 mM $MgCl_2$

PBS (10x)
  75.97 g NaCl
  12.46 g Na_2HPO_4 (dihydrate)
   4.68 g NaH_2PO_4 (dihydrate)
    add 500 ml H_2O
    adjust to pH 7.4
    add H_2O to final volume of 1 l

TE (pH 8.0)
  50 ml 1M Tris-HCL (pH 8.0)
   2 ml 1M EDTA
    add H_2O to final volume of 1 l

Washing buffer 1 (for dot blot)
  0.01 M Tris buffer (pH 7.5)
  0.5 M NaCl
  0.5 % Triton X 100
  0.03 % BSA

Washing buffer 2 (for dot blot) = SSC (2x)
  0.3 M sodium chloride
  0.03 M sodium citrate

2.3.1.4 Solutions for plasmid rescue

CIA
  $CHCl_3$: Isopenthanol = 24 : 1

EDTA (1 M)
  186.1 g EDTA
    dissolve in 500 ml H_2O

Homogenisation buffer
  1 ml 5 M NaCl
  2.5 ml 2 M Tris (pH 8.0)
  10 ml 0.25 M EDTA
  1.25 µl 20 % SDS
    add H_2O to final volume of 50 ml

    before use add:
    RNase A (10 mg/ml) 5.5 µl/ml
    Protease K (10 mg/ml) 20 µl/ml

KAc (8 M)
  235.5 g Potassium acetate
    add H_2O to final volume of 300 ml

Ligation buffer (10x)
  2.5 ml 1 M Tris-HCl (pH 7.6)
  0.5 ml 1 M DTT
  0.4 ml 0.25 M rATP
  0.5 ml 1 $MgCl_2$
  250 µl 10 mg/ml BSA
  850 µl H_2O

NaAc (3 M)
  40.82 g Sodium acetate
    dissolve in 60 ml H_2O

TE (pH 8.0)
  50 ml 1M Tris-HCL (pH 8.0)
   2 ml 1M EDTA
  948 ml H_2O

2.3.1.5 Solutions for single fly PCR

dNTP

    10 µl 100 mM dATP
    10 µl 100 mM dCTP
    10 µl 100 mM dTTP
    10 µl 100 mM dGTP
      add H_2O to final volume of 500 µl

Squishing buffer (SB)

    2 ml 0.5 M Tris
  0.2 ml 0.5 M EDTA
    1 ml 2.5 M NaCl
      add H_2O to final volume of 100 ml
      before use add:
      20 µl Proteinase K (10 mg/ml)

2.3.1.6 Solutions for Southern blot

Denaturation solution

    0.5 M NaOH
    1.5 M NaCl

Depurination solution

    0.25 N HCl

Filter washing buffer

    20 ml 20x SSC
    10 ml 20 % SDS
      add 2 l H_2O

Hybridisation buffer

    50 % Formamide
     5x Denhardts
     5x SSPE
    0.1 % SDS
100 mg/ml Dextransulfate
 100 µg/ml salmon sperm DNA
 (boil DNA just before use for 5 min)

Neutralisation solution

    0.5 M Tris-HCl (pH 7.5-8.0)
    1.5 M NaCl

SSC (20x)

  175.3 g NaCl
   88.2 g Na Citrate
      adjust pH to 7.0
      add H_2O to final volume of 2 l

2.3.1.7 Solutions for glycerine stock

Glycerine stock

  0.3 ml medium
  0.7 ml 50 % Glycerine

2.3.1.8 Solutions for Agarose gelelectrophoresis

Loading buffer

  0.25 % Xylene cyanol FF
  0.25 % Orange B
    30 % Glycerol in H_2O

Marker

    2 µl Ladder
    2 µl 10x buffer
    1 µl loading buffer
  10 µl H_2O

TBE (10x)

  500 mM Tris-base
  500 mM Boric acid
  2.5 mM EDTA
    in 2.5 l H_2O

2.3.2 Kits

Table 2: Kits used for molecular experiments.

Method	Kit	company
Gel extraction	QIAquick Gel Extraction Kit	QIAGEN
	QIAEXII Gel Extraction Kit	QIAGEN
PCR purification	QIAquick PCR Purification Kit	QIAGEN
Plasmid Midi preparation	QIAGEN® Plasmid Midi Kit	QIAGEN
	Concert™ Rapid Plasmid Midiprep System	GibcoBRL
Plasmid Mini preparation	QIAGEN® Plasmid Mini Kit	QIAGEN
	Concert™ Rapid Plasmid Miniprep System	GibcoBRL
	QIAprep Spin Mini Kit	GibcoBRL
5´RACE	GeneRacer™ Kit and TOPO TA Cloning Kit for Sequencing	Invitrogen
RT-PCR	Oligotex mRNA Mini Kit	QIAGEN
	SUPERSCRIPT™ First Strand Synthesis System for RT-PCR	GibcoBRL

2.4 Enzymes

Table 3: Enzymes used for molecular experiments.

enzymes	company
DNA Polymerase I Klenow Fragment	Amersham, Gibco BRL
Restriction enzymes	Gibco, MBI Fermentas, New England Biolabs, Amersham, Stratagene, Promega
RNaseA	Sigma
Taq-polymerase	Eppendorf
T4-DNA-Ligase	GibcoBRL

2.5 Oligonucleotides

Table 4: Table includes nucleotides used for plasmid rescue and cDNA sequencing.

Oligonucleotide	Oligonucleotide sequence	company
T7	AAT ACG ACT CAC TAT AGG	
PCR1	CGA CGG GAC CAC CTT ATG TT	Gibco BRL
PM001	CGT TAG AAC GCG GCT ACA AT	
PCR2	TCA CTC AGA CTC AAT ACG ACA	Gibco BRL
Pout	CGA CGG GAC CAC CTT ATG TTA TTT CAT CAT	Gibco BRL

Table 5: Table shows oligonucleotides for characerization of *ign^{P1}*, cDNAs SD05277, GH21818, GH08264 and deletion lines of *ign^{P1}*. Primer name, primer binding site, nucleotides and direction (f = forward, r = reverse) are indicated. All oligos were ordered from Invitrogen.

Primer no.	scaffold position	nucleotides	direction
1	27853	GAG AAT GAT TTG GCC CGT G	f
2	28537	ACC CAG ACA GCG TTT TTG	r
3	27172	TTG CTG CTC CGC ATT GTT G	f
4	27512	GCA GGG AAA CCA GAG AAA TC	r
5	27411	TCT CCC TAC TTC CGA TTT CAC	f
6	27758	GCT GAA TAC GCA CAG TAA AAA C	r
13	28945	CAA GGC AGT ACA GAA ATG GAC	f
14	29540	GCA GAA ATG ACA GAG ACC AG	r
15	26133	ACC AAT CAG CGG CAA AAT C	f
16	26727	AAG GAA GTC ATC AAG GAG GG	r
17	29602	CAG GCA AAT GAG GAG AAC AG	f
18	29929	GGT GGA TAA GCA AGC GAT AAG	r
19	25158	GCC GCA TAC TGG CAT ATA ATA TC	f
20	25460	CAG CAT CCA CAT CCA CTT C	r
21	31253	TTT CCA CTG TCC CAA GTC C	f
22	31569	GTT CCC CAA TAC GAC CTT TTC	r
23	23869	ATA TAG ATG CCC CGC ACA G	f
24	24338	GCA GCA GAA TCA CAT CTC C	r
25	25245	TTG TCC TTA ACA CCG CGC TG	f
26	25918	GCA CTG CTT TTT TGC CAC CAT C	r
27	24693	ACC TCG GGA GCC ACA AAA TTG G	f
28	25459	AGC ATC CAC ATC CAC TTC TGC C	r
29	27729	CAG CCG ATG TTT TTA CTG TG	f
30	28314	GCC TAA TTT TTG CCC TGT TTC	r
31	25411	CAT AAT CTC CAC CTC CTC CC	f
32	26071	CCG CCA GAG ACT ATG AAT C	r
33	24285	CGG AAA GTG GCA TCA ACA G	f
34a	24913	AGC CAA AGT TCC ATC CTA TTG	r
34	22640	GCA CAC ACA CAA GCT CGC AAA C	f
35	23262	CCG ACG TTC TTT CCA ACA ACT GC	r
36	21208	GGC AAC TGA TAA GAA ACA CAA G	f
37	21969	CAC AAA AAG GAC AGA GAC AAC	r
38	19876	AAT GAC GCC GTT TCA CGC ACC	f
39	20181	ATT GAG CAC GTT GAC CGC TTC C	r
40	25945	ACC CGC ACC CAA ACG ATT CTG	f
41	26159	AGG GAC ACG ATT TTG CCG CTG	r
42	26141	GCG GCA AAA TCG TGT CCC TTT C	f
43	26445	CTG GAT TTT CTT CGT GGC GGT G	r
44	24804	ACC AAC AAG AAA TAC TCG CAC	f
45	25235	CGG TAG CAT ATC TCC ATG AAC	r
46	27758	GCT GAA TAC GCA CAG TAA AAA C	r
47	15789	TGC TTT TCC CGT CAC ATC	f
48	15897	ACT ATT CGT CGT CTG CCT C	r
49	16546	GGA TTC AGC TTA CCC CAT TG	f
50	17327	GGC TGT GGA AAT AAG CGA G	r
51	42505	TCT AAT TAA AGC GGC GTC C	f
52	42610	ATA GCG CAC AAC ACA TCG	r

53	27053	CCA CTC TCA TCG TCC TC	f
54	26422	ATC ACC GCC ACG AAG AAA ATC C	f
55	26948	TCG AGC AGC ATC ACA TCT CAC C	r
56	26834	TGG GAC TCG GAA TCA CTC AG	f
57	27380	TGG AAA CGC AGG GGG AAC	r
58	23810	CGA ATC CTA AAG CAA GGG C	r
59	24038	ACA AAC GCT GGG CAA TC	r
60	27441	TCT CCC TAT TCC GAT TTC AC	f
61	27938	GTT TCG TGA CGA CGT TTT C	r
62	26493	CGA TGA AAG CAT GAC CCA C	f
63	27588	CGA AGC GGA TAG TAA AGC AG	r
64	21887	GGG GAA TTT AGT CGA GAG TTG	f
65	22000	CGA ACG GTC TCT TAC AAA AAT G	r
66	26842	GGA ATC ACT CAG CTC CAT AAG	f
67	27749	GCA CAG TAA AAA CAT CGG C	r
68	25460	CAG CAT CCA CAT CCA CTT C	r
69	25419	CCA CCT CCT CCC AAC AAT C	f
70	24304	GCT GTT GAT GCC ACT TTC C	r
71	22617	GAA GCA CAC ACA CAG CC	f
483lacWf	483	AAC GTG ACT GTG CGT TAG	f
699lacWr	699	CTC TTC GCT ATT ACG CCA G	r
918lacWr	918	AAC AAA CGG CGG ATT GAC	r
3860lacWf	3860	TGT TCT CGC TAT TAT TCC AAC C	f
4251lacWr	4251	GTT TTT AAG CAA ACT CAC TCC C	r
5664lacWr	5664	AGG CAA GGG CAT TCA GCA A	r
5820lacWr	5820	GGA AAA TCA GGT GTT CCC TGG C	r
5904lacWf	5904	AGC AAA TGT CAG CAC ACG	f
5921lacWr	5921	CGT GTG CTG ACA TTT GCT GAG	r
5922lacWr	5922	TCG TGT GCT GAC ATT TGC	r
5980lacWr	5980	GCC AGA CGC TTC CTT TCT CC	r
6249lacWr	6249	AAA CAC ATC GAA CTC ACT AGG	r
6453lacWr	6453	CAA CAA CTG CTC CAT ATC CC	r
7436lacWf	7436	GAT TAA CCA ATG GGC GGA C	f
7864lacWr	7864	GTA AGG TAT GCA GGT GTG TAA G	r
9259lacWf	9259	TGG ATG GAG GCG GAT AAA G	f
9834lacWr	9834	CTA CGG CTA CAC TAG AAG GAC	r
10031lacWf	10031	ACA CCG AAC TGA GAT ACC TAC	f
10252lacWf	10252	TGG AAA AAC GCC AGC AAC	f
10444lacWr	10444	CAT CAA CTC CAT CAC TGT CC	r

2.6 Radionucleotide

For radioactive labeling the radionucleotide [α-^{32}P]-dCTP (3000 Ci/mMol) from Amersham was used.

2.7 Size standard

All used size standards were ordered from GibcoBRL:

1 kb plus DNA Ladder
100 bp DNA Ladder
High DNA Mass™ Ladder

2.8 Bacteria strain

For transformation expeiments the bacterial strain DH5α with the genotype deoR, endA1, gyrA96, hsdR17 (r_k- m_k+) recA1, relA1, supE44, thi-1, Δ(lacZYA-argFV169 was used.

2.9 Clones

2.9.1 *Drosophila* BAC clone

BACCR05K22(AC011760)

2.9.2 *Drosophila* EST clones

Table 6: Table shows the names of EST clone, cloning vector and antibiotic resistance.

EST clone	vector	resistance
GH08264	pOT2a	chloramphenicol
GH21818	pOT2a	chloramphenicol
LD42024	pOT2a	chloramphenicol
SD05277	pOT2a	chloramphenicol

2.10 Vectors

placW
pOT2a
pBluescript KS (+)
pW8

pCR^{R}4-TOPO^R

2.11 Specific software

Corel Draw Version 8
(Corel)

Excel Version 97
(Microsoft)

GCG Version 8
(Genetics Computer Group)

Microsoft Word Version 97
(Microsoft)

Photoshop Version 5.5
(Adobe Systems Incorporated)

Statistica Kernel-Version 5.5
(StatSoft Incorporation)

Turbo Pascal Version 6.0

2.12 Technical devices

Centrifuge:
    Centrifuge Model J2-21 (Beckman Instruments)
    Centrifuge 5414C (Eppendorf)

DNA / Protein amount:
    DU-40 Photospectrometer (Beckman Instruments)
    BioPhotometer (Eppendorf)

Electrotransformation:
    Electroporator (constructed by the workshop of the genetic department)

Gel electrophoreses:
    Electrophorese chamber (Biorad „DNA Sub cellTM")

PCR / Sequencing:
    Thermocycler (Omnigene)
    Mastercycler$^®$ Gradient (Eppendorf)

Southern blotting:
    UV-Stratalinker (Stratagene)

2.13 Histochemical Methods

2.13.1 *In situ* Hybridisation

In situ hybridisation on polytene chromosomes gave the approximate locus of the P-elements in mutant lines.

2.13.1.1 Preparation of chromosomes of the salivary glands

Salviary glands of 3rd instar larvae of *Drosophila melanogaster* were prepared in *Drosophila*-Ringer and fixed with a tiny drop of a mixture containing lactic acid / water / acetic acid (1 / 2 / 3) for 4 minutes. After transfer onto a slide, salivary glands were squeezed by knocking carefully with a preparation needle to destroy the tissue and spread the chromosomes. The preparation was kept at room temperature for 1 hr and then stored at 4 °C overnight (o/n).
Next morning, the slides were frozen in liquid nitrogen. After removing the cover slip, each slide was transferred to ethanol at –70 °C for 3-4 hr and then dried at room temperature.

2.13.1.2 Biotin labeling and dot blot

Biotin labeling reaction:

> 74 µl H_2O
> 6 µl DNA (0,37 µg/µl = 2 µg DNA)
> 10 µl 10x dNTP Mix (Gibco Kit)
> 10 µl 10x Enzyme Mix (Gibco Kit)
> 100 µl final volume, store 1 hr at 16 °C

A reaction volume of 100 µl is sufficient to label ten slides. The reaction is stopped by applying 10 µl of loading buffer (GibcoKit), followed by ethanol precipitation and resuspension in 50 µl TE.
To control for the successful labeling with biotin, a dot blot was performed. For this, 1 µl of the probe was twice applied to a nylon membrane and fixed via auto-crosslinking with UV light. The nylon membrane was kept for 15 min in blocking buffer at 37 °C and subsequently exposed to an avidin complex for 1 hr. After several washing steps with washing buffer 1 and 2, a staining reaction was started by incubation in 3 % H_2O_2 in 1 ml DAB and stopped with a washing step after 10 min.

Brown staining was observed at the application site of the probe with succesful labeling.

2.13.1.3 Preparation of polytene chromosomes

After biotin labelling, the slides were incubated in 2x SSC at 68 °C for 30 minutes and then acetylated for 10 min in a solution of 200 ml 0.1 M Triethanolamine-HCl and 250 µl acetic acid anhydride. Next, they were four times washed in 2x SSC for 4 min and then denatured in 0.07 N NaOH for 3 min. After a final washing step in 2x SSC, the slides were dehydrated with ethanol and dried.

2.13.1.4 Hybridisation

For hybridisation, the probe was boiled for 5 min, then instantly placed on ice and provided with 100 µl hybridisation buffer. 18-19 µl of probe was pipetted onto each slide and the slides afterwards covered with a cover slip and sealed with nail polish. The slides were incubated in a humid chamber o/n at 58 °C.

2.13.1.5 Signal detection

The cover slips were removed the following day. Several washing steps were followed:

2x SSC	15:00 min	53 °C
2x SSC	15:00 min	53 °C
2x SSC	15:00 min	53 °C
2x SSC	2:00 min	25 °C
0.05 % TritonX100/ PBS (pH 7.4)		
	5:00 min	25 °C
1x PBS	2:00 min	25 °C

When all washing steps were finished, 90 µl of DETEK Hrp complex dilution buffer / DETEK Hrp complex (100 / 1) was applied to each slide and a cover slip added. After incubation for 2 hr at 37 °C in a humid chamber, the cover slips were removed again and the slides washed three times for 5 min in 1x PBS at room temperature. Then, staining was performed, applying 190 µl of DAB 3 % H_2O_2 (100 / 1) onto each slide. 10 min later, the reaction was stopped by washing the slides three times with H_2O. After drying the slides,

the chomosomes were stained for 30 sec in Giemsa solution (Ashburner, 1989). Giemsa was removed by a washing step in water. The slides were dried and covered with a cover slip after applying DePeX (Ashburner, 1989). Evaluation of *in situ* hybridisations was performed at the microscope.

2.14 Molecular techniques

Standard molecular methods such as preparations of competent cells, restriction digests, other enzymatic reactions, PCR reactions, DNA cloning techniques, and DNA preparations were performed according to the methods described in Ausubel (Ausubel et al., 1994) and Sambrook (Sambrook et al., 1989).

2.14.1 Plasmid rescue

2.14.1.1 Isolation of genomic DNA

Genomic DNA of 50 adult flies was isolated in the following way:

1. 50 flies were homogenized in 500 µl homogenisation buffer using tight fitting glass homogenizers
2. Incubation of the homogenate at 68 °C for 30 min
3. Protein / SDS precipitation with 75 µl 8 M KAc
4. DNA precipitation with 1 ml 100 % ethanol
5. Pellet was washed with 500 µl 70 % ethanol and resuspended in 360 µl TE
6. 1 µl RNaseA (10 mg/ml) was added for 10 min at 37 °C
7. 40 µl 3 M NaAc was added
8. Extraction with 500 µl phenol, organic phase was removed
9. 500 µl CIA was added, organic phase was removed
10. Steps 8 and 9 were repeated
11. DNA precipitation with 1 ml 100 % ethanol
12. Pellet was washed with 500 µl 70 % ethanol and resuspended in 50 µl TE

approximate yield: 50 flies $\approx$ 15 µg gDNA

2.14.1.2 Restriction digest of genomic DNA

Isolated genomic DNA was digest with restriction enzymes (see table below).
For digest:

      15 µl genomic DNA
       2 µl restriction enzyme
       2 µl respective buffer
       1 µl H$_2$O
      20 µl final volume of digest,
           incubate at 37 °C for 2 hr
           apply 7 µl of digest to a test gel

Table 7: Enzymes used for plasmid rescue:

P-element	enzymes plasmid rescue (3′ end)	enzymes plasmid rescue (5′ end)
PlacW	EcoRI, SstII	XbaI, BamHI

2.14.1.3. Preparation and ligation of genomic DNA

1. 257 µl TE and 30 µl 3M NaAc were added to remaining 13 µl of digest
2. Extraction with 500 µl phenol, organic phase was removed
3. 300 µl CIA was added, organic phase was removed
4. Step 3 was repeated
5. DNA precipitation with 750 µl 100 % ethanol
6. Pellet was washed with 500 µl 70 % ethanol and resuspended in 100 µl H$_2$O
7. Recipe for ligation:

      100 µl digested DNA
       40 µl 10x ligation buffer
      256 µl H$_2$O
        4 µl T4 DNA ligase
      400 µl final volume of ligation
           o/n at 18 °C

8. 400 µl phenol / CIA (1 / 1) was added, organic phase was removed
9. 44 µl 3M NaAc and 1 ml 100 % ethanol were added
10. Pellet was washed with 500 µl 75 % ethanol and resuspended in 10 µl H$_2$O

2.14.1.4 Transformation

Generation of electrocompetent cells and electrotransformation were performed according to the methods described in Walter (Walter, 1991). After electrotransformation, cells were spread on LB-agar plates with 50 µg/ml Carbenicillin and incubated o/n at 37 °C.

2.14.1.5 Plasmid Mini preparation

Colonies of transformants were picked and transferred to master plates. Mini prep DNA was extracted by alkaline extraction procedure following the method of Birnboim and Doly (Birnboim and Doly, 1979). In this direction, 2 ml of an o/n culture of single colonies (LB + carbenicillin) were used.
Alternatively, plasmid Mini preparation was performed using reagents and protocol of QIAGEN® Plasmid Mini Kit from QIAGEN, Concert™ Rapid Plasmid Miniprep System from GibcoBRL or QIAprep Spin Mini Kit from GibcoBRL.

2.14.1.6 Plasmid Midi preparation

Plasmid Midi preparations were performed using the reagents and the protocols provided with QIAGEN® Plasmid Midi Kit from QIAGEN or Concert™ Rapid Plasmid Midiprep System from GibcoBRL. A volume of 50 ml o/n culture (LB + carbenicillin) was used for each reaction. The final DNA pellet was resuspended in 300 µl H_2O and Plasmid DNA concentration was determined by OD-measurements at 260 nm.

2.14.1.7 Determination of DNA concentration

The DNA to be measured was diluted by a factor of 40 (390 µl H_2O and 10 µl probe) and the plasmid DNA concentration afterwards determined at an absorption of 260 nm.
As reference 400 µl H_2O were used.

Convertion of the measured OD-value:
c = absolute value * 40 df * 50 [µg/ml]

40 df = dilution factor

50 µg/ml factor for double-stranded DNA

In case of single-stranded DNA a factor of 33 µg/ml is used

2.14.1.8 Sequencing

Sequencing reactions were performed in a Mastercycler®Gradient from Eppendorf or in a Thermocycler from Omnigene. In case the latter was used, the reactions were covered with 50 µl of mineral oil. Proceeding from midi preparations, 300-400 ng DNA was used. Alternatively, proceeding from PCR reactions, 80 ng DNA was necessary.

Sequencing reaction:
    2 µl ABI PRISM™ *BigDYE*™
            Terminator Cycle Sequencing
            Ready Reaction Kit
    4.5 µl 2 µM Primer
300-400 ng DNA
            add H_2O to final volume of 10 µl

Reaction cycles.

step	temp	duration	cycle no.
Denaturation	96.0 °C	0:15 min	
Annealing	50.0 °C	0:01 min	25 cycles
Elongation	60.0 °C	4:00 min	

The reaction was purified following the protocol of ABI PRISM™ for ethanol precipitation. Analysis was performed using the ABI PRISM 310 Genetic Analyzer at the Biocenter sequencing facility.

2.14.2 Single fly PCR

2.14.2.1 Fly homogenate

Fly homogenates were generated by smashing single flies in 50 µl SB and incubating the homogenates for 30 min at 37 °C. Proteinase K was then inactivated by heating to 95 °C for 1-2 min. The homogenates could be stored at 4 °C for several months.

2.14.2.2 PCR reaction

For PCR reaction:

x µl DNA (100 ng DNA)

41-x µl H_2O

boil together for 5 min,
chill on ice, spin briefly
then add

1 µl 2.5 mM $MgCl_2$

1 µl 2 mM dNTP

1 µl primer (1pmol/µl)

1 µl primer (1pmol/µl)

5 µl 10x PCR buffer

50 µl final volume

PCR reactions were performed in a thermocycler with the following program:

step	temp	duration	cycle no.
Denaturation	94.0 °C	5:00 min	1 cycle
add 0.2 µl Taq-Polymerase after 1 min			
Denaturation	94.0 °C	0:30 min	26 cycles
Annealing	50.0 °C	0:30 min	
Elongation	72.0 °C	1:00 min	
Final Exension	72.0 °C	5:00 min	1 cycle
Final Soak	4.0 °C	∞	

The reaction was covered with a layer of 50 µl mineral oil when a thermocycler without heated lid was used (e.g. thermocycler from Omnigene). PCR purification was done using the reagents and the protocol provided with the QIAquick PCR Purification Kit from QIAGEN. When several PCR products were obtained, I performed gel extraction using reagents and protocol delivered with the QIAquick Gel Extraction Kit from QIAGEN or with the QIAEXII Gel Extraction Kit from QIAGEN.

2.14.3 RT-PCR

2.14.3.1 Isolation of total RNA

To isolate total RNA for RT-PCR, 100 flies (50 % males, 50 % females) were homogenized in a glass homogenizer with 1 ml TRIzol Reagent and kept at room temperature for 5 min. The homogenate was then tansferred to an eppendorf tube to perform chloroform extraction and isopropanol precipitation. The pellet was air dried and resuspended in 100 µl DEPC-water.

2.14.3.2 Isolation of poly(A)$^+$ mRNA

Isolation of poly(A)$^+$ mRNA from total RNA was performed with Oligotex mRNA Mini Kit from QIAGEN following the protocol.

2.14.3.3 First-Strand Synthesis using Oligo (dT) and Random Hexamers

SUPERSCRIPT^{TM} First Strand Synthesis System from GibcoBRL was optimized to synthesize first-strand cDNA from varying amounts of purified poly(A)$^+$ or total RNA. After determination of poly(A)$^+$ mRNA concentration, RT-PCR was started with 100-500 ng poly(A)$^+$ mRNA and random hexamers as described in the protocol.
PCR followed using primer pairs 1/3Edi, 2/3 Edi, 15/16, 19/20 and 23/24.

PCR reaction:

1 µl cDNA

40 µl H_2O

1 µl 2.5 mM $MgCl_2$

1 µl 2 mM dNTP

1 µl primer (1 pmol/µl)

1 µl primer (1 pmol/µl)

5 µl 10x PCR buffer

50 µl final volume

PCR reactions were performed in a Mastercycler$^®$ Gradient from Eppendorf using the following program:

step	temp	duration	cycle no.
Denaturation	94.0 °C	5:00 min	1 cycle
add 0.2 µl Taq-Polymerase after 1 min			
Denaturation	94.0 °C	0:30 min	26 cycles
Annealing	51.0 °C	0:30 min	
Elongation	72.0 °C	1:00 min	
Final Exension	72.0 °C	5:00 min	1 cycle
Final Soak	4.0 °C	∞	

2.14.4 5´ RACE

For full-length RNA ligase-mediated rapid amplification of the 5´ cDNA ends the GeneRacer[TM] Kit and TOPO TA Cloning Kit for Sequencing from Invitrogen were used (Fig. 10). I followed their protocols, except for the mentioned steps.

RLM-RACE includes:
1. Generation of polyA$^+$-mRNA
2. Dephosphorylation of non-mRNA or truncated mRNA
3. Removal of the 5´ cap structure from full-length mRNA
4. Ligation of the GeneRacer[TM] RNA Oligo to the 5´ end of full-length mRNA
5. Reverse-transcribing of mRNA into cDNA
6. Amplification of cDNA ends

PCR reaction:
    1 µl 5´-cDNA CantonS
    5 µl Taq buffer
    1 µl 2 mM dNTPs
    1 µl MgCl$_2$
    1 µl gene specific primer (3, 5, or 53)
    1 µl Gene Racer 5´-primer
    <u>40 µl DEPEC-H$_2$O_____</u>
    50 µl final volume

add 0.3 µl Taq Polymerase

PCR program:

temp	duration	cycle no.
94.0 °C	0:30 min	5 cycles
72.0 °C	1:00 min	
94.0 °C	0:30 min	
70.0 °C	0:30 min	5 cycles
72.0 °C	1:00 min	
94.0 °C	0:30 min	
58.0 °C	0:30 min	20 cycles
72.0 °C	1:00 min	
72.0 °C	10:00 min	1 cycle

7. Purifying of the PCR products by gel extraction

TOPO TA Cloning includes:
1. Cloning of cDNA ends into pCR® 4-TOPO® vector
2. Chemical transformation into TOP10 One Shot® Chemically Competent *E. coli*
3. Mini preparation
4. Restriction digest
5. Sequencing with M13 forward or M13 reverse
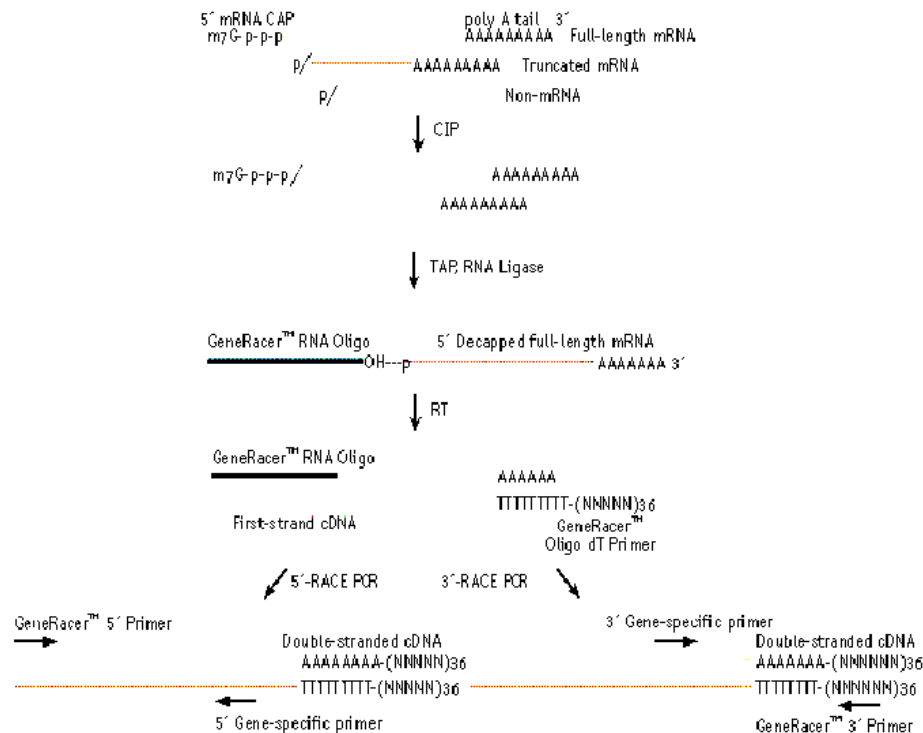
Figure 10: Schematic diagram of RLM-RACE. Figure from Invitrogen GenRacer[TM] protocol.

2.14.5 Southern blot

2.14.5.1 Generation of the DNA probe

Probes for Southern blotting were obtained in two different ways. The probe was either generated by PCR and purification or by isolation of genomic DNA, preparative digest, and gel extraction.

For preparative digest:
        10 µl DNA (2 µg DNA)
         2 µl restriction enzyme
         4 µl respective buffer
         1 µl RNase (1mg/ml)
        23 µl H$_2$O_____
        40 µl final volume,
                incubate at 37 °C for 2 hr

After the digest, 60 µl H$_2$O was added. The DNA was cleaned with a phenol wash and precipitated with ethanol. The pellet was resuspended in 20 µl TE, 4 µl loading buffer was added and applied to an analytical gel. Gel extraction started from gels of 0.7 % using reagents and protocols from QIAEXII when large DNA fragments were used as probes, whereas gels of 1.0 % and Gel Extraction Kit from QIAGEN were used when small DNA fragments were extracted.

When PCR was used for generating the probe, PCR purification was done using the reagents and the protocol provided with the QIAquick PCR Purification Kit from QIAGEN. If several PCR products were obtained, gel extraction of a single product was performed as described.

2.14.5.2 Labeling of the DNA probe

The DNA probe for Southern blotting was labeled with [α-^{32}P]-dCTP after a modified protocol of the Multiprime labeling System from Amersham (Feinberg and Vogelstein, 1983).
100 ng DNA in a volume of 14 µl H$_2$O was denatured at 100 °C for 10 min and afterwards immediately placed on ice for 1-2 min. After brief centrifugation, the following reaction was started.

Labeling reaction:
        14 µl denatured DNA
         5 µl labeling buffer
        2.5 µl Primer / BSA-solution
        2.5 µl [α-^{32}P]-dCTP (25 µCi)
        0.7 µl Klenow-Polymerase_____
                incubate for 30-40 min at 37 °C

After incubation, the reaction was stopped with 5 µl 200 mM EDTA and H$_2$O was added for a final volume of 70 µl. To test for the incorporation of radionucleotides, 990 µl 3.5 % perchloric acid (PCA) / 100 mM NaPPi solution and 10 µl Carrier DNA were applied to a 1 µl labeling reaction. The separation of radionucleotides which were not incorporated was achieved by vacuum filtration. Radioactivity of the filter paper (GF52, Schleicher & Schuell) used was measured with a Geiger counter. Succesfully labeled probes were denatured again for 10 min at 100 °C.

2.14.5.3 Southern blotting

DNA was transferred onto a Nylon Membrane by vacuum blotting at 55 mbar using the following solutions:

1. Depurination solution
    0.25 N HCl   12:00 min
2. Denaturation solution
    0.5 N NaOH, 1.5 M NaCl   12:00 min
3. Neutralisation solution
    0.5 M Tris, 1.5 M NaCl (pH8.0) 12:00 min
4. Transfer solution
    20x SSC   2:00 hr

The gel was washed with H$_2$O between the different steps to remove remaining solutions. After the transfer the DNA was covalent bound to the membrane by UV-crosslinking.

2.14.5.4 Hybridisation and washing

The blots were prehybridised for 2 hr at 42 °C with hybridisation buffer. Afterwards, the labeled and denatured probe was added and the blot hybridised o/n at 42 °C. The next morning, the membrane was washed 4 times for 20 min with a washing buffer (0.2x SSC / 0.1 % SDS) at 68 °C. After 24-48 hr of exposure at –80 °C, the x-ray films were developed.

3 RESULTS

3.1 Characterization of memories in the *Drosophila* heat-box conditioning paradigm

3.1.1 Influence of age, sex, and larval density on test performance

To gain a better understanding of heat-box conditioning, I searched for optimal rearing and training conditions. Age and gender of flies as well as larval density were the first variables investigated. If not mentioned differently, measurements were performed using the modified version of the heat-box.

3.1.1.1 Age and Sex

Wild-type CantonS flies of different ages were tested to examine the influence of age and gender on the performance of flies in heat-box conditioning. Each animal was trained for 4 minutes with the standard protocol and was only tested once. Comparing 1-min test performances of males and females of the same age, I did not find any difference between gender (Fig. 11; U-tests, p=n.s.) and, therefore, pooled data in further analysis.

The next question was whether age could influence the performance of flies. Age of tested flies ranged from 2 to 29 days (d) after eclosion. The results show that increasing age does not lead to a decrease in test performance as 4 week old flies still performed well. In this series, 1-min test performance of 5 and 9 d old flies was significantly reduced compared to the performance of younger and older flies (Anova, H=50.67, p<0.000; U-test, see Appendix Table 1). However, remeasurements of 5 to 9 d old flies did not confirm these results (data not shown). The described low performance cannot be explained by differences in daily performance (e.g. weather) as all groups were measured in parallel and for several days.
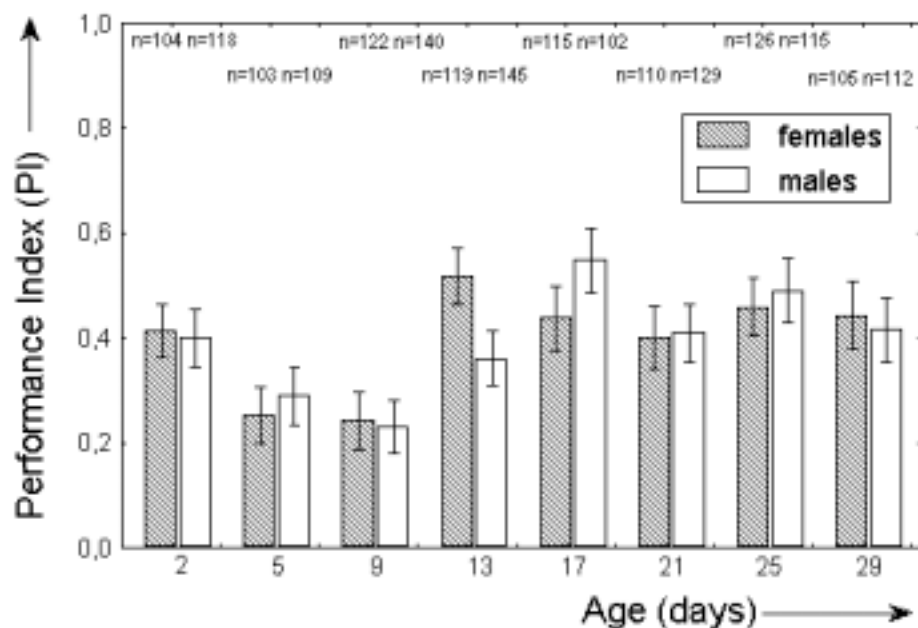
Figure 11: Standard learning experiment of wild-type CantonS flies of different ages. Figure shows 1-min test performance after 4-min training. Males (empty bars) and females (hatched bars) were measured separately 2 days (d) to 29 d after eclosion. Each fly was only tested once.
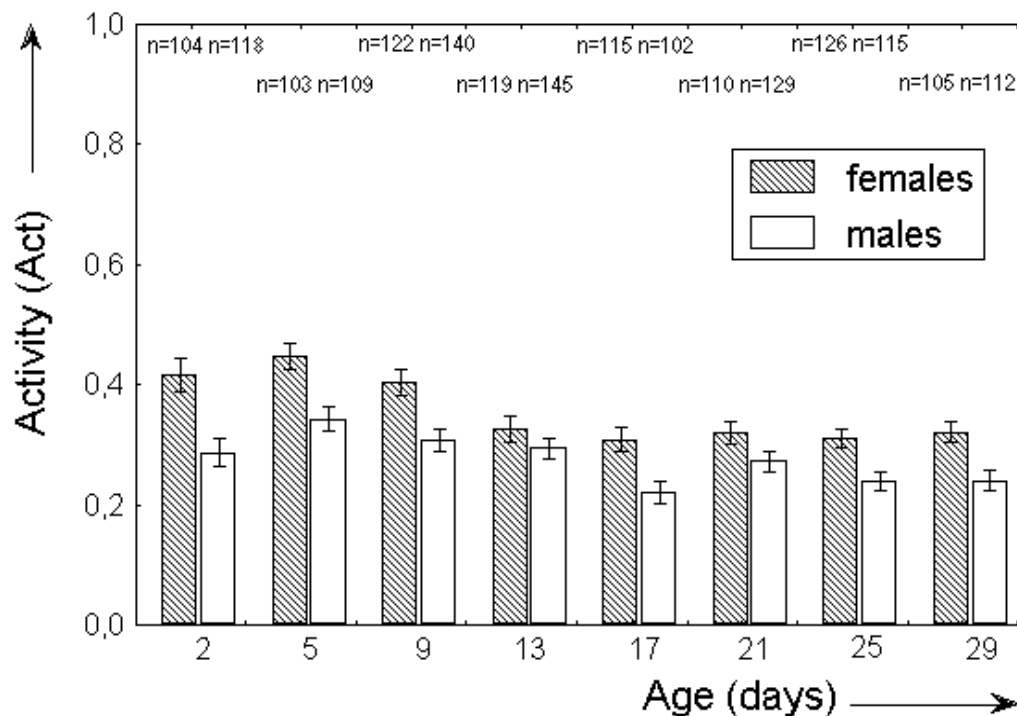
Figure 12: Walking activity of wild-type CantonS flies of different ages in the standard learning procedure (same flies as Fig. 11). Figure shows 1-min tests after the standard training procedure of 4 min. Males (empty bars) and females (hatched bars) were measured in separate groups. Age of tested flies ranged from 2 to 29 d with each animal being tested only once.

It may have resulted from bacterial infection or an attack of mites, which have a negative effect on the state of health of the flies. Reduced test scores could also be due to strongly increased walking activity. Hyperactive flies might pay less attention to the contiguity of behavior and reinforcement and, therefore, obtain low performance values. To test the latter idea, the walking activity of the flies was analysed: Figure 12 shows the 1-min test activity corresponding to the performance values in Figure 11.

In general, females were more active than males of the same age (for 13 and 21 d old flies this difference was not significant; U-tests, see Appendix Table 2). Thus, activity data for males and females were analysed separately. Males at an age of 5 and 9 d were more active than older ones (Anova, $H_{males}=26.90$, p<0.001; U-tests, see Appendix Table 3), in females this tendency was even stronger (Anova, $H_{females}=39.68$, p=0.000; U-tests, see Appendix Table 4). The finding that

5 to 9 d old flies, which were characterized by reduced test performance, at the same time have an increased walking activity raises the question whether walking activity and performance are negatively correlated. A comparison of walking activity and performance of all tested flies in the memory test showed a weak negative correlation for the two parameters (Pearson Korrelation, r=-0.39; p<0.01; n=1874). The correlation is already visible in the pretest (see next page Fig. 13; Pearson Korrelation, r=-0.13; p<0.01; n=1874).

From the results, I conclude that sex and age themselves are not critical parameters influencing performance of wild-type CantonS flies in the heat-box, whereas activity of experimental flies should be considered in data interpretation. For the behavioral characterization of *Drosophila* mutants, however, it might be interesting to analyse performance of males and females separately to control for sex specific influences of genes.
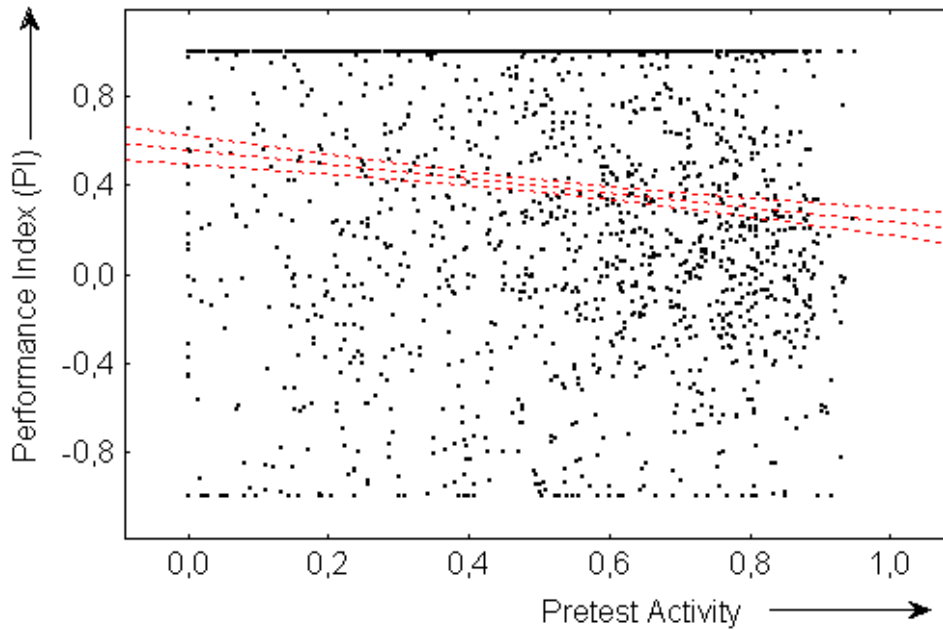
Figure 13: Correlation of the pretest walking activities versus the 1-min test performances of wild-type CantonS flies in the standard learning procedure (same flies as Fig. 11 and 12; n=1874). Figure also shows regression line and SEM line.

3.1.1.2 Larval density

High larval density leads to undernurished larvae and finally small adults. To test whether suboptimal raising conditions impair the performance of flies in operant conditioning two experimental groups were established: In one group, about 50-100 parental flies were allowed to lay eggs in fresh food vials over night, while in another group the same number of flies were given an egg laying period of three days. In the latter group, the same flies were allowed to lay eggs for three days to mimic a situation where offspring are raised after uncontrolled egg laying periods of the parents. Thus, I did not control for the age of the mothers. Offspring of both groups were reared and subsequently measured in the heat-box (Fig. 14).

After a 2-min training period, males (U-test, Z=1.03, p=n.s.) and females (U-test, Z=1.46, p=n.s.) raised in overcrowded vials obtained test scores comparable to that of flies raised at low larval density. Larval density is, therefore, not a critical parameter for heat-box experiments. Nevertheless, in all following behavioral experiments larval density was controlled to enable optimal food supply for larvae and adults.
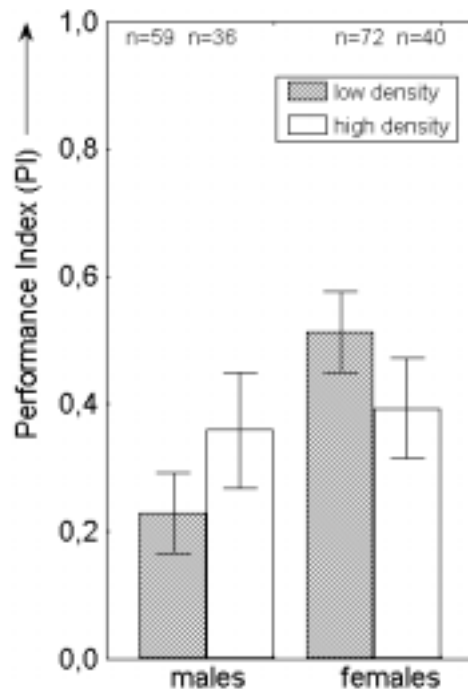
Figure 14: Performance of wild-type CantonS flies grown at low (hatched bars) and high (empty bars) larval density in a learning experiment. Figure shows the 1-min test scores.

3.1.2 Influence of training procedures on test performance

3.1.2.1 Improved memory after intermittent training

The standard 4-min of training in the heat-box leads to a final avoidance of PI=0.60 +/- 0.04 and a 3-min memory score of PI=0.35 +/- 0.03 (Fig. 15A; see also Zars et al., 2000a). If the training is extended to 12 minutes a final avoidance of PI= 0.85 +/- 0.02 and a memory score of PI=0.56 +/- 0.02 is obtained (Fig. 15B). As spaced training in other learning paradigms has been shown to generate a more robust memory (Tully et al., 1994; Xia et al., 1997a), I investigated whether splitting the training session into several cycles of training and intermittent test phases might further increase performance. In Fig. 15C, training consists of six 2-min periods separated by 1-min test phases. Flies of Fig. 15B and C were taken from the same batches. Trained intermittently they show higher PIs during the training and test phases than with continuous training (U-test, Z=4.34, p<0.001 for final 3-min memory score). Also, memory decay is slightly slower after intermittent training which is evident in the slope (Fig. 16; same data as Fig. 15B, C).
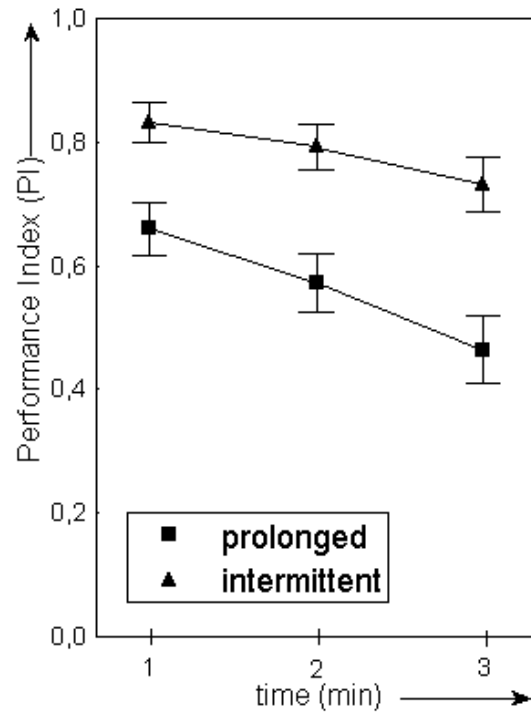
Figure 16: Extinction during the final test is slower with intermittent (triangles, n=145) than with continuous training (squares, n=146). Figure shows PIs of memory tests of Fig. 15 binned to 1-min blocks.
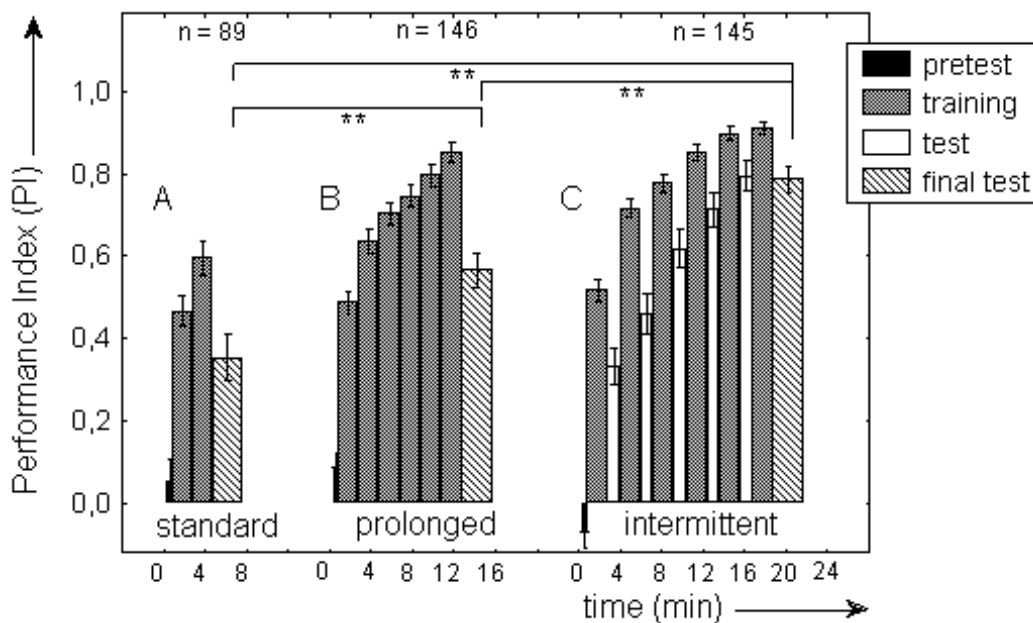
Figure 15: Continuous (A, B) versus intermittent (C) training in CantonS flies. Performance Index (PI) includes 30-sec pretest (black bars), training (densely hatched bars, 2 min each), intermittent test phases (only in C, empty bars, each 1 min) and final test (broadly hatched bars, 3 min). Error bars are SEMs; n indicates number of flies; * p<0.05, ** p<0.01, *** p<0.001, as in all subsequent figures.
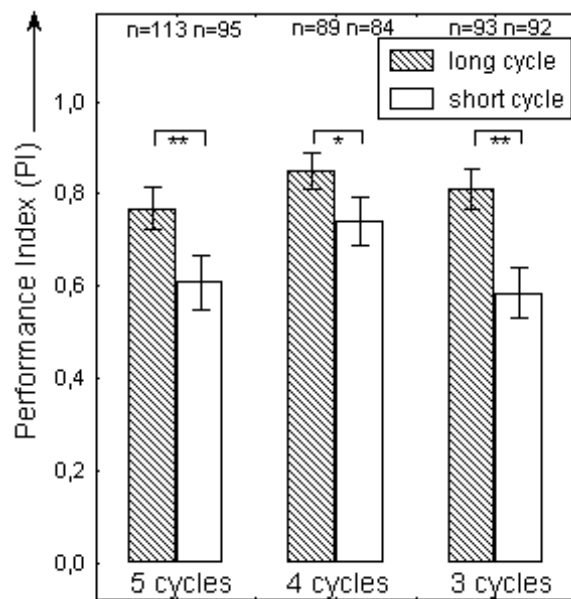
Figure 17: Different training regimes. Flies were trained either 5, 4 or 3 times with either short cycles of 2-min training / 30-sec intermittent test (empty bars) or long cycles of 4-min training / 1-min test (hatched bars). Figure shows PIs of the final 3-min tests for all six groups.

3.1.2.2 Influence of cycle number and duration of training

To optimize the memory score, I performed a parametric study of increasing cycle number (from 3 to 5) for short cycles of 2-min training and 30-sec test as well as for cycles of 4-min training and 1-min test. After training, all six experimental groups received a 3-min memory test. Figure 17 shows that the duration of cycles influences test performance. In all groups, from 3 to 5 cycles, long cycles lead to a significantly higher test performance than short cycles (U-tests, 3 cycles: $Z=4.13$, $p< 0.001$; 4 cycles: $Z=2.31$, $p<0.05$; 5 cycles: $Z=3.02$, $p<0.01$). In contrast, the number of long cycles does not significantly influence test performance (Anova, $H=1.97$, $p=$n.s.). Comparing the test performance of short cycles, 4 cycles lead to a higher test performance than 3 cycles (Anova, $H=7.18$, $p<0.05$; U-test, 4 versus 3 cycles: $Z=2.68$, $p<0.01$), whereas 5 cycles give no significantly better result than 4 or 3 cycles. The data show that long training / test cycles lead to better test performances than short ones. Whether this difference is due to the total training time or to an inter-trial-interval effect remains open. In any event, based on the above experiments, four long cycles were used in most of the following experiments including intermittent training, as they seem to yield asymptotic values.

3.1.3 Separation of two memory components

In the heat-box a fly can avoid the 'punished' side because it can switch off the heat. If a fly has no control of the heat punishment it does not develop a side preference. This obvious effect can be visualized in a 'yoked control' experiment (see next page Fig. 18A). Flies were treated with seven training/test cycles (2-min of training and 1-min test). One group was able to control chamber temperature by its position in the chamber (experimental) while in the other group each fly experienced a temporal sequence of hot and cold temperatures generated by one of the flies in the first group, but had no influence on the temperature (yoked control).

Experimental flies reach a performance index of $PI = 0.84$ +/- 0.06 in the test after 12 minutes of training while yoked flies have no positive avoidance or memory scores. Interestingly, flies that have the possibility to control the chamber temperature, reduce their locomotor activity more than flies that have no influence on the temperature (Fig. 18B). Already in the second training/test cycle, the performance value of experimental flies is significantly reduced compared to yoked flies (U-tests, second training period: $Z=3.10$, $p< 0.01$; second test period: $Z=2.95$, $p< 0.01$). Figure 18C shows this difference. The standard training procedure with experimental and yoked control groups gives a similar result (U-tests; performance: first training min: $Z=6.87$, $p< 0.001$; final test: $Z=4.77$, $p< 0.001$; walking activity : last training min: $Z=-2.00$, $p< 0.05$; final test: $Z=-2.40$, $p< 0.05$; see next page Fig. 19).
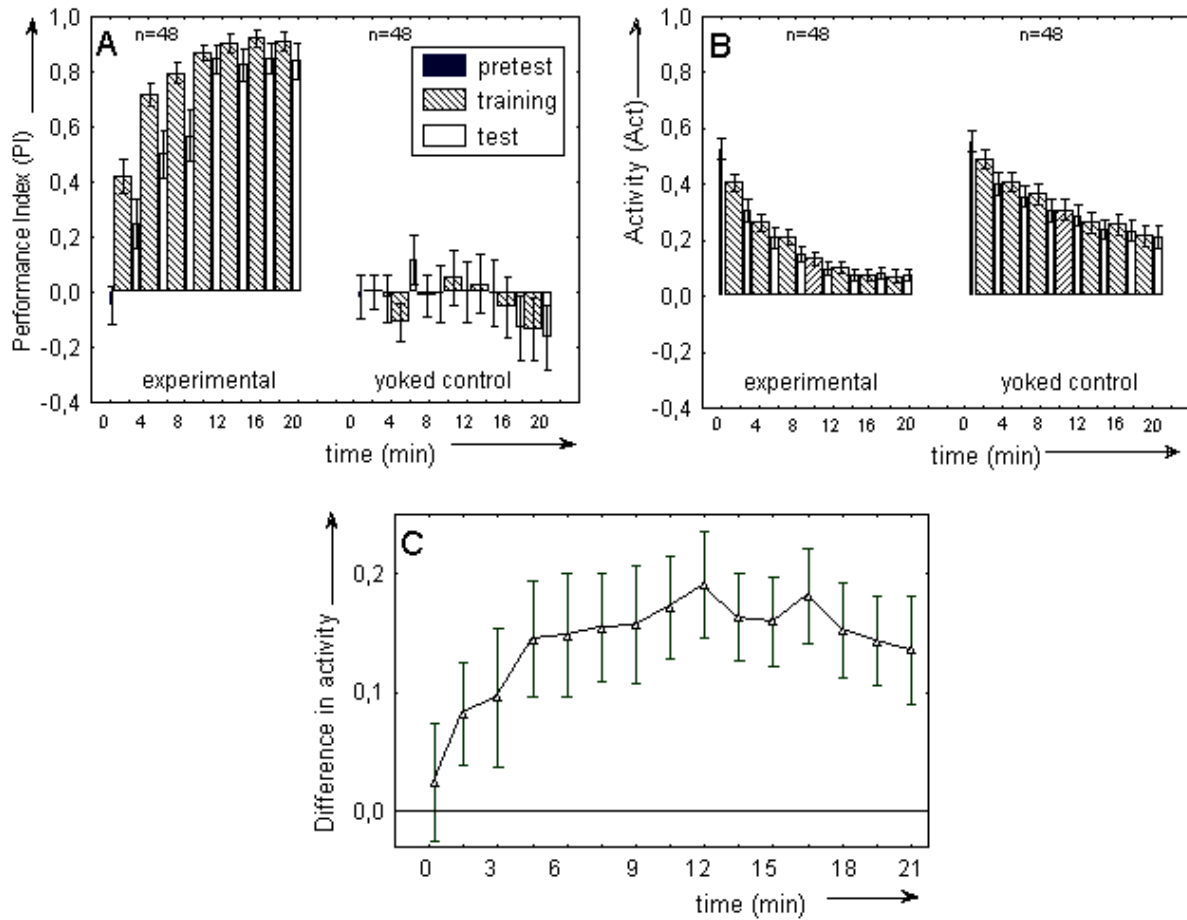
Figure 18: Yoked control experiment. Experimental flies have the possibility to control heat punishment. Each fly of the yoked control group gets the same heat regime as a particular fly of the experimental group, independently of its behavior. A) Performance Index of the experimental group (experimental) with intermittent training versus the yoked control group (yoked control). Performance Index (PI) includes pretest (pre, black bars, 30 sec), training (tr, hatched bars, each 2 min), test phases (te, empty bars, each 1 min), B) locomotor activity of experimental and yoked flies (same experiment as in A); C) difference in locomotor activity between experimental and yoked control group.
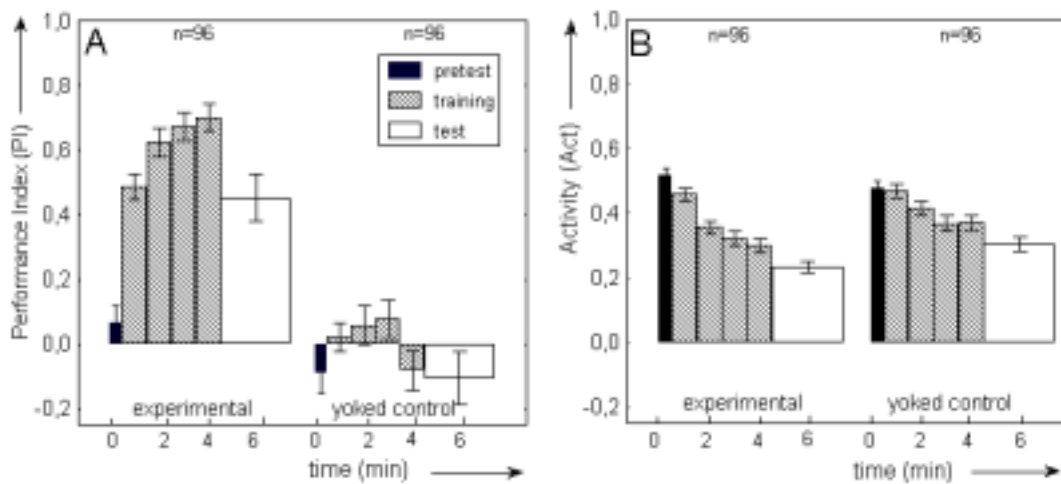
Figure 19: Yoked control experiment. A) Performance Index of the experimental group (experimental) with 4-min training versus the yoked control group (yoked control). Performance Index (PI) includes pretest (pre, black bars, 30 sec), training (tr, hatched bars, each 1 min) and final test (te, empty bars, 3 min), B) locomotor activity of experimental and yoked flies (same experiment as in A).

Several explanations can account for the additional decrement in locomotor activity in experimental versus yoked control animals. One possibility is that experimental flies utilise activity reduction to avoid the heat. They might learn that with heat off, slow / no walking is a successful strategy (contributing to a 'stay-where-you-are' effect). Another explanation takes the temporal patterns of spontaneous locomotor activity into account (Martin et al., 1999). Flies have their individual schedule of activity and rest periods. Activity bouts and pauses are not synchronised between flies. During training the flies in the experimental group can follow their endogenous temporal pattern with minimal adjustments, whereas in the yoked flies the heat pulses during rest periods may induce additional activity bouts.

As mentioned in the introduction, the memory test in the present paradigm immediately follows the training phase and is, therefore, not a pure measure of the fly's preference for one or the other half of the chamber. It includes an aftereffect of heat avoidance at the end of the training period that leaves most of the flies on the unpunished side. The contribution of this effect is difficult to assess directly.
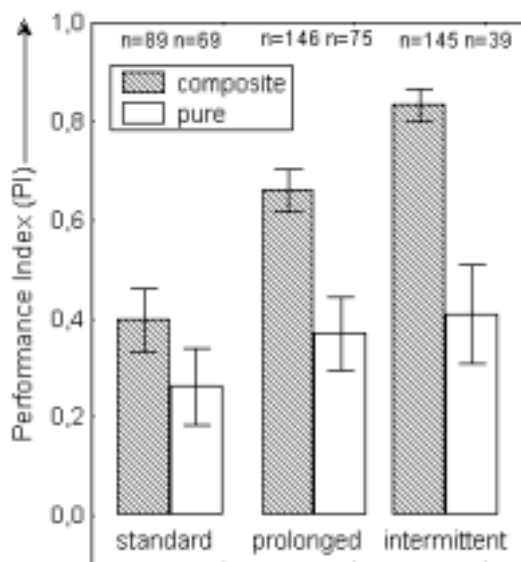
Figure 20: 1-min test scores starting immediately after training (composite; hatched bars) versus conservative estimates (pure; empty bars) after different training regimes (4 min, 12 min continuous, and 12 min intermittent training; same data as Figs. 15). Note different numbers of flies in composite and pure scores.

A lower estimate of the true spatial memory component can be obtained by starting the memory test for each fly only after the first midline crossing (Fig. 20; Wustmann et al., 1996). This evaluation excludes flies that after training stay on the unpunished side for the whole test period (stay-where-you-are).

After continuous 12-min training this low estimate during the first minute of the evaluated test phase is PI=0.37 +/-0.06, after intermittent training it is PI=0.41 +/- 0.08 (Fig. 20). This small difference suggests that most of the memory increment of the intermittent training over the continuous training is due to an increasing fraction of flies spending the whole test period on the formerly unpunished side. Taking into account that the early part of the test is discarded we conclude that the spatial choice component accounts for at least half of the total memory score.

3.1.4 Investigation of memory retention

3.1.4.1 Associative memory after transfer

Flies were trained intermittently with four cycles of 4-min, removed from their chamber and immediately transferred to a new chamber where they received a 30-sec reminder training during which they had to experience heat at least once to be included in the ensemble average (experimental design: Fig. 4a). In the subsequent 6-min test without heat punishment they showed a small but significant memory score (Wilcoxon, p<0.01) as observed before under slightly different conditions (Wustmann and Heisenberg, 1997). They were compared to control groups of naïve flies and to flies that had been kept in the chamber for 20 min just like the first group but without training, at a constant low temperature (see next page Fig. 21; experimental design: Fig. 4a-c). Neither the naïve group nor the exposed group showed a significantly positive PI in the test. This result demonstrates that after a short training of 30 seconds the *stay-where-you-are* effect is minimal. In all transfer experiments we, therefore, disregarded the *stay-where-you-are* effect and directly used the memory scores for further evaluation.
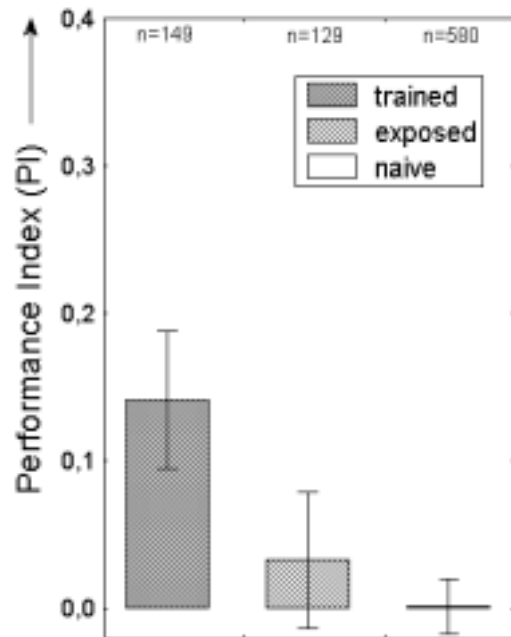
Figure 21: Direct transfer between two chambers. Flies are either trained (trained; densely hatched bar) or just kept in the first chamber for the corresponding time without heat punisment (exposed; broadly hatched bar). After tranfer, all flies receive a short training of 30 sec and finally their memory was tested for 6 min. Control animals (naïve; empty bar) underwent only the 'reminder' training and the final test. Figure shows PIs of the final 6-min tests.

Figure 22: Yoked control experiment with direct transfer. Flies of the experimental group can control heat punishment during intermittent training, while flies of the yoked control group can not. Immediately after training flies were transferred to another heat chamber where they received a 30-sec reminder training and a 6-min test. Both groups can control heat punishment during the reminder training. Figure shows the PIs of final 6-min memory tests for the experimental and yoked control groups.

To test whether memory scores after transfer are the result of an operant associative learning process or are due to a motivational change, a yoked control experiment was performed. One group of flies was able to control during training the temperature by its position in the chamber (experimental group), while the other group received the heat 'punishment' independently of its behavior (yoked control group). The flies were subsequently transferred to a new chamber where all of them received a 30-sec reminder training and were finally tested. Yoked control flies had a significantly decreased test performance compared to experimental flies (Fig. 22; U-test, Z=2.76, p< 0.01). Their memory score was statistically indistinguishable from zero (Wilcoxon, p=n.s.). Thus, I conclude that positive performance values of flies after transfer are the result of an associative learning process in the heat-box.

3.1.4.2 Two-hour memory

How long does the fly retain the link between its position in the chamber and temperature? To measure memory retention without extinction training in the time interval between memory acquisition and test, flies must be kept in a different environment during that period. Flies were transferred after the training first to a food vial for various intervals (either 1- 3 min, 30 min, 2 hrs, or 4 hrs) and afterwards back into a chamber for reminder training and test (see next page Fig. 23, squares; experimental design: Fig. 4d). They showed PIs significantly different from zero for retention intervals of up to 2 hrs (Wilcoxon, 1-3 min: p<0.01; 30 min: p< 0.001; 2 hr: p<0.05; 4 hr: p= n.s.).
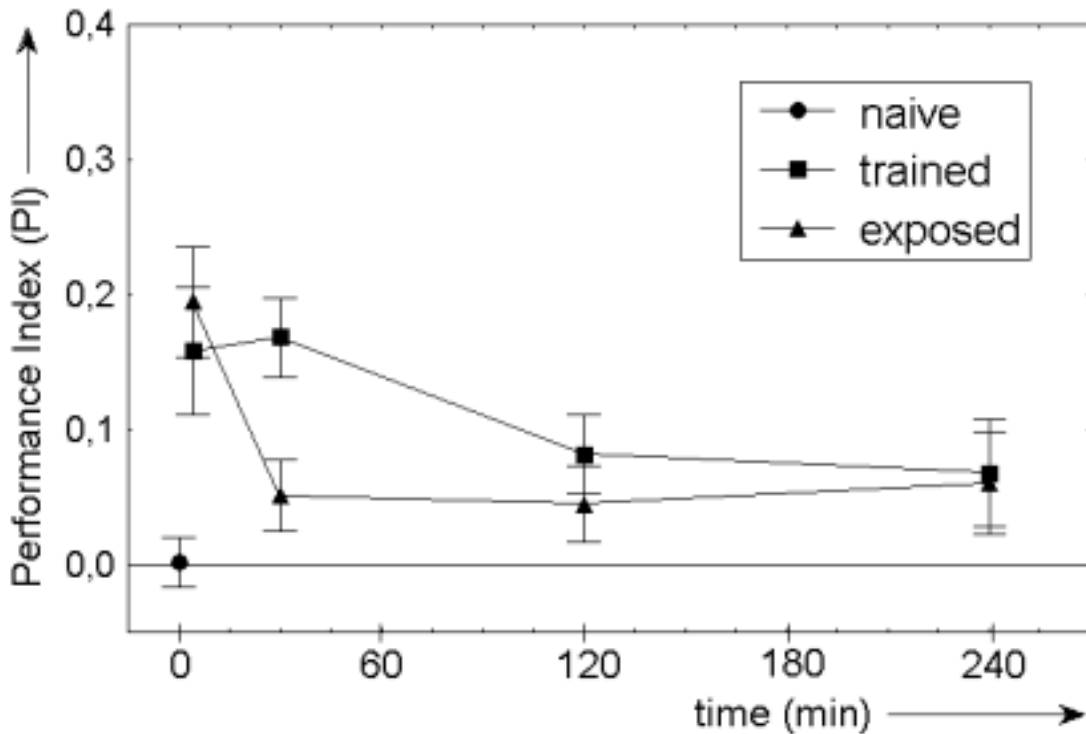
Figure 23: Indirect transfer. Flies are either trained (squares) or just exposed to the chamber without heat (triangles). Between conditioning and test periods flies were first transferred to a food vial for the indicated time and then back into a new chamber where all flies underwent a short training of 30 sec and a final 6-min memory test. Control animals (naïve; filled circle) underwent only the 30-sec training and the final test. Each group includes about 200 flies. Figure shows PIs of the final 6-min test phases.

Control flies were kept in the chamber without any heat punishment for the same amount of time before the double transfer (20 min; experimental design: Fig. 4e). Surprisingly, they also showed a significantly positive PI for the 1- 3 min retention interval similar to that of the trained flies (Fig. 23, triangles). The mere exposure to the chamber improves acquisition during the reminder training. This effect lasts only briefly, though. Already for the 30 min retention interval the test PIs in merely 'exposed' flies were significantly lower than in trained flies (U-test, $Z=2.70$, $p< 0.01$) and at 2 hrs were not significantly different from zero. In naïve flies, as shown before (Fig. 21), the reminder training in itself did not lead to PIs significantly different from zero (Wilcoxon-matched pairs test, $p=$ n.s.). Hence, with the double transfer another type of aftereffect is observed: a contextual memory relating to characteristics of the situation in the chamber (exposure effect) rather than to the heat/position contingency. It should be noted that without the short intermission in the food vial this exposure effect is not observed.

In the experiment of Fig. 23, for the 1-3 min retention interval memory scores of trained flies are not larger than those of merely exposed flies. Since trained flies are necessarily also 'exposed' one can ask whether their memory reflects the heat/position contingency or only the situation in the chamber as in the merely exposed animals. To answer this question, a yoked control experiment was again performed which deviated from the yoked experiment in Fig. 22 only in that the flies were kept in a food vial for 1 min between conditioning procedure and reminder training (see next page Fig. 24). Test performance of the yoked control group was significantly lower than that of the experimental group (U-test, $Z=2.01$, $p< 0.05$) and statistically not different from zero (Wilcoxon, $p=$n.s.), indicating that the experimental flies remember an association between punishment and behavior from the operant conditioning procedure. The exposure effect seems to be suppressed by the heat punishment in trained and yoked control animals, at least for the 1-3 min retention

interval. If this applies also for the 2-hour retention interval, the 2-hour memory of the trained group can also be regarded as a memory of the heat/position contingency. In the following experiments, I address the issue of what is learned during exposure to the chamber without heat.
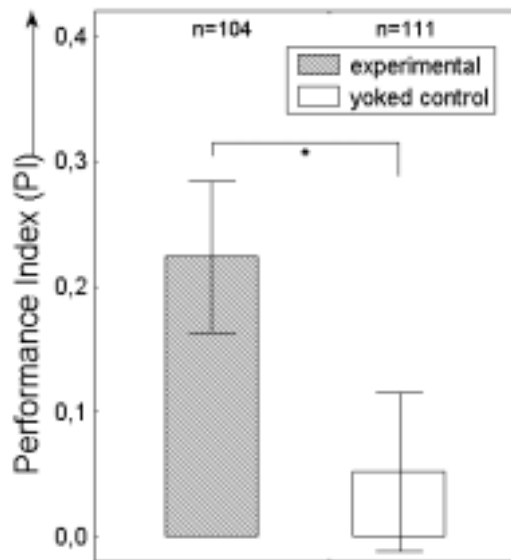
Figure 24: Yoked control experiment with indirect transfer. Flies of the experimental group can control heat punishment during intermittent training, while flies of the yoked control group can not. Immediately after training flies are transferred to a food vial for 1 min, then to a new chamber where they received a 30-sec reminder training and a 6-min test. Both groups could control heat punishment during the reminder training. Figure shows the PI of the experimental versus yoked control groups in the final 6-min test. Flies of the experimental group, and also the flies of the yoked control group were kept together in a food vial during the transfer. We, therefore, could not follow individual flies. As some flies escaped during the experiment, we obtain different sample size for the two groups.

3.1.4.3 Analysis of the exposure effect

No contribution of handling

In the transfer experiments above, each fly is sucked into and blown out of the aspirator three times: at the transfer from the home vial to the chamber, from the chamber to the food vial, and from the food vial to the new chamber. To investigate whether this handling might contribute to the exposure effect, I reduced the period in the chamber to a few seconds (handling control; experimental

design: Fig. 4f). Afterwards, flies were treated just like animals of the trained and exposed groups. They stayed in the food vials for 1 min, were transferred back to the new chambers and, after the reminder training, were tested for 6 min with the heat off. Only flies of the training and exposed group showed significantly positive PIs in the final test (Fig. 25; Wilcoxon, trained group: p< 0.01; exposed group: p<0.01). Flies that had received the full handling but had spent only a few seconds in the chamber showed no significant memory (Wilcoxon, handling control: p= n.s.). Both, trained (Anova: H=7.15, p<0.05; U-test, trained versus handling: Z=2.35, p<0.05) and exposed groups (U-test, exposed versus handling: Z=2.00, p<0.05) had a significantly higher test performance than the handling control. Apparently, handling *per se* does not contribute to the exposure effect. It is the experience of the 20-min period in the chamber that enhances the effectiveness of the reminder training in building up a memory.
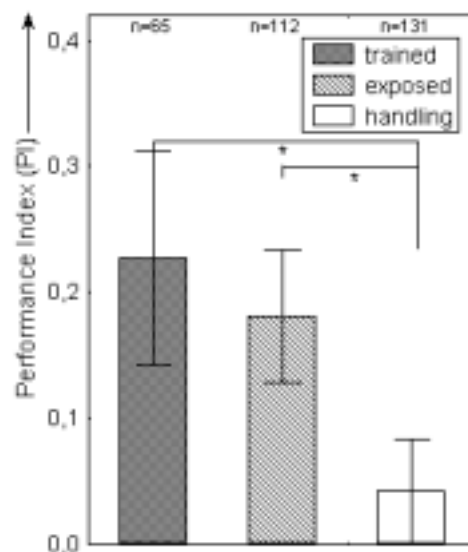
Figure 25: Handling does not cause the exposure effect. Prior to the transfer to the food vial, flies are kept in the chamber for only a few seconds ('handling group') but receive the same handling as those in the 'trained' and 'exposed' groups. After 1 min in the food vial, flies are transferred to the chamber where they undergo a 30-sec reminder training and a 6-min memory test. Only final memory scores are shown.

Isolation and chamber characteristics contribute to the exposure effect

The experiment of Fig. 25 indicated that during exposure the flies learned characteristic

features of the chamber enabling them afterwards to acquire the heat/position contingency more readily during the reminder training. This is not the only interpretation, however. With their first transfer to the heat-box they are separated from their home vials and their sibling flies for the first time in their life. I, therefore, asked whether the flies during the exposure to the chamber just learned to cope with isolation in a strange environment, rather than memorizing specific properties of the geometry and material of the chamber. Before the transfer, flies were kept one by one for a 20-min time period in transparent small plastic vials (ϕ 22.0/ 63 mm; experimental design: Fig. 4g). A group of flies exposed to the heat-box before the transfer and a group of naïve flies, both from the same culture vials as the experimental animals, served as controls. After the exposure, all groups received the same treatment in that they were transferred to a food vial, after 1 min were transferred back to a chamber, received reminder training, and were finally tested (Fig. 26).

test, exposed versus vial: Z=2.85, p<0.01), indicating that the flies learned characteristics of the situation in the chamber. Additionally, however, flies kept in vials showed significantly larger PIs than naïve flies (U-test, vial versus naïve: Z=2.29, p<0.05), arguing that chamber-independent aspects of the exposure such as isolation may facilitate acquisition during the reminder training.

Length of chamber is not critical

I next investigated whether chamber length was a critical parameter learned during exposure. Flies were kept in chambers of either full length, half, or quarter length by using stoppers which filled part of the chambers. After transfer into a food vial for 1 min and back to the chambers, flies were tested in full-size chambers. If chamber length was learned, a decrement in the test scores of flies exposed to smaller sized chambers was expected. As Fig. 27 shows, this was not observed. There was no significant difference in test performance between the three groups (Anova, H=0.49, p=n.s.), all of which showed positive PIs significantly different from zero (Wilcoxon, 1 chamber: p< 0.001; ½ chamber: p< 0.01; ¼ chamber: p< 0.01). I conclude that chamber length is not a critical feature of the memory in the exposure effect.

Figure 26: Chamber-specific and chamber-independent components of the exposure effect. Flies of the experimental group were exposed to plastic vials (vial) before the transfer. Control groups included flies that were exposed to the heat chamber (exposed) or naïve flies (naïve). All flies had a 1-min rest period in the food vial before being transferred to the chamber to undergo a 30-sec training and a 6-min memory test. Figure shows the PIs of the memory tests.

Figure 27: No influence of chamber length on exposure effect. Flies were exposed to chambers of different length (full length, half, or quarter length). After exposure, flies were transferred to the food vial for 1 min and subsequently to chambers of normal size. Figure shows the PIs during the 6-min memory test after the second transfer and 30-sec training.

Flies kept in plastic vials showed significantly smaller PIs in the test than those of the exposed group (Anova, H=29.85, p<0.001; U-

3.1.4.4 Mushroom bodies are not required for training or exposure effects

As a first step towards identifying the neural substrate of the training and exposure effects, we investigated whether mushroom body-less flies still showed any of these types of memory. Heat-box learning with the standard procedure is independent of the mushroom bodies (Wolf et al., 1998), but the transfer experiment used to document the training and exposure effects involves severe context changes (chamber/ food vial/ chamber) to which in a different learning experiment flies without mushroom bodies have shown to be more sensitive than normal control animals (Liu et al., 1999).

Flies treated as 1st-instar larvae by hydroxyurea (HU), and flies treated the same but without HU (HU controls) were either trained or merely exposed to the chamber. After being transferred from the chambers to a food vial for 1 min, they were transferred back to the chambers for reminder training and test. Brain sections of tested HU flies gave 90 % of animals with total loss of postembryonic mushroom bodies. In less than 10 % one tiny mushroom body was left.

Neither for the trained group, nor for the exposed group were significant differences between HU and HU control flies observed (Fig.28; U-tests, p= n.s). All groups gave a positive 6-min memory score (Wilcoxon, trained group HU: p<0.05; trained group HU control: p<0.001; exposed group HU: p<0.05; exposed group HU control: p<0.01). The mushroom bodies that are not necessary for heat-box learning are also dispensable for the associative and non-associative memories in the transfer experiments.
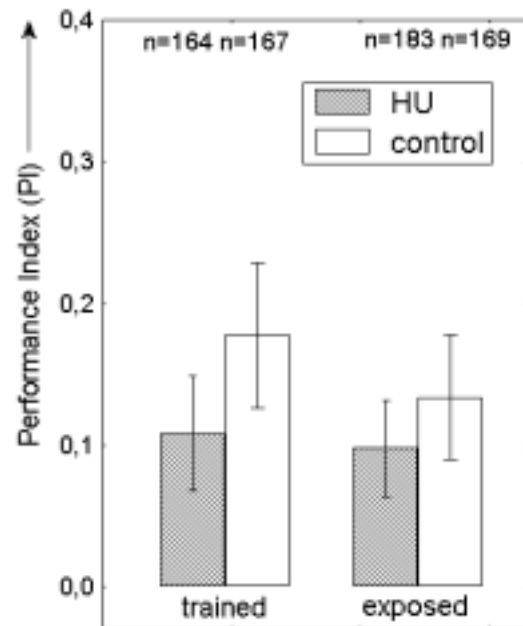
Figure 28: No requirement of the mushroom bodies for training and exposure effect after indirect transfer. Hydroxyurea (HU) treated and control flies were compared in the indirect transfer experiment for training and exposure for 1-3 min retention interval. Figure shows the 6-min memory tests after transfer and reminder training.

3.1.5 Performance of classical learning and memory mutants

Most studies of learning and memory processes in the past concentrated on classical conditioning. They demonstrated that the cAMP signaling cascade has an important role in learning and memory processes in invertebrates (Davis et al., 1995; Fagnou and Tuchek, 1995) as well as vertebrates (Mayford and Kandel, 1999). This conservation between species might indicate a more general role of the cAMP signaling cascade in learning and memory. An important question, therefore, is whether *Drosophila* mutants which affect the cAMP signaling cascade and which are known to be defective in classical conditioning like *dunce (dnc), rutabaga (rut),* and *amnesiac (amn)* also have a reduced perfomance in operant conditioning in the heat-box (Tully and Quinn, 1985). The *dnc* gene encodes a cAMP specific phosphodiesterase, while *rut* encodes an adenylate cyclase and *amn* a neuropeptide hormone (FlyBase Report).

Wustmann and colleagues (Wustmann et al., 1996) already reported that mutant lines *rutabaga (rut¹)* and *dunce (dnc¹)* which are deficient in classical odor avoidance learning (Dudai et al, 1976; Aceves-Pina et al., 1983), in conditioned courtship suppression (Gailey et al., 1984), and visual pattern discrimination learning (Eyding, 1993) also show defects in operant conditioning. Experiments were performed in the original heat-box version using cantonized rut^1 and dnc^1 flies. I was interested in testing whether measurements of different mutant alleles in the modified heat-box version give results comparable to Wustmann´s data. Thus, dnc^{ML}, rut^{2080}, and amn^1 mutants were chosen to be tested in the standard experiment (Fig. 29).
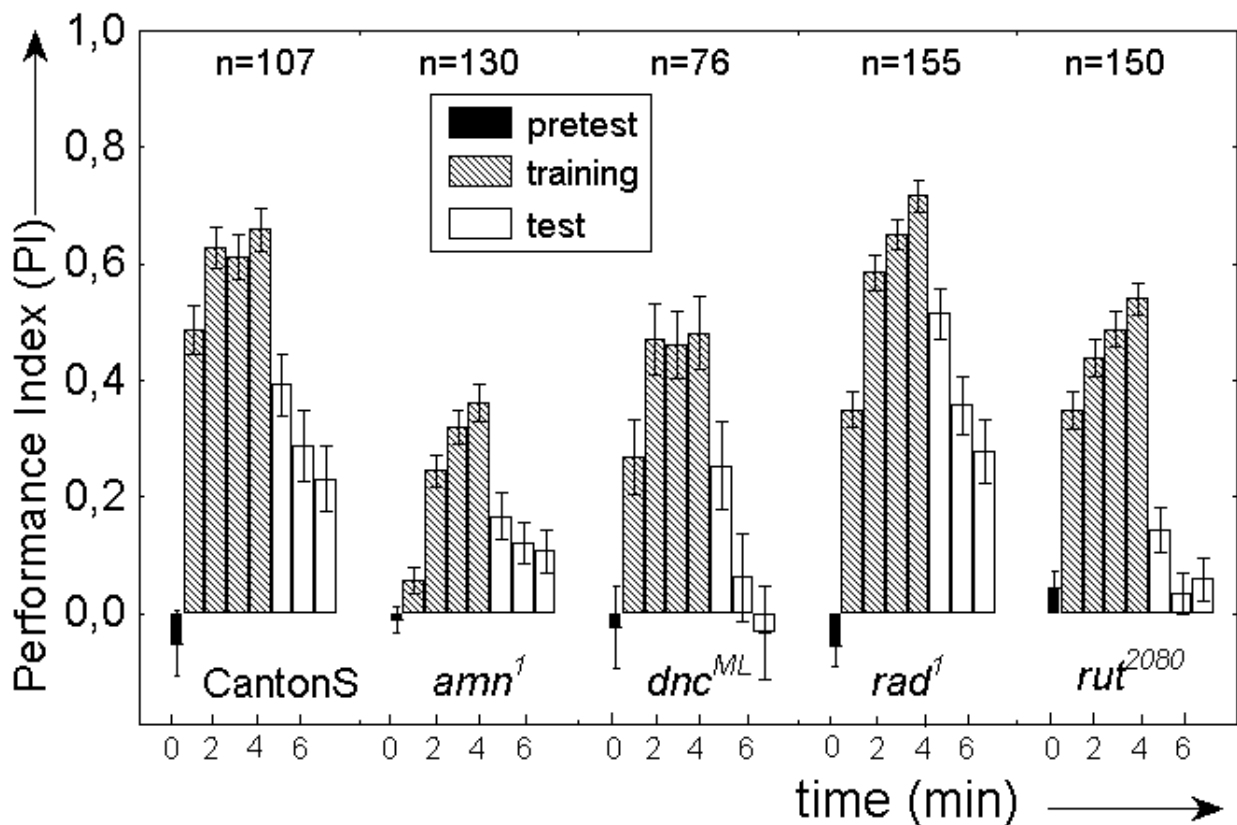
Figure 29: Mutant flies *amnesiac (amn¹), dunce (dncML), radish (rad¹)* and *rutabaga (rut²⁰⁸⁰)* in the standard learning experiment. Figure shows pretest (pre, black bars, 30 sec), training binnned to 1-min blocks (tr, hatched bars) and memory test binned to 1-min blocks (te, empty bars). CantonS flies were used as wild-type comparison.

Another investigated line was *radish*, which was also shown to be defective in classical conditioning (Folkers et al., 1993). Although it was recently shown that atypical PKM is sufficient to enhance memory of *radish* mutants, the mutation is not molecularly characterized yet. Thus, there is no proof for the *radish* gene to be implicated in the cAMP signaling cascade (Drier et al, 2002). As the contribution of the *stay-where-you-are* effect in the standard experiment was negligible (Fig. 20), the composite memory score was calculated in all following experiments. Using this evaluation, longer test periods and, thus, memory decay can be studied. For analysis of the memory decay, the memory test is binned in 1-min blocks.

Drosophila dnc[ML] mutants had a defect in heat avoidance and a rapid memory decay (U-tests, Appendix Table 5). There was no further increase in performance from the second minute until the end of the training period. This result is similar to that of Wustmann who found that *dnc*[1] mutants avoided the heat during 4-min training, but did not show an improvement in heat avoidance during that period (Wustmann et al., 1996). However, differing from *dnc*[ML] mutants, *dnc*[1] flies already in the first minute of training reached a PI which was significantly higher than that of control flies, while *dnc*[ML] mutants never performed better than control line CantonS in the experiment. Also in the memory test, the two mutants behave differently. While a significantly reduced performance is not found in *dnc*[ML] mutants for the first test minute, *dnc*[1] mutants had a performance lower than 0.1 already in the first minute of the memory test. Performance of *rut*[2080] flies was reduced during all training and test phases. It increased during 4 min of training, but did not reach wild-type level. In *rut*[1] mutants, heat-avoidance started at the same level as CantonS and stayed at that level (PI ~0.3) for the last 3 min of training (Wustmann et al., 1996). In the subsequent memory test, both lines failed. Despite described minor differences between Wustmann´s and my results, there is a general agreement that *rut* as well as *dnc* flies are defective in operant conditioning in the standard experiment. Observed differences might result from the fact that different strains were tested. While Wustmann focussed on alleles *dnc*[1] and *rut*[1], I tested mutant alleles

dnc[ML] and *rut*[2080]. *dnc*[1] und *dnc*[ML] are described as hypomorpic alleles (Nighorn et al., 1991; Salz and Kiger, 1984). *rut*[1] is a point mutation in the cyclase catalytic domain (Levin et al., 1992), while *rut*[2080] is hypomorphic with respect to transcript levels and a size defect (T. Zars, pers. comm.). Alternatively, deviating results can be explained by differences between the two heat-box versions which were used. Wustmanns experiments were performed in the original heat-box with colored diodes as orientation cues for the flies using a temperature range of 28 °C (±2 °C) to 45 °C (±2 °C) degree, while I performed experiments in the modified heat-box in complete darkness in the temperature range of 20 °C (±1 °C) to 40 °C (±1 °C). Higher temperatures in Wustmann´s experiments might account for a better heat avoidance of *dnc* flies in his experiments. The phenotype of mutant flies might, thus, be specific for the paradigm in which they are tested. Another explanation for described deviations might be differences in daily performance (e.g. weather). In my experiments, those influences are eliminated as measurements were performend in parallel and for several days. For Wustmann´s data, there is no detailed information about the data collection procedure. However, low sample sizes might point to the fact that his experiments were not performed for three days, but in a more narrow time range. Therefore, his results might indeed be influenced by daily variances. Performance of *amn*[1] flies, which were not tested by Wustmann, was even worse than that of *dnc* and *rut* mutants. They showed reduced performance during all training and test phases with a training PI of less than 0.4 after 4 min of training. Also in the memory test, *amn*[1] mutants obtained very weak scores. However, memory decay was slower compared to that of *rut* and *dnc* flies. The *rad*[1] mutants learn normally but display abnormally rapid memory decay after training in the olfactory discrimination test of Tully and Quinn. While STM and LTM are intact, the flies are lacking anesthesia-resistant memory (ARM; Tully, 1995). In the heat-box, mutant line *rad*[1] is neither defective in training nor in the test.

3.2 Behavioral screen for mutants in heat-box conditioning

3.2.1 Behavioral results

Until now, we only have hints at which genes and cellular processes are involved in operant conditioning in the heat-box and which brain regions are essential (Zars et al., 2000a). To address those questions, we performed a behavioral screen with a collection of P-element insertion lines provided from Dr. Ulrich Schaefer (Max Planck Institute in Goettingen) searching for genes which result in learning and memory defects when they are mutated. The screening procedure was done in collaboration with Dr. S. Kramer and the technical assistance of S. Flurschuetz-Twardzik.

All investigated strains have an insertion of p[lacW] and are viable in the homozygous state. As the transposon carries the mini-white gene (Bier et al., 1989), Schaefer and colleagues used white-eyed flies as a starter line for the mutagenesis to be able to control for the P-element insertion. Progeny which carried the P-element could then easily be detected by selecting for red-eyed virgin females. The genetic background of the investigated mutant lines was not uniform due to several crossing steps during mutagenesis.

3.2.1.1 Behavioral results of original P-element mutants

The screen consisted of several steps. First, 1221 P-element mutant lines were measured in the original heat-box (Wustmann and Heisenberg, 1996) with a protocol including a 30-sec pretest, 3-min training and 3-min test. Experiments were performed in complete darkness. About 25 flies per P-element line were tested.

We were interested in two classes of behavioral phenotypes: The first were flies with low performance during training indicating a defect in learning (H). As we also select for flies with defective thermoreception by this criterium, we later tested for intact thermoreception. The second class were flies with low performance in the test indicating a defect in memory (M). Criteria are described in detail in Table 8. *Drosophila* lines of the P-element collection which performed well were used as a control for the optimal functioning of the apparatus.

4 % of all tested lines fulfilled mutant criteria (Appendix, Table 6). Among the 49 selected lines, 17 were defective in heat avoidance and 32 in the following memory test. To test reproducibility of the behavioral phenotype, each of the candidate lines was remeasured in at least two consecutive generations with varying training durations. Lines characterized by reduced training performance were tested for defects in thermosensitivity by Dr. S. Kramer. As none of the lines showed abnormal heat avoidance compared to wild-type CantonS flies (data not shown), we could exclude impaired thermosensitivity as a reason for the failure of the flies in operant conditioning.

The next step included repeated measurements of the 49 candidates with the modified apparatus and their final classification. Measurements were performed with the standard protocol and resulted in 29 candidate lines of first choice or second choice, depending on the consistency of their learning / memory phenotype. 10 lines fell in the category of heat avoidance candidates, 19 in the category of memory candidates.

Table 8: Criteria for mutant selection

classification	criteria
heat avoidance candidate (H)	Performance during last minute of training < 0.4
memory candidate (M)	Performance during last minute of training > 0.4
	Performance during first half minute of test < 0.3

Six of the original heat avoidance candidates were now classified as memory candidates and two lines originally found to be defective in the memory test already failed during training in the modified heat-box. There are at least three possible explanations for the fact that only 57 % of the candidate lines also showed a phenotype in the modified heat-box version and that the behavioral phenotype changed for another 26 % of the 29 candidates. One possibility is that an increased sample size per measured line resulted in more accurate values. As there are differences between the two heat-box versions (e.g. temperature range) a second explanation is that the phenotype of candidates is specific for the paradigm in which they were originally identified. Experiments were performed within the temperature range of 25 °C (±2 °C) to 37 °C (±2 °C) in the original heat-box, while in the new apparatus a range of 20 °C (±1 °C) to 40 °C (±1 °C) was used. Stronger punishment might be sufficient for some candidates to compensate their learning / memory deficit which was evident with less severe heat punishment. Another chance is that candidates which lost their phenotype accumulated genetic modifiers since they were identified. Such modifications are frequently observed in structural brain mutants (Heisenberg, 1980) where they mask the anatomical defect and might also mask the behavioral phenotype. In the case of anatomical defects, mutant lines were outcrossed from time to time to keep their phenotypes.

3.2.1.2 Performance of candidate lines in a uniform genetic background

As mentioned, tested flies did not share a uniform genetic background. Thus, there was no appropriate control line to compare selected mutant lines with. To enable such a comparison, eight of the most interesting candidate lines were crossed into a w^{1118} Berlin background (Table 9; crossing protocol see chapter 2.2.2., Materials & Methods).

Table 9: Results of isogenised P-element mutants, control w^{1118} Berlin and wild-type CantonS in the standard experiment. Table includes P-element line (line), date of measurements (date), performance in the last training minute (tr) and the first test minute (te 1, te 2, each 30 sec), sample size (n) and classification of the candidates (class, memory candidate (M), heat avoidance candidate (H), excluded by criteria (ex)). Measurements were either performed in the range of 19°C to 39°C or within a more narrow range of 22°C to 37°C (temp). ‚sex' indicates measurements with separate analysis of males (m) and females (f).

standard experiment								
line	date	tr	te 1	te 2	n	class	temp	sex
5054	0222-0224	0.544	0.253	0.260	78	M	19°C-39°C	
8522	1202-1206	0.247	-0.021	0.022	72	H	22°C-37°C	m
	1207-1208	0.416	0.081	0.099	74	M	19°C-39°C	m
8570	1202-1206	0.253	0.074	0.052	75	H	22°C-37°C	m
	1207-1208	0.415	0.226	0.191	71	M	19°C-39°C	m
8631	1202-1206	0.232	0.050	0.005	74	H	22°C-37°C	m
	1207-1208	0.386	0.100	0.184	76	H	19°C-39°C	m
8657	1207-1208	0.504	0.235	0.188	73	M	19°C-39°C	m
	0222-0224	0.431	0.326	0.141	83	M	19°C-39°C	
9530	1202-1206	0.181	0.088	0.025	68	H	22°C-37°C	m
	1207-1208	0.292	0.087	0.132	68	H	19°C-39°C	m
9690	1202-1206	0.272	0.136	0.130	71	H	22°C-37°C	m
	1207-1208	0.328	0.074	0.199	75	H	19°C-39°C	m
9885	1202-1206	0.218	0.070	0.014	69	H	22°C-37°C	m
	1207-1208	0.401	0.062	0.070	72	M	19°C-39°C	m
w^{1118}Berlin	1202-1206	0.338	0.200	0.217	77	H	22°C-37°C	m
	1202-1206	0.361	0.225	0.130	69	H	22°C-37°C	f
	1207-1208	0.344	0.105	0.089	73	H	19°C-39°C	m
	1207-1208	0.363	0.131	0.084	76	H	19°C-39°C	f
CantonS	1202-1206	0.665	0.470	0.347	93	ex	22°C-37°C	m
	1207-1209	0.627	0.432	0.198	63	ex	19°C-39°C	m

As repeated outcrossing of the mutants to w^{1118} *Berlin* flies exchanged the autosomes and a large part of the X-chromosome, they were genetically very similar except for the regions directly surrounding the P-element insertion site. Thus, w^{1118} flies were used as control. Furthermore, a behavioral phenotype caused by a second site mutation is expected to disappear after that procedure. All berlinised lines were retested for defects in operant conditioning (see previous page Table 9). In case the P-element and not the genetic background was responsible for the learning / memory defect, the behavioral phenotype should be reproducible. Measurements were performed in the temperature range of 25 °C (±1 °C) to 37 °C (±1 °C) to accomodate conditions of the two heat-box versions and in the range of 19 °C (±1 °C) to 40 °C (±1 °C). Using both ranges, I tested whether the strength of the reinforcer influenced the performance of the flies. CantonS was used as control for optimal functioning of the apparatus. All eight investigated P-element lines fullfilled candidate criteria, although classification (H or M) of three lines varied between measurements (Table 9). Stronger punishment in those lines resulted in better performance during training, while performance in the test was still poor. Punishing with 40 °C was, hence, sufficient for the flies to learn to avoid the heat, but not to remember the task.

Unfortunately, control line w^{1118} *Berlin* which was outcrossed to wild-type Berlin did not perform well (Table 9). A comparison of control w^{1118} *Berlin* to wild-type CantonS flies showed reduced performance of w^{1118} *Berlin* flies in the last training minute (Fig. 30, U-test, Z= 9.30, p= 0.00) and in the memory test (Fig. 30, first min: U-test, Z=4.40, p= 0.00).
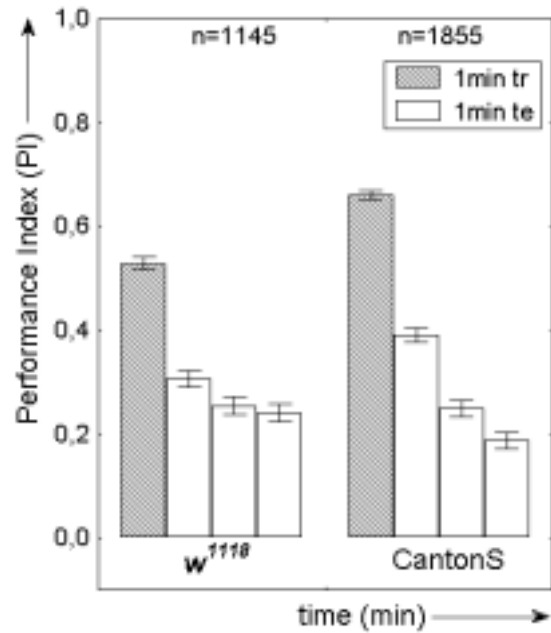
Figure 30: Performance of control line w^{1118} *Berlin* versus wild-type CantonS in the standard experiment. Figure shows Performance Index of the last training minute (tr, hatched bars) and memory test binned to 1-min blocks (te, empty bars, each 1 min).

For this reason, 19 new control lines were generated by establishing single pair matings of the original w^{1118}*Berlin* stock and selected for wild-type behavior in the heat-box. After repeated measurements two lines, w^{G8} and w^{G11}, were chosen which consistently performed well in the standard experiment. 4-min training resulted in a Performance Index of PI=0.55 +/- 0.04 for w^{G8} flies and a PI=0.61 +/- 0.04 for w^{G11} flies (see next page Fig.31) compared to wild-type CantonS with a training score of PI=0.66 +/- 0.01 (Fig. 30). Also in the memory test performance of both control lines was comparable to that of wild-type CantonS flies (Fig. 30 and 31; w^{G8}: PI=0.35 +/- 0.05; w^{G11}: PI=0.46 +/- 0.06; CantonS: PI=0.39 +/- 0.01).
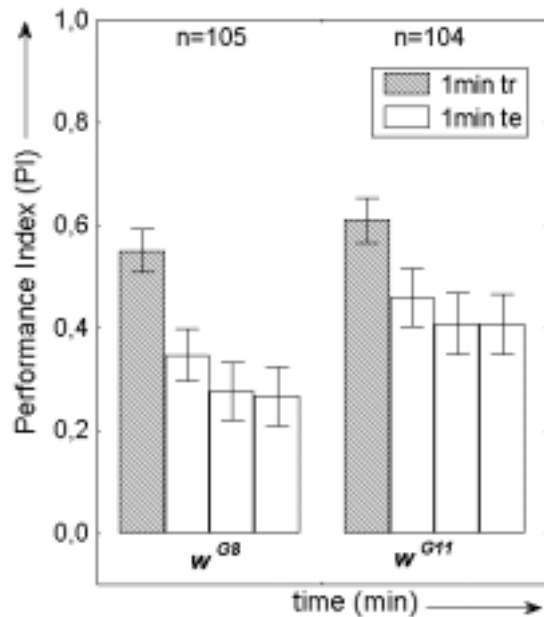
Figure 31: Performance of control lines w^{G8} and w^{G11} in the standard experiment. Figure shows Performance Index (PI) of the last training minute (tr, hatched bars, 1 min) and memory test binned to 1-min blocks (te, empty bars, each 1 min)

Seven interesting P-element lines from the screen were then outcrossed to w^{G8} and w^{G11} for six generations and afterwards again tested in the heat-box using w^{G8} and w^{G11} as appropriate control lines (Table 10). Performance values of control lines were in the wild-type range. In the new genetic background, six of seven P-element lines were still found to be defective either in heat avoidance or in the memory test and could be classified as candidates. This is an indication that the behavioral phenotype in these lines was likely produced by a lesion near the P-element insertion site. The mutant phenotypes sometimes varied, depending on the genetic background. This can be explained either by the influence of two genetically different backgrounds or by variances between measurements. 8631 was the only line which fulfilled candidate criteria in only one of the genetic backgrounds.

Table 10: Performance of P-element mutant lines in a w^{G8} and w^{G11} background in the standard experiment. *Drosophila* strains w^{G8} and w^{G11} were used as control lines. Table includes P-element line (line), date of measurements (date), performance in the last training minute (tr) and in the first test minute (te 1, te 2, each 30 sec), sample size (n) and classification of the candidates (class, memory candidate (M), heat avoidance candidate (H), excluded by criteria (ex)). Measurements were performed in the range of 19°C to 39°C.

line	background w^{G8}						background w^{G11}					
	date	tr	te 1	te 2	n	class	date	tr	te 1	te 2	n	class
5054	0619-0621	0.397	0.177	0.187	68	H	0629-0701	0.53	0.276	0.106	70	M
8522	0619-0621	0.359	0.245	0.154	68	H	0629-0701	0.456	0.195	0.088	73	M
	0622-0624	0.391	0.304	0.235	62	H						
8570	0629-0701	0.468	0.292	0.101	66	M						
8631	0619-0621	0.347	0.218	0.014	70	H	0629-0701	0.728	0.572	0.439	71	ex
	0622-0624	0.392	0.166	0.035	62	H						
8657	0629-0701	0.336	0.143	0.127	74	H	0629-0701	0.321	0.026	0.158	70	H
9690	0619-0621	0.386	0.221	0.269	61	H	0629-0701	0.382	0.154	0.207	74	H
9885	0619-0621	0.360	0.153	0.028	67	H	0629-0701	0.487	0.179	0.202	70	M
	0622-0624	0.329	0.206	0.097	62	H						H
control w^{G8} **and** w^{G11}	0619-0621	0.58	0.431	0.305	68	ex	0629-0701	0.531	0.316	0.237	73	ex
	0622-0624	0.665	0.447	0.412	62	ex						
	0629-0701	0.605	0.391	0.335	66	ex						
CantonS	0619-0621	0.694	0.516	0.466	58	ex	0629-0701	0.669	0.433	0.283	66	ex
	0622-0624	0.621	0.432	0.577	55	ex						

3.2.2 Localisation of the P-element insertion

P-elements of all investigated lines were inserted on the X-chromosome (pers. comm. to U. Schaefer). To determine the approximate P-element insertion site on the X chromosome of several interesting candidates and the number of P-element insertions, *in situ* hybridisations of polytene chromosomes were performed. I found that all investigated P-element lines carried only one p[lacW] insertion.

My next aim was to distinguish the exact P-element insertion site. The transposable portion of p[lacW] contains a bacterial origin of replication and the ß-lactamase gene coding for ampicillin resistance at its 3´ end. This feature allows cloning of DNA flanking the insertion site of p[lacW] (Cooley et al., 1988). Therefore, I performed plasmid rescue and subsequently sequenced the clones (Table 11). Plasmid rescue was successful in eight out of ten investigated P-element lines.

In three lines, I obtained sequences corresponding to both the 5´ and 3´ junctions between the inserted element and genomic DNA. Because the P-element insertion generates an 8 bp target site duplication, it was possible to assemble these sequences to reconstruct a contiguous sequence of genomic DNA spanning the insertion site (Liao et al., 2000; O´Hare and Rubin, 1983). These direct repeats found flanking the transposable element are thought to be produced by a staggered cut made at the site of integration with a 5´ overhang. The 8 bp single-stranded parts are then filled in upon insertion, resulting in the duplication (Engels W., pers. comm.). In another four lines, I isolated the sequence flanking the 3´ end of p[lacW]. Although this method did not work in lines 8657 and 9690, I could determine the approximate locus of the transposon in both lines by *in situ* hybridisation. 8657 has its P-element insertion in region 14B, 9690 in region 3B-3C close to the *dunce* locus (Qiu and Davis, 1993).

Table 11: Summary of results obtained from plasmid rescue and *in situ* hybridisations. Table indicates P-element line (line), name of sequence reaction (sequence), restriction enzyme, primer used, and sequence length (length). Table also shows results of *in situ* hybridisation, indicating the approximate location of the P-element insertion and results of NCBI blast searches with the obtained sequences (gene hit and location of the P-element insertion)

line	sequence	enzyme	primer	length	*in situ*	blast result
5054	GPu 20	EcoRI	PCR2	228 bp	18F-19A	*amn* (19A1)
6139	GPu 02	EcoRI	PCR2	342 bp	7E-7F	*inaF* (10D5-10D6)
8466	GPu 19	EcoRI	PCR2	292bp	--	*NetB* (12F)
8522	GPu 10 GPu 34 GPu 35 **composite sequence**	EcoRI BamHI BamHI	PCR2 Sp1 Pout	278 bp 284 bp 324 bp **604 bp**	20B-20D	*S6KII* (20A1)
8570	GPu 12	EcoRI	PCR2	362 bp	19F	*amn* (19A1)
8631	GPu 06 GPu 40 **composite sequence**	EcoRI XbaI	PCR2 SP1	293 bp 121 bp **414 bp**	14A	*CG6340* (13 D2-13D4)
9530	GPu 07	EcoRI	PCR2	334 bp	6E-6F	*inx2* (6E4-6E7)
9885	GPu 01 GPu 26 GPu 27 **composite sequence**	EcoRI BamHI BamHI	PCR2 Sp1 Pout	350 bp 251 bp 376 bp **726 bp**	18C-18D	*CG14207* (18D7-18D9)
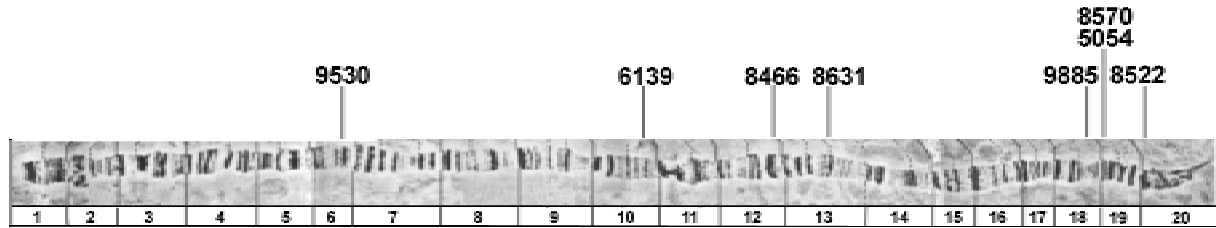
Figure 32: *Drosophila*'s X-chromosome from GeneSeen. Figure indicates P-element insertion sites of investigated mutant lines.

All obtained sequences are listed below. They were analysed in a NCBI blast search for sequence similarity to the *Drosophila* genome. In many cases, where a cytological localisation of a P-element by *in situ* hybridisation was performed the localisation was identical to that obtained from sequence comparison. I could further confirm the finding that P-elements preferably insert in the 5´ expressed region of genes from our results (Spradling et al., 1995). In many cases, I found homology to already identified genes or ,expressed sequence tags' (ESTs). Figure 32 shows the distribution of the P-element insertion sites on the X-chromosome in all investigated lines. The numbers below the arm indicate the cytological divisions.

3.2.2.1 P-element line 5054

In situ hybridisation revealed the approximate location of the P-element in region 18F-19A.

By plasmid rescue the sequence flanking the 3´ end of p[lacW] was determined. I obtained a sequence of 228 bp length (GPu 20, Fig. 33). Sequence comparison showed that the P-element had inserted in region 19A1 in the *amnesiac* (*amn*) ORF at position 2604/2605 (see also *Drosophila melanogaster* genomic scaffold 142000013386053 section 30, position 187378/187379, AE003513).

amn flies are reported to be defective in classical and operant conditioning. In the heat-box, the *amn*[1] mutant was defective in training and the subsequent test in the standard experiment. Line 5054, however, was characterized as a memory candidate (see Appendix Table 6). Diverse phenotypes could result from the fact that different alleles are affected in *amn*[1] and P-element line 5054. Isolation of *amn* alleles in the mutant screen confirmed that learning and memory mutants could be found using the described criteria for mutant selection.

```
         1            15 16         30 31          45 46          60 61         75 76          90
1 5054 TAAGTGGATGTCTCT TGCCGACGGGACCAC CTTATGTTATTTCAT CATGGTTTGGGCCAA CACTTGTCGCCTCTT GGCCATTATGCAACG        90


        91           105 106        120 121        135 136        150 151        165 166         180
1 5054 ACAGCGAAACGAAGG TGCTCACGAAGTGGC CAAGCTGCTCTCTGA TTGGTCGGCGGTCAG TGCCACGAGGCCAGC CGAAGTTCTCTCGCG        180


        181          195 196        210 211        225 226        240 241        255 256         270
1 5054 AGAACCCAAGAGCAC TCTCTCCCTCTCTTT TGGGAGAGATGAGAT AGC          228
```

Figure 33: Result of plasmid rescue of P-element line 5054 and subsequent determination of the sequence flanking the 3´ end of p[lacW]. Blue letters indicate sequence of p[lacW], red letters indicate 8 bp duplication.

```
          1             15 16            30 31              45 46              60 61              75 76              90
1 6139 TAAGTGGATGTCTCT TGCCGACGGGACCAC CTTATGTTATTTCAT CATGCATTAGGCGAT CTTCGCTTTTAATTT CATTCATAAAATCCG     90


          91           105 106           120 121            135 136            150 151            165 166            180
1 6139 TCTCTCTCACTCGCG CTCATTTGCATTTTC GGCTGCCCACGCTCA TTGTTGTTTCTTCTG CTTCTTTTTTTTTGT GTTCTTTTCATTATT    180


          181          195 196           210 211            225 226            240 241            255 256            270
1 6139 TCGTGGGCATTCCTT GAAGGCGAAATAAAA ACAAAAACAAACCGC ACGTCGGCACGTTGC CGCTCTTTGTGTGTC AAGTGCAGTTTTCAC    270


          271          285 286           300 301            315 316            330 331            345 346            360
1 6139 TCCCCCGAAAATCAG CGCTCCCCTTTTTGC GTTCGATTTACATGC GGAAATTAAAATGCG CGCTTGAATTAA     342
```

Figure 34: Result of plasmid rescue of P-element line 6139 and determination of the sequence flanking the 3´ end of p[lacW]. Blue letters indicate sequence of p[lacW], red letters indicate 8 bp duplication.

3.2.2.2 P-element line 6139

In situ hybridisation showed that the P-element had inserted in region 7E-7F. Plasmid rescue and sequencing of the obtained clone resulted in a 342 bp sequence (Fig. 34, Gpu 02). The NCBI blast search of GPu 02, however, revealed that the P-element is inserted in region 10D5-10D6 in the intron of *inaF* (*CG2457*; AE003487, *Drosophila melanogaster* genomic scaffold 142000013386053 section 4, position 25411/25412).

InaF has a transcript of 3123 bp (CT8105) and encodes a calcium channel regulator involved in the maintenance of rhodopsin mediated signaling. It interacts genetically with *transient receptor potential* (*trp*) and *trp-like* (*trpl*). *InaF* mutants cause a reduction in retinal degeneration in *trp* mutants, while isolated loss-of function mutations affect the photoreceptor cell and visual behavior (Li et al., 1999; Flybase report).

3.2.2.3 P-element line 8466

As *in situ* hybridisation did not give clear results, the location of the P-element was determined by plasmid rescue and subsequent sequencing. Homology comparison of the obtained sequence (GPu 19, length of 292 bp, Fig. 35) revealed that the transposon had inserted in region 12F, 88 bp upstream of the *NetrinB* transcription start site (*CG10521*, AE003496, *Drosophila melanogaster* genomic scaffold 142000013386053 section 13, position 260748/260749). BAC clone BACR08K05 (AC008334) includes the genomic region.

```
          1             15 16            30 31              45 46              60 61              75 76              90
1 8466 TAAGTGGATGTCTCT TGCCGACGGGACCAC CTTATGTTATTTCAT CATGGTCGAAACTGT CTTGCGTTTGCTTTG CTTTGCTTTTGGTTT     90


          91           105 106           120 121            135 136            150 151            165 166            180
1 8466 GCCAGAGAGAGGGCA ACATTTGATGATGCT GCTTGGCAACATTTT AGCTGCCGCAACATT GATGCAGACTGAATG TCACAAGATGATGCT    180


          181          195 196           210 211            225 226            240 241            255 256            270
1 8466 GGAAATTCTCGAAAG AAAGTTCATTTAACT CTTATTCAAAAATAT TAAATTACATGTATA TTCTATTAGTTTATT TTTAATGTGTAATGC    270


          271          285 286           300 301            315 316            330 331            345 346            360
1 8466 ATTTCTAACTTATTC TTAAGCC     292
```

Figure 35: Result of plasmid rescue and subsequent sequencing of line 8466. Figure shows region flanking the 3´ end of the transposon. Blue letters indicate sequence of the transposon p[lacW], red letters indicate 8 bp target site duplication.

```
⌐ GPu 35
        1            15 16          30 31          45 46          60 61          75 76          90
1 8522  TGTGAGGCAACTGCT CCTAGCGGTTCCTTT GTTTCCAGATCCTGA TGCACAATGCTAGAG CCCGAGTTCCCCCTG CGTTTCCAACTCCTC   90
                                                    ∟ GPu 34
        91          105 106         120 121         135 136         150 151         165 166        180
1 8522  CTGCTGCTCCTCCTC CACCGAACTCTCCCT ACTTCCGATTTCACT TTTCTTTTTTATTAC TTCCGCTGCGTGGGC GTCGCAGTCGACTGC   180

        181         195 196         210 211         225 226         240 241         255 256        270
1 8522  GCTGTTGCCTCTCCG GATTTCTCTGGTTTC CCTGCTGCTCCTACG GCCAACAAGCAGCGC TTTCCTAATGCGCCC ACGTTTCGTACGCCG   270
                                                          GPu 35 ⌐
        271         285 286         300 301         315 316         330 331         345 346        360
1 8522  TCTGCTTTACTATCC GCTTCGCAATCCGCA CAGCTGTGTTGCTTC TTATCACTATT↓ATTT ACACATTCACAATAA AGTTTTTTTAGAGCT   360
                                               GPu 34 ∟∟ GPu 10
        361         375 376         390 391         405 406         420 421         435 436        450
1 8522  GCAAAAAAATCGATG TTACCATCGATTACA TCGATAGTTTCAGTT TTCCGAAAACATCGA TTTACTTTTTGCAGC CGATGTTTTTACTGT   450

        451         465 466         480 481         495 496         510 511         525 526        540
1 8522  GCGTATTCAGCAAAT TTTTGTTTACACTTT TAATACATTCTTTCG GTTTTAAAAGTCTAT ATAACTCAATTAAAA AAAAAAAAACATATA   540

        541         555 556         570 571         585 586         600 601         615 616        630
1 8522  AATATAGTTTTATTC TGAGAATGATTTGGC CCGTGTGACCGTGTC TAGCAATATACCAAA CGCG    604
                                               GPu 10 ↵
```

Figure 36: Result of plasmid rescue and sequencing of P-element line 8522. The P-element insertion site is indicated by a red arrow. Figure shows region flanking the 3´ end and 5´ end of p[lacW]. 8 bp which were duplicated due to the insertion of p[lacW] are marked in red. Range of sequences GPu 35, GPu 34 and GPu 10 are indicated by black arrows.

3.2.2.4 P-element line 8522

Netrin-B (*NetB*) is expressed in subsets of muscles and encodes a product involved in motor axon targeting. Ectopic expression and loss of funcion analysis in both the CNS and peripery demonstrates that the pattern of netrin expression is crucial to the correct patterning of axons, providing evidence that netrins function as instructive guidance cues (Mitchell, 1996).

The approximate locus of the P-element insertion obtained by *in situ* hybridisation is region 20B-20D. Plasmid rescue was performed with an EcoRI digest in 3´ direction and a BamHI digest in 5´ direction of p[lacW] (Fig. 36). Clones of the EcoRI digest were sequenced with primer PCR2 (GPu 10, 278 bp), those of the BamHI digest either with primer SP1 (GPu 34, 284 bp) or primer Pout

```
        1            15 16          30 31          45 46          60 61          75 76          90
1 8570  TAAGTGGATGTCTCT TGCCGACGGGACCCC TTATGTTATTTCATC ATGGTTTGGGCCAAC ACTTGTCGCCTCTTG GCCATTATGCAACGA   90

        91          105 106         120 121         135 136         150 151         165 166        180
1 8570  CAGCGAAACGAAGGT GCTCACGAAGTGGCC AAGCTGCTCTCTGAT TGGTCGGCGGTCAGT GCCACGAGGCCAGCC GAAGTTCTCTCGCGA   180

        181         195 196         210 211         225 226         240 241         255 256        270
1 8570  GAACCCAAGAGCACT CTCTCCCTCTCTTTT GGGAGAGATGAGATA GCAAAGAAAAGCCAA AACACTAGAACAATC CAGAAGCCAAGTTTT   270

        271         285 286         300 301         315 316         330 331         345 346        360
1 8570  CCGGCTTGCACATAC GCCGTATAATCCGCA TTTTTTTGCGAACTCT AAACTCGAGTCTATC AGCTATAATATTACA ATTCGTTCACTATAA   360

        361         375 376         390 391         405 406         420 421         435 436        450
1 8570  AC    362
```

Figure 37: Result of plasmid rescue and determination of the sequence flanking the 3´ end of p[lacW] of P-element line 8570. Sequence of P-element p[lacW] is indicated in blue, red letters indicate 8 bp target site duplication.

```
ATG CGC AGT TTT TGT TGT TGT TTT TAT CCG GCT GCT GTG GCG TTG
 M   R   S   F   C   C   C   F   Y   P   A   A   V   A   L

CAC TCG GTA TTA CTG TTT TAC ACT TTT TTT TTA CTT TTT AGA GCG
 H   C   V   L   L   F   Y   T   F   F   L   L   F   R   A

TCC GCG TTG CGG CGA CGC GTT GTA AGC GGT TCG AAA GGT AGC GCA
 S   A   L   R   R   R   V   V   S   G   S   K   G   S   A


GCA CTG GCG CTC TGC CGC CAA TTC GAA CAG CTG AGC GCC AGC CGA
 A   L   A   L   C   R   Q   F   E   Q   L   S   A   S   R


AGA GAG AGA GCC GAA GAG TGC CGA ACG ACG CAG CTC CGC TAC CAC
 R   E   R   A   E   E   C   R   T   T   Q   L   R   Y   H


TAC CAC CGC AAT GGC GCT CAG TCG CGG TCG CTG TGC GCT GCT GTG
 Y   H   R   N   G   A   Q   S   R   S   L   C   A   A   V


CTC TGC TGT AAG CGC TCT TAC ATT CCG CGC CCG AAT TTT TCG TGC
 L   C   C   K   R   S   Y   I   P   R   P   N   F   S   C


TTT TCC CTT GTT TTC CCT GTG GGC CAG CGT TTC GCT GCT GCT CGG
 F   S   L   V   F   P   V   G   Q   R   F   A   A   A   R
       8570/5054 ▼   ▼ 9725
ACT CGG TTT GGG CCA ACA CTT GTC GCC TCT TGG CCA TTA TGC AAC
 T   R   F   G   P   T   L   V   A   S   W   P   L   C   N

GAC AGC GAA ACG AAG GTG CTC ACG AAG TGG CCA AGC TGC TCT CTG
 D   S   E   T   K   V   L   T   K   W   P   S   C   S   L

ATT GGT CGG CGG TCA GTG CCA CGA GGC CAG CCG AAG TTC TCT CGC
 I   G   R   R   S   V   P   R   G   Q   P   K   F   S   R

GAG AAC CCA AGA GCA CTC TCT CCC TCT CTT TTG GGA GAG ATG AGA
 E   N   P   R   A   L   S   P   S   L   L   G   E   M   R

TAG
 *
```

Figure 38: Insertions of p[lacW] in the *amnesiac* ORF. Figure shows nucleotides 187209 through 187751 of reverse and complement *Drosophila* genomic scaffold 142000013386053 (AE003513). Arrows indicate the P-element insertion site of lines 5054, 8570 and 9725.

(GPu 35, 324 bp). From sequencing results, I obtained a composite sequence of 604 bp. The P-element is inserted in region 20A1 in the first exon of *p90 ribosomal S6 kinase* (*S6KII*; *CG17596*; AE003574, *Drosophila melanogster* genomic scaffold 142000013386033 section 1, position 96833/96834; Putz et al., 2000). Concluding from ORF finder results, this region is transcribed but not translated. In chapter 3.3.2.1 the P-element insertion site is described in detail.

3.2.2.5 P-element line 8570

The approximate insertion site of p[lacW] in line 8570 was determined to region 19F by *in situ* hybridisation. Plasmid rescue and sequencing resulted in a sequence of 362 bp (GPu 12, see previous page Fig. 37). The P-element insertion site is identical to that of line 5054 with the P-element insertion in the *amn*

ORF at position 2604/2605 (see also AE003513, *Drosophila melanogaster* genomic scaffold 142000013386053 section 30, position 187378/187379). Line 8570, was isolated as memory candidate with a defect only in the memory test, which is consistent with the phenotype of line 5054. A third line 9725, molecularly characterized by Dr. S. Kramer, has the P-element insertion also in the *amn* ORF at position 2608/2609 (Fig.38). The behavioral phenotype of this line was not consistent, but varied between heat-avoidance and memory candidate (Appendix, Table 6).

3.2.2.6 P-element line 8631

The approximate locus of the P[lacW] insertion which was obtained by *in situ* hybridisation is region 14A. By plasmid rescue and sequencing, I determined the sequence

```
        1              15 16            30 31            45 46            60 61            75 76            90
1 8631  AGTCAAAAACAAAAA ACGAAAACAAACCTG ACCGACGATAATTGA CGATAGCCCGATCGT AGTAATCGCTCGATT ACCAATAGCTTCTTC   90
        └ GPu 40

        91            105 106           120 121           135 136           150 151           165 166           180
1 8631  GATAGTGCGACAATC GATCAAATTTGTTAT GACCACCAC↓TGTGTG TGTAGTCCTGTGTGC GACTCTGTGGCTGTG TTGGTGGGTGTGTGT   180
                GPu 40  ↓└ GPu 06

        181           195 196           210 211           225 226           240 241           255 256           270
1 8631  GAGCCACTGAAAAAG GTAAAAGCAAATAGA ACGATCACACTCACC ACACACATGCCTGCC CGCACACACACACAC ACACAACCACCCACC   270

        271           285 286           300 301           315 316           330 331           345 346           360
1 8631  CATCCACCCACTCAC TCACTCACTGGTGGC GCGAAAGCGAGAGAG AGAGAGAGAGAGAGA TAATGCGAAAGAGAG ATCAAGGCGCACGAA   360

        361           375 376           390 391           405 406           420 421           435 436           450
1 8631  AGTGAGTATACAAAA AGCGATCAAATAGAA CCTAACAACTCTCTA AGCAATCTA     414
                                                        GPu 06 ↓
```

Figure 39: Result of plasmid rescue and sequencing of P-element line 8631. The P-element insertion site is indicated by a red arrow. Figure shows region flanking the 3´ end and 5´ end of p[lacW]. The 8 bp which were duplicated due to the P-element insertion are marked by red letters. Sequence results of GPu 40 and GPu 06 are indicated by black arrows.

flanking the 3´ end (GPu 06, 293 bp) and 5´ end of the transposon (GPu 40, 121 bp). A NCBI search blast with the combined sequence of 414 bp (Fig. 39) shows that the transposon has inserted in the intron of gene *CG6340* in region 13D2-13D4 (AE003499, *Drosophila melanogster* genomic scaffold 142000013386053 section 16, position 80479/80480). *CG6340* has two predicted transcripts of 1619 bp (CT19841) and 641 bp (CT38120) length. Two bac clones include the P-element insertion site, BACR36D15 (AC010706) and BACR02B12 (AC011070). So far, there is no information available on the function of gene *CG6340*.

3.2.2.7 P-element line 9530

The approximate location of the transposon in region 6E-6F was determined by *in situ* hybridisation. Subsequently, sequence GPu 07 corresponding to the 3´ junctions between the inserted element and genomic DNA was isolated (334 bp, Fig. 40). The P-element is inserted in 6E4-6E7 128 bp upstream of the gene *inx2* (*CG4590*, AE003439, *Drosophila melanogaster* genomic scaffold 142000013386054 section 23, position 156153/156154).

```
        1              15 16            30 31            45 46            60 61            75 76            90
1 9530  TAAGTGGATGTCTCT TGCCGACGGGACCAC CTTATGTTATTTCAT CATGACTCCATTGTT AAACAAGATCCACCC CAAAAAGATTGCGCT   90

        91            105 106           120 121           135 136           150 151           165 166           180
1 9530  CTCCCGCTCTCTGAA TCTCTCCGCTTCGGC GACGGCGTTTAGCGC AGCCAAGCGACTTGT CCCAGCGGAAAGCCA AACAGTTTGAATTTT   180

        181           195 196           210 211           225 226           240 241           255 256           270
1 9530  CTATCCGAAGAGTGC GCTTAGTGCCGCTGC TGCGACGGCGTTTAG ACTTCCACCTGGGTA ACCGTCGCCCCGCC GCCCCCTTGAAACAC   270

        271           285 286           300 301           315 316           330 331           345 346           360
1 9530  AAGTGGTTTTGTTTT TGTTGTCGCGTTGGT GTTGAGAGCGCGAAA CGAACTCGAACCGAA CAGA     334
```

Figure 40: Result of plasmid rescue and sequencing of the region flanking the 3´ end of the transposon of P-element line 9530. Sequence of P-element p[lacW] is shown in blue letters, red letters indicate 8 bp target site duplication.

Inx2 has a transcript of 1819 bp and is categorized as neurotransmitter transporter, which interacts genetically with *innexin3* (*inx3*). The protein innexin2 is a component of the gap junction (Stebbings et al., 2000). Mutations have been isolated which are recessive lethal (Bourbon et al., 2002). Bac clone BAC RP98-17C9 (AC023698) is identified in that region.

3.2.2.8 P-element line 9885

Using *in situ* hybridisation, I could restrict the insertion site of p[lacW] to region 18C-18D. Plasmid rescue in 3´ direction of the P-element was performed with an EcoRI digest and sequencing with primer PCR2 (GPu 01, 350 bp), while a BamHI digest was performed to determine the sequence flanking the 5´ end of p[lacW]. Following the BamHI digest, I sequenced either with primer SP1 (GPu 26, 251 bp) or with primer Pout (GPu 27, 376 bp). For evaluation of sequence data, a composite sequence of 726 bp was created (Fig. 41). The P-element is inserted 169 bp upstream of the predicted gene *CG14207* which is located in 18D7-18D9 (AE003512, *Drosophila melanogaster* genomic scaffold 142000013386053 section 29, position 206784/206785) and which is predicted to be a chaperone (Flybase report). Two bac clones, BACR33M08 (AC010671) and BACR10M08 (AC010847), are available in that region.

```
            1            15 16          30 31          45 46          60 61          75 76          90
1 9885 TTCTCTGTTCTGTTC TGTTCTGCTCAACGC GCTTTCGAGCTTGTT CACTTGCTAATCGAC GGAGCAACCAAAAAA AAAGGGAACTCGATG   90
       ↳ GPu 27

            91          105 106        120 121         135 136         150 151         165 166         180
1 9885 GGTTTTCTATATGTA TTCGAAACTCAAGAC GTTCAAGTGTGTGAT TTCGGTTGGACGTAT CGCACAGATAGCCGC GTTTCAATTTCTGAA   180
                                        ↳ GPu 26

            181         195 196         210 211         225 226         240 241         255 256         270
1 9885 CTGTTTTGCATTCAC AGTGTGTCTTTGCTT TTATAGCTTTCGTAC CGATTGTACTTCGAT TGCCGCTGCGCAGCA GTGTGGCGATATCGA   270

            271         285 286         300 301         315 316         330 331         345 346         360
1 9885 TAGACCGATGAAGCC GCATTTAGTCATCGA TTTGCGGATTGGCGG TAATTTGATTATGTT TCTATTGCCATTGGA TCAAAAAGAACTTCT   360

            361         375 376         390 391         405 406         420 421         435 436         450
1 9885 ATCCGCAAAGGGAAT TGTTTAAGC↓GTACTT TAACCACTAATCGTT AGCGGACCACCCAAA ACACTATATTCGCTG TATTGATTTTGTATT   450
       GPu 26 / GPu 27 ↓↳ GPu 01

            451         465 466         480 481         495 496         510 511         525 526         540
1 9885 TTATGGAAATAATGA CGCACGGGGNAACAC ATTTAGAAGTGAAAC AATAGTTGTAAGACA AATTTGGTTTATACA AAATGACCGAAAACA   540

            541         555 556         570 571         585 586         600 601         615 616         630
1 9885 AGGAAGCCATGATTT ATTTTCCGTTAATAA GCTAGTAATTCATAA ATGCAATGAAATAAC ATAACACATTTCAAA GCTCCTAAAAAACTT   630

            631         645 646         660 661         675 676         690 691         705 706         720
1 9885 TCTAGGAACAAGTGT TCAATTAGCAACAAT TAATTAGTTGATGAT CAACTTGATCTTGAT AAGTATGCTTAAAAC TTCTCGCGTGGGAGC   720

            721         735 736         750 751         765 766         780 781         795 796         810
1 9885 CATTCA      726
       GPu 01 ↓
```

Figure 41: Result of plasmid rescue and sequencing of P-element line 9885. The P-element insertion site is indicated by a red arrow. Figure shows region flanking the 3´ end and 5´ end of p[lacW]. 8 bp which were duplicated due to the insertion of p[lacW] are marked in red. Range of sequences GPu 26, GPu 27 and GPu 01 is indicated by black arrows.

3.3 Molecular and behavioral characterization of ign^{P1} (8522)

Behavioral data from the screen as well as molecular data from plasmid rescue resulted in the selection of three lines, 8522, 9885, and 8631 for further investigation. P-element mutant line 8522 seemed to be the most promising line, as the gene affected in this strain was known to be involved in long-term memory in vertebrates (see Introduction). I called the line ign^{P1} and characterized the behavioral defect in more detail.

3.3.1 Behavioral characterization of mutant ign^{P1}

3.3.1.1 *Drosophila* mutant ign^{P1} is defective in operant conditioning

To characterize the learning and memory defect of the ign^{P1} P-element line, males and females were tested in the heat-box with the standard protocol. Figure 42 shows the last minute of training and test. As ign^{P1} was outcrossed to w^{G11} this line was used as the appropriate control.
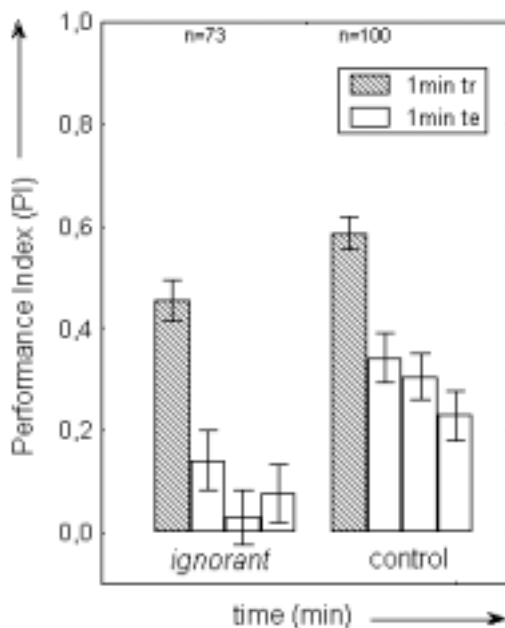
Figure 42: Performance Index (PI) of ign^{P1} versus w^{G11} in the standard experiment. Figure shows last minute of training (tr, hatched bars) and memory test binned to 1-min blocks (te, empty bars).

The mutant line ign^{P1} is characterized by reduced training performance (U-test, Z=2.48, p<0.05) and a reduced test score compared to control flies (U-test, 1st min, Z=2.09, p<0.05; 2nd min: Z=3.45, p<0.001, 3rd min: Z=2.03, p<0.05). Analysis of males and females separately showed that the observed phenotype is stronger in males than in females (Fig. 43). Training performance is significantly reduced only in males (U-test, Z=2.28, p<0.05), which also have a reduced test performance (U-test, 1st min, Z=2.77, p<0.01; 2nd min, Z=2.14, p<0.05; 3rd min, Z=2.69, p<0.01). Females show normal memory in the first minute but then a rapid decay (2nd min, Z=2.44, p<0.05).
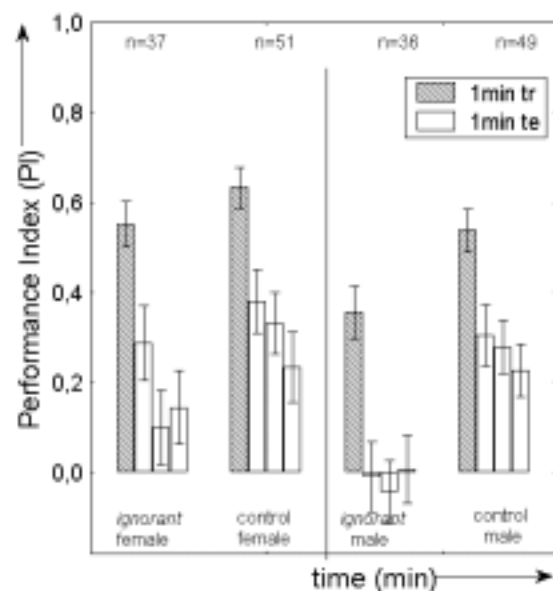
Figure 43: Same data as Fig. 42 but males and females evaluated separately. Figure shows last minute of training (tr, hatched bars) and memory test binned to 1-min blocks (te, empty bars).

To test whether performance can reach wild-type level when training duration is increased, measurements with 8 min and 10 min of training were performed (see next page Fig. 44). A training period of 8 min still resulted in reduced performance in ign^{P1} males in the last 2 min of training (U-test, Z=2.40, p<0.05) while ign^{P1} females performed well. After another 2 min of training, there was no longer a statistically significant difference between mutant and control males.
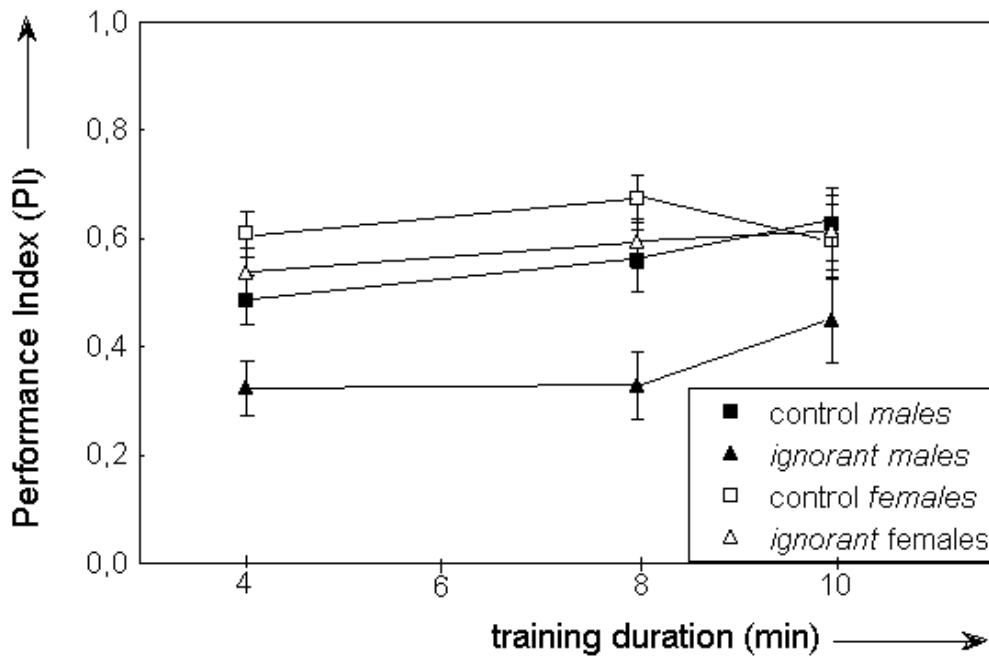
Figure 44: Performance Index of mutant ign^{P1} versus control w^{G11} in a learning experiment with training periods of 4 min, 8 min, or 10 min. Performance Index (PI) shows the last two minutes of training. Data from males and females are presented separately. Each group includes about 40 flies.

As Figure 45 shows, results are different for the test period. Only after 4 min of training, ign^{P1} males show a significantly reduced memory score. The deficit in ign^{P1} males may be due to a difficulty in learning the task as quickly as control flies, rather than to a general inability to learn it.
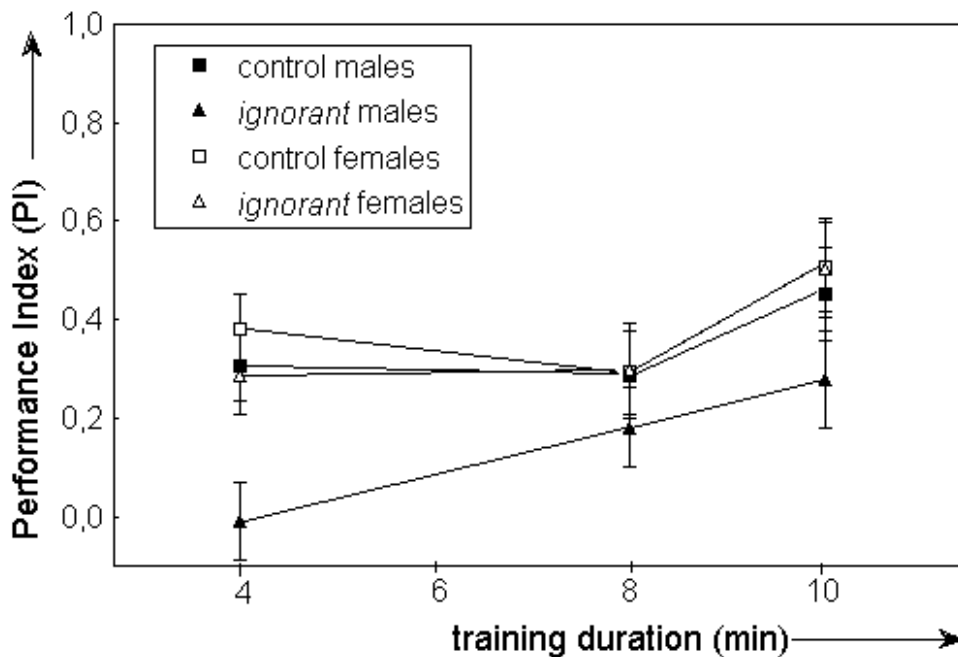
Figure 45: Performance Index of ign^{P1} flies versus control w^{G11} in a learning experiment with training periods of 4 min, 8 min or 10 min. Performance Index (PI) indicates the first test minute with males and females being tested separately. Each group includes about 40 flies.

3.3.1.2 *Drosophila ign^PI* flies show no defect in thermosensitivity

In the standard experiment, *ign^PI* males showed a reduction in heat avoidance during the conditioning procedure. To test whether this phenotype resulted from a difficulty in learning an association between their behavior and a reinforcer or only from reduced thermosensitivity, *ign^PI* flies were tested for potential defects in thermoreception. In the thermosensitivity assay (for details see chapter 2.1.4, Materials & Methods) wild-type flies obviously show an avoidance of the heated side, while flies which are defective in thermoreception (e.g. *ss^aristaepedia*, *bizarre*) are not able to avoid the heated part of the chamber in the lower temperature range (Sayeed and Benzer, 1996; Zars, 2001). As the thermosensitivity assay was performed before outcrossing the lines to *w^1118 Berlin,* Canton S was chosen as the control line (Fig. 46). Thermosensitivity of *ign^PI* males and females was intact over the entire temperature range of 24 °C to 45 °C (repeated measures Anova, $F=0.93$, p = n.s.). From these results, I conclude that the phenotype of *ign^PI* flies does not result from defects in thermosensation, but from difficulties in learning the task.
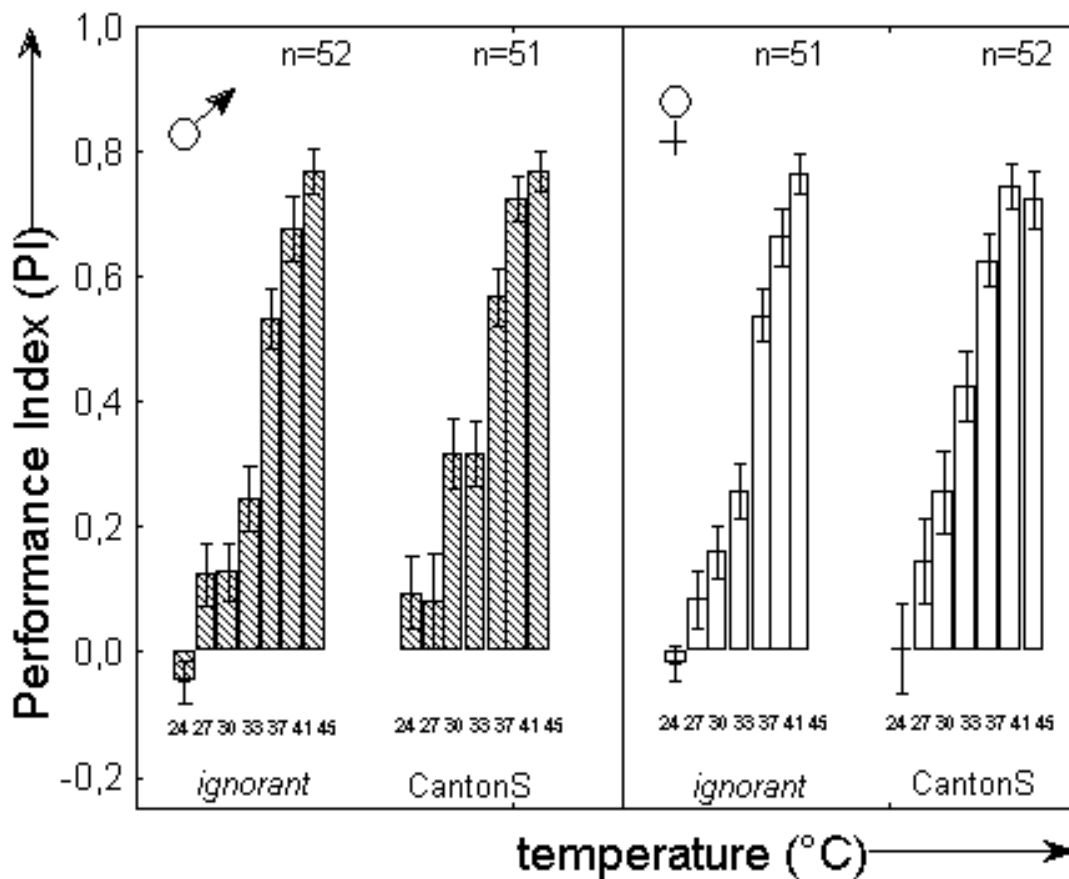
Figure 46: Performance Index of *ign^PI* versus wild-type CantonS flies in a thermosensitivity assay before outcrossing mutant flies into a uniform genetic background. Results are shown separately for males (hatched bars) and females (open bars). Each bar represents a 1-min test phase. During the test, temperature of one chamber half is stepwise elevated from 24 °C to 45 °C, while the other chamber half is kept at 24 °C. A positive value indicates that the flies spent more time in the 24 °C area.

3.3.2 Molecular characterization of mutant line *ign^P1* and jumpout lines

In situ hybridisation and plasmid rescue of the *ign^P1* line with subsequent sequencing and comparison of the obtained sequences to the *Drosophila* genome project showed that the P-element was inserted in the first exon of *S6KII*. The gene is flanked by gene *CG17602*, which is located upstream of *S6KII*, and *CG17598*, which is located downstream of *S6KII*. While the function of *CG17602* is still unknown, *CG17598* is predicted to code for a protein serine / threonine phosphatase.

To simplify the handling of nucleotide positions in the genomic region surrounding *S6KII*, I chose a genomic fragment of 50 kb of the genomic scaffold AE003574 (*Drosophila melanogaster* genomic scaffold 142000013386033 section 1) competely including the genomic region and defined the positions 1-50000 (Appendix Fig. 1). Position 1 was identical to scaffold position 150001 and position 50000 identical to scaffold position 200000. As numbering of the scaffold in Flybase changed, position 1 now refers to scaffold position 124458 and position 50000 to scaffold position 74458. Using a fragment of the genomic scaffold enabled easier nomenclature assignment of primers, with primer names referring to the primer binding site. The position of the P-element insertion in the genomic fragment is 27623/27624. In the following, all position numbers refer to the 50 kb genomic fragment.

3.3.2.1 Analysis of the structure of the *Drosophila S6KII* gene

Digest and complete sequencing of three ordered EST clones SD05277, GH08264 and GH21818 confirmed Flybase information about the structure of *S6KII*, predicting two exons and one intron. Sequences are shown in Figures 2 to 4 in the Appendix. Based on the sequencing results of SD05277, I conclude that exon 1 has a length of at least 2527 bp (nucleotide position 25124-27650) and is separated from exon 2 (nucleotide position 23534-24770) by an intron of 353 bp (nucleotide position 24771-25123). The second exon consists of 1237 bp.

Predicted open reading frames of the sequenced ESTs and the published mRNA of *S6KII* were obtained using the ORFfinder (Appendix Fig. 5 to 7). For all sequences a peptide of 911 aa (nucleotide position 27276-24190) was predicted. This protein has two kinase domains, a N-terminal kinase domain (aa 195 to 460, nucleotide position 26691-25894) and a C-terminal kinase domain (aa 560 to 840, nucleotide position 24401-24772, 25123-25596).

Additionally, I showed that the Flybase prediction for another clone LD42024 to match the *S6KII* gene was incorrect. Digest and PCR, however, gave a match of LD42024 to the surrounding *CG17602* and *CG17600* annotations (confirmed by pers. comm. with Sima Misra).

Sequencing cDNA SD05277, GH08264, and GH21818 revealed deviations from the published mRNA of *S6KII* (3137 aa). In 8 out of 12 cases (Appendix, Fig. 8) nucleotide exchanges were identical in at least two of three sequenced EST clones (see next page Table 12). Among the 12 nucleotide exchanges in the ORF of *S6KII*, only two resulted in an exchange of an amino acid and one in an additional three nucleotides resulting in an extra Glycine. There are several possible explanations for the described deviations. First, they could be attributed to sequencing errors in my experiments. This possibility is unlikely for deviations which were identical in all investigated cDNAs (found in 75 % of all nucleotide exchanges). A second possibility is that there are sequencing errors in the published mRNA of *S6KII*. A third explanation might be polymorphic changes in *S6KII*.

The most interesting sequencing result was the finding that the P-element insertion in *ign^P1* is not located upstream of *S6KII*, but is inserted in the first exon. I obtained this information from sequence comparison of SD05277 with the *Drosophila* genome. The P-element insertion is located at position 27/28 of clone SD05277 referring to Figure 8 in the Appendix.

Table 12: Summarised results of sequence comparison. Table shows deviations between mRNA of *S6KII* (1), SD05277 (2), GH08264 (3) and GH21818 (4). Position number refers to nucleotides of EST clone SD05277 which, with 3763 bp, is the largest sequence. Sequence and alternative sequence indicate where sequence deviations occured. Amino acid and alternative amino acid show corresponding nucleotide triplets and amino acids.

position	sequence	amino acid		alternative sequence	alternative amino acid
426-428	1,2,4	Q= Gln (cag)	⇔	3	Q= Gln (caa)
429-431	1	– – –	⇔	2,3,4	Q= Gln (cag)
444-446	1,2,4	S= Ser (tcc)	⇔	3	S= Ser (tct)
447-449	1,2,4	S= Ser (tcc)	⇔	3	P= Pro (ccc)
588-590	1	E= Glu (gaa)	⇔	2,3,4	D= Asp (gat)
849-851	1	G= Gly (gga)	⇔	2,3,4	G= Gly (ggg)
886-888	1	T= Thr (aca)	⇔	2,3,4	T= Thr (acc)
1086-1088	1,3	L= Leu (ctt)	⇔	2,4	L= Leu cta)
1196-1198	1	L= Leu (ctt)	⇔	2,3,4	L= Leu (ctc)
1744-1746	1	F= Phe (ttc)	⇔	2,3,4	F= Phe (ttt)
1869-1871	1	P= Pro ccc)	⇔	2,3,4	P= Pro (ccg)
2946-2948	1	C= Cys (tgc)	⇔	2,3,4	G= Gly (ggc)

The sequenced clones might not be complete at the 5´ end. To determine the 5´ end of *S6KII* mRNA, RNA ligase-mediated rapid amplification of the 5´ end (RLM-RACE) and five-minute cloning of Taq polymerase-amplified PCR products were performed. Two PCR steps were necessary to obtain enough amplified product for the cloning procedure. The first PCR was done using Gene Racer 5´ Primer and a gene specific primer (Primer 3, for details see chapter 2.14.4, Materials & Methods). The obtained PCR product was then used for a nested PCR using Gene Racer 5´ Nested Primer and another gene specific primer (Primer 5). Subsequent seqencing revealed five different 5´ ends, all confirming that the P-element was inserted in the first exon of *S6KII*. All different ends are in the range of 16 bp to 70 bp (nucleotide position 27694) upstream of the P-element insertion.

3.3.2.2 Generation of excision lines

In a next step, I wanted to show that the P-element insertion was responsible for the behavioral defect in *ign[P1]* flies. Additionally, I wanted to investigate whether the P-element insertion *ign[P1]* is a hypomorphic allele and partial or complete loss of *S6KII* might cause an even more severe behavioral phenotype. A strategy to address both questions was the remobilisation of the P-element by crossing a stable transposase source into the genome of *ign[P1]* flies (Robertson et al., 1988).

In 1983, O´Hare and Rubin (1983) already demonstrated that precise excisions occur and that they are accompanied by loss of both, the P-element and one copy of the 8 bp duplication precisely restoring the wild-type sequence at the insertion site. In case the P-element is responsible for the behavioral phenotype, a precise jumpout of the transposon should result in wild-type behavior of those flies. To obtain precise jumpouts, I performed excisions in females where the presence of a homologous chromosome increases the frequency of precise excision events (see chapter 2.2.2, Materials & Methods). I also remobilised p[lacW] in males to create imprecise excisions at higher frequency in search of null mutants (Engels et

al., 1990). Generation of jumpout lines resulted in 128 lines generated from jumpouts in females and 227 from jumpouts in males. The lines were then investigated molecularly and behaviorally.

In a first screen, I tested 13 jumpouts via Southern blot to select for potential precise jumpout lines. I isolated two lines, $ign^{\Delta 1P1}$ and $ign^{\Delta 2P1}$, completely restoring wild-type sequence at the P-element insertion site. Line $ign^{\Delta 1P1}$ does not show any nucleotide changes close to this site (Appendix, Fig. 9), while line $ign^{\Delta 2P1}$ has several nucleotide changes surrounding the P-element insertion site in the untranslated region (Appendix, Fig. 10).

In a second screen, 355 jumpout lines were screened by PCR and revealed 9 excision lines with deletions of about 1kb (2.5 %) and 4 lines with a loss of more than 2 kb of the *S6KII* gene (1.1 %; lines $Df(1)ign^{\Delta 30/2}$, $Df(1)ign^{\Delta 53/1}$, $Df(1)ign^{\Delta 58/1}$, and $Df(1)ign^{\Delta 67/1}$; Fig. 47). Three potential excision lines of each group were characterized in more detail by sequencing (see next page Table 13). All sequenced deletion lines have a loss of nucleotides at the sequence flanking the 5´ end of the transposon and are homozygous viable. I confirmed that deletion lines $Df(1)ign^{\Delta 4/1}$, $Df(1)ign^{\Delta 24/3}$, and $Df(1)ign^{\Delta 37/1}$ have a loss of about 1 kb of genomic sequence in the 5´ region of *S6KII* removing part of the first exon (Table 13). A complete loss of the first exon was found in line $Df(1)ign^{\Delta 30/2}$ which has a deletion of 2197 bp. In excision lines $Df(1)ign^{\Delta 67/1}$ and $Df(1)ign^{\Delta 58/1}$ the *S6KII*-coding region is completely removed.

Among the six investigated deletions lines, 4 lines still have part of the 10691 bp p[lacW], ranging from 16 bp to 5.5 kb. In two of those lines, I found 2 to 52 nucleotides which neither had homology to the P-element nor to the genomic sequence of *S6KII*. These nucleotides are situated between the remaining nucleotides of p[lacW] and the sequence of *S6KII*.
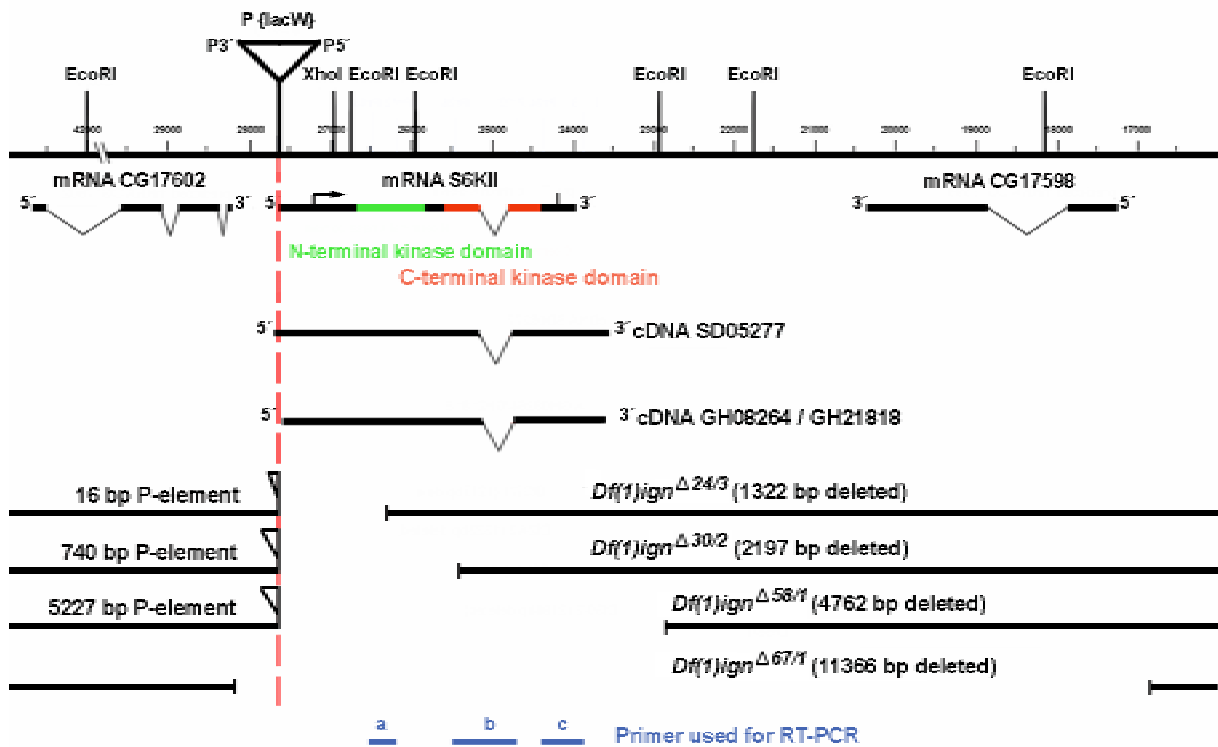
Figure 47: Molecular map of P-element line ign^{P1}. Figure shows restriction sites, insertion site of p[lacW], predicted mRNA of *S6KII* and mRNA of neighbouring genes, structure of sequenced cDNAs (SD0522, GH21818, and GH08264), range of deletion in line $Df(1)ign^{\Delta 24/3}$, $Df(1)ign^{\Delta 30/2}$, $Df(1)ign^{\Delta 58/1}$, and $Df(1)ign^{\Delta 67/1}$ and the expected product length resulting from RT-PCR with primer pairs 42/43 (a), 27/28 (b), and 23/24 (c).

Table 13: Summary of sequencing results of excision lines $Df(1)ign^{\Delta4/1}$, $Df(1)ign^{\Delta24/3}$, $Df(1)ign^{\Delta37/1}$, $Df(1)ign^{\Delta30/2}$, $Df(1)ign^{\Delta58/1}$, and $Df(1)ign^{\Delta67/1}$. Table shows remaining nucleotides of p[lacW], position and number of deleted nucleotides, number of bases located between P-element and genomic sequence which showed no homology to (unknown bp) the *Drosophila* genome.

Line	P[lacW] rest	pos. of deleted nucleotides	deleted genomic sequence	unknown bp
$Df(1)ign^{\Delta4/1}$	5 bp	26301-27623	1323 bp	2
$Df(1)ign^{\Delta24/3}$	16 bp	26302-27623	1322 bp	52
$Df(1)ign^{\Delta37/1}$	16 bp	26408-27623	1216 bp	--
$Df(1)ign^{\Delta30/2}$	740 bp	25427-27623	2197 bp	--
$Df(1)ign^{\Delta58/1}$	5227 bp	22862-27623	4762 bp	--
$Df(1)ign^{\Delta67/1}$	--	16868-28233 16284-16559 16690-16751	11366 bp 276 bp 62 bp	--

3.3.2.3 Identification of two null mutants

To test whether ign^{P1} and excision lines with a loss of more than 1 kb genomic fragment are null mutants, I tested for transcripts via RT-PCR (see chapter 2.14.3, Materials & Methods). The quality of isolated cDNA was tested by amplification of the rp49 ribosomal gene using primers rp49 and rp49r (GCGGGTGCGCTTGTTCGATCC and CCAAGGACTTCATCCGCCACC, from T.

Zars). RT-PCR showed that the P-element line as well as deletion lines $Df(1)ign^{\Delta24/3}$, $Df(1)ign^{\Delta30/2}$, and $Df(1)ign^{\Delta53/1}$ still have transcript in the region where genomic DNA is intact (Table 14). Jumpout line $Df(1)ign^{\Delta67/1}$ with a deletion of 11366 bp and line $Df(1)ign^{\Delta58/1}$ with a loss of 4762 bp completely remove the *S6KII* coding region and were null mutants via RT-PCR (see next page Fig. 48).

Table 14: Results of RT-PCR with line ign^{P1}, deletion lines line $Df(1)ign^{\Delta24/3}$, $Df(1)ign^{\Delta30/2}$, $Df(1)ign^{\Delta53/1}$, $Df(1)ign^{\Delta58/1}$, and $Df(1)ign^{\Delta67/1}$, and wild-type CantonS (CS). „+" indicates positive and „--" negative result of RT-PCR with mentioned primer pairs. Negative control (neg) was performed without DNA. Primer and reverse primer (r) used are indicated. Numbers below primer name indicate primer binding site and expected length of the amplified product. Letter next to primer name is used as abbreviation in later experiments. In cases of empty cells, RT-PCR was not performed. Positive control with rp49/rp49r was only performed in RT-PCR of excision lines where no internal positive result was expected. Primer pair 27/28 amplifies across the intron and was used as control for contaminations with genomic DNA by differences in expected PCR product sizes.

line	15/16 26727-26133r 595 bp	42/43 (a) 26445-26141r 305 bp	19/20 25460-25158r 303 bp	27/28 (b) 25459-24693r 413 bp	23/24 (c) 24338-23869r 470 bp	rp49/rp49r pos contr 152 bp
ign^{P1}	+		+	+	+	
CS	+	+	+	+	+	+
$Df(1)ign^{\Delta24/3}$	--		+	+	+	
$Df(1)ign^{\Delta30/2}$	--		+	+	+	
$Df(1)ign^{\Delta53/1}$	--			--	+	
$Df(1)ign^{\Delta58/1}$	--	--		--	--	+
$Df(1)ign^{\Delta67/1}$	--	--		--	--	+
neg	--	--	--	--	--	--

Figure 48: Results of RT-PCR with line $Df(1)ign^{\Delta 58/1}$ and $Df(1)ign^{\Delta 67/1}$ compared to wild-type CantonS and a negative control where no cDNA was used. Primer pairs 42/43 (a), 27/28 (b), and 23/24 (c) bind within the coding region of *S6KII* (Fig. 47). The positive control (pos contr) was performed with primer pair rp49/rp49r. A 1 kb DNA ladder was used as size standard.

The P-element line ign^{P1} had a transcript in all investigated genomic regions of the *S6KII* gene and, thus, cannot be considered a null mutant. Whether the mutation leads to the generation of a nonfunctional S6 kinase or the absence of a product must be investigated. The same applies to deletion lines $Df(1)ign^{\Delta 24/3}$, $Df(1)ign^{\Delta 30/2}$, and $Df(1)ign^{\Delta 53/1}$ with a partial loss of the coding region and transcript. The deletions are expected to result in a truncated protein or no protein. However, it is neither known whether such a protein is made, nor whether it is functional.

3.3.3 Behavioral characterization of jumpout lines versus P-element line ign^{P1}

3.3.3.1 Performance of jumpout lines in heat-box conditioning

Subsequent to molecular characterization, the original P-element line ign^{P1}, deletion lines $Df(1)ign^{\Delta 24/3}$, $Df(1)ign^{\Delta 30/2}$, $Df(1)ign^{\Delta 58/1}$, and $Df(1)ign^{\Delta 67/1}$, including deletions from 1kb to 12 kb, as well as precise jumpout lines $ign^{\Delta 1P1}$

and $ign^{\Delta 2P1}$ were chosen for behavioral investigation. Unfortunately, control line w^{G11} showed a decrease in performance in the heat-box after several generations of consistently good performance. For this reason, I chose wild-type CantonS as a more stable genetic background. First, I initiated recombination of the w^{+} gene onto the X-chromosome (see Materials & Methods, chapter 2.2.2) and controlled for successful recombination events by PCR. In parallel, flies carrying the balancer FM7a were outcrossed to wild-type CantonS for six generations. Finally, selected lines carrying w^{+} were outcrossed to the cantonized FM7a flies for six generations. Performance of males and females in the standard experiment was then measured (following page Fig. 49).

Test performance of all cantonized lines did not significantly deviate from wild-type CantonS flies. Focussing on the last training minute, I could confirm a defect in ign^{P1} males also in the cantonized mutants (U-test, Z= 3.57, p< 0.001), while ign^{P1} females performed well. This result is consistent with earlier measurements. Males and females of precise excision lines $ign^{\Delta 1P1}$ (U-test, males: Z=1.08, p= n.s.; females: Z=-0.77, p= n.s) and $ign^{\Delta 2P1}$ (U-test, males: Z=0.64, p= n.s; females:
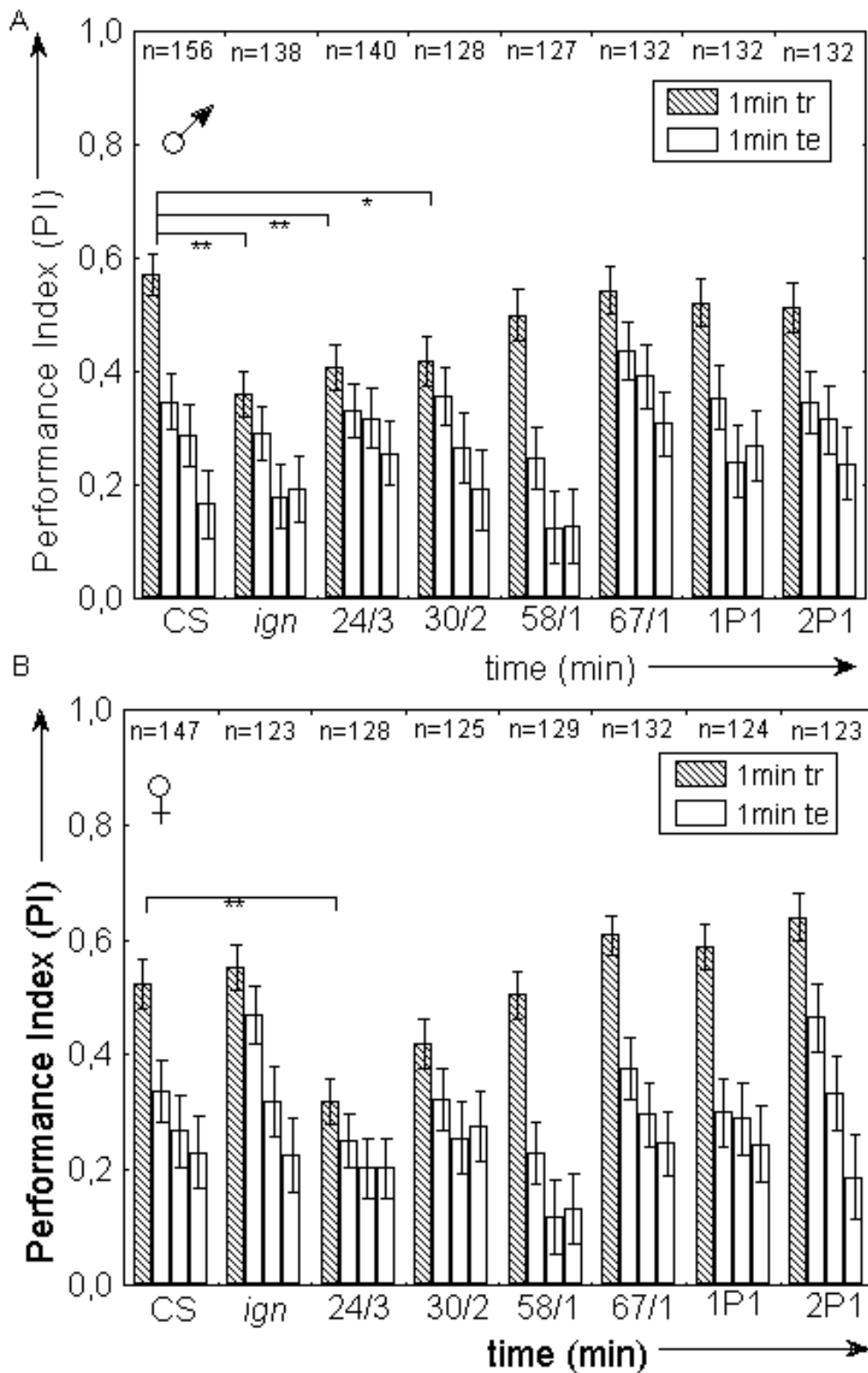
Figure 49: Performance Index (PI) of cantonized P-element line ign^{P1}(ign) deletion lines $Df(1)ign^{\Delta24/3}$ (24/3), $Df(1)ign^{\Delta30/2}$ (30/2), $Df(1)ign^{\Delta58/1}$ (58/1) $Df(1)ign^{\Delta67/1}$ (67/1), and precise jumpouts $ign^{\Delta1P1}$ (1P1) and $ign^{\Delta2P1}$ (2P1) versus wild-type control CantonS (CS) in the standard experiment. Males (A) and females (B) are shown separately. Figure shows last minute of training (tr, hatched bars) and all 3 min of the memory test binned to 1-min blocks (te, empty bars).

Z=-1.91, p= n.s) both perform well in the last minute of training and thus revert the phenotype observed in *ign^P1* flies.

Flies with a deletion of about 1 kb of the coding region of *S6KII* have a reduced performance in males and females (U-test, males: Z=2,97, p< 0.01; females: Z=3.73, p< 0.001), whereas a deletion of about 2 kb (*Df(1)ign^Δ30/2*) results in a statistically significant defect only in males (U-test, males: Z=2.50, p< 0.05). Females of this line still show a tendency to perform less well than CantonS females. As the P-element line, but also deletions *Df(1)ign^Δ24/3* and *Df(1)ign^Δ30/2* still make some transcript, the phenotype could be due to the production of a truncated protein and its interaction with other peptides. Results are discussed in more detail in chapter 4.2. Surprisingly, removal of the complete coding region of *S6KII* does not have an effect on operant conditioning in the heat-box (U-test,

males: Z=1.24, p= n.s.; females: Z=0.34, p= n.s.). Also, additional removal of the neighbouring gene *CG17598* does not lead to a defect in performance (U-test, males: Z=0.90, p= n.s.; females: Z=-0.73, p= n.s.). The behavioral defects of the mutants generated are consistent with gain-of-function phenotypes. Whether this is a consequence of *S6KII* signaling or not, however, cannot be answered yet.

3.3.3.2 Jumpout lines show no defect in thermosensitivity

To exclude the possibility that reduced training performance of *ign^P1* and deletion lines *Df(1)ign^Δ24/3* and *Df(1)ign^Δ30/2* in heat-box conditioning resulted from defective thermosensitivity, all cantonized jumpout lines were tested for intact thermoreception (Fig. 50 and 51).
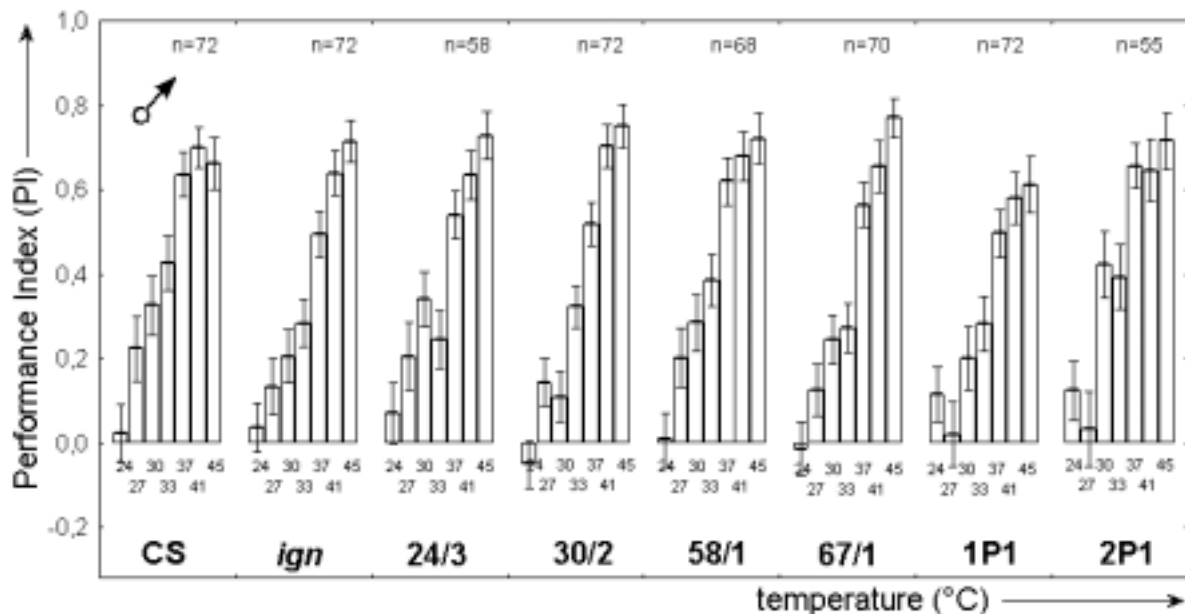
Figure 50: Performance Index of *ign^P1* and jumpout lines versus wild-type CantonS flies in the thermosensitivity assay. Figure shows results of males. Each bar represents a 1-min test phase. During the test, temperature of one half of the chamber is elevated stepwise from 24 °C to 45 °C, while the other chamber half is kept at 24 °C. The side of the chamber set to the reference temperature changes after 60 sec. A positive value indicates that the flies spent more time in the 24 °C area.
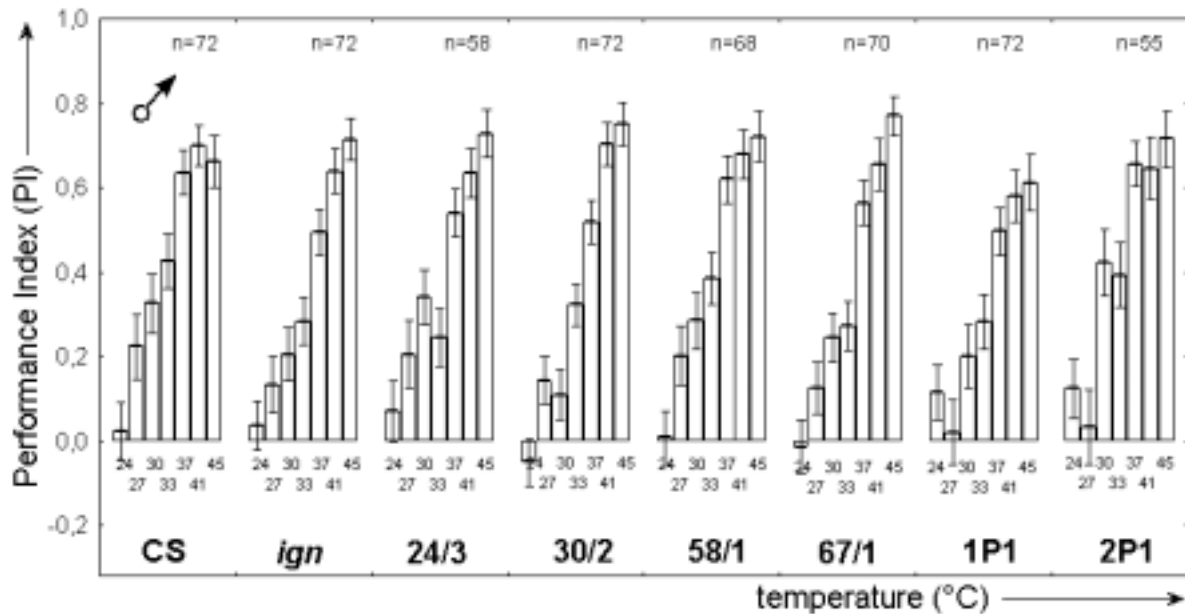
Figure 51: Performance Index of *ign*^{P1} and jumpout lines versus wild-type CantonS flies in the thermosensitivity assay. Figure shows results of males. Each bar represents a 1-min test phase.

Males and females were tested separately. Neither in males (repeated measures Anova, F=0.93, p = n.s.) nor in females (repeated measures Anova, F=0.77, p = n.s.) a defect in thermoreception was found for any of the investigated lines, supporting the idea that the observed low performance in operant conditioning reveals a learning deficit.

3.3.4 Anatomical and histological characterization of *ign*^{P1}

3.3.4.1 No obvious structural brain defects in *ign*^{P1} flies and null mutants

Brain structure mutants like *mbm* are known to show behavioral defects (de Belle and Heisenberg, 1996). To test whether the behavioral phenotype of *ign*^{P1} resulted from a change of brain structures, paraffin sections were generated by Eike Kiebler. Brains of *ign*^{P1} in different genetic backgrounds (undefined, *w*¹¹¹⁸ *Berlin* or *w*^{G11}) did not reveal obvious structural defects. Also excision lines *Df(1)ign*^{Δ30/2} and *Df(1)ign*^{Δ67/1} with the recombined *w*⁺ gene on the X-chromosome did not show any obvious changes in brain structure. Thus, there is no indication that the defect in operant conditioning of *S6KII*

mutants is due to stuctural changes in the brain, although this possibility cannot be fully excluded due to the coarseness of the assay.

3.3.4.2 Mutant line *ign*^{P1} has normal body size

Besides *S6KII*, there is another gene in *Drosophila* called *S6k* which is localised in chromosome region 64 F1-2 encoding a p70 ribosomal S6 kinase. RPS6-p70-protein kinase regulates cell size in a cell-autonomous manner. Flies deficient in this gene have a severely reduced body size (Montagne et al., 1999). To test for a similar phenotype in *S6KII* mutants, length (see next page Fig. 52A) and surface area (Fig. 52B) of the wings were measured in *ign*^{P1} mutants and wild-type CantonS flies as an indicator of body size. Compared to control flies, I neither found a difference in wing length, nor in surface area between mutant and wild-type flies (U-tests, p= n.s.). *S6KII* mutants do not share the anatomical phenotypes of *S6k* mutants. Between males and females of the same genotype, I found expected differences for wing length (U-tests, *ign*^{P1}, Z=3.78, p<0.001; CantonS, Z=3.22; p<0.001) and surface area of the wings (U-tests, *ign*^{P1}, Z=3.79, p<0.001; CantonS, Z=3.02; p<0.01).
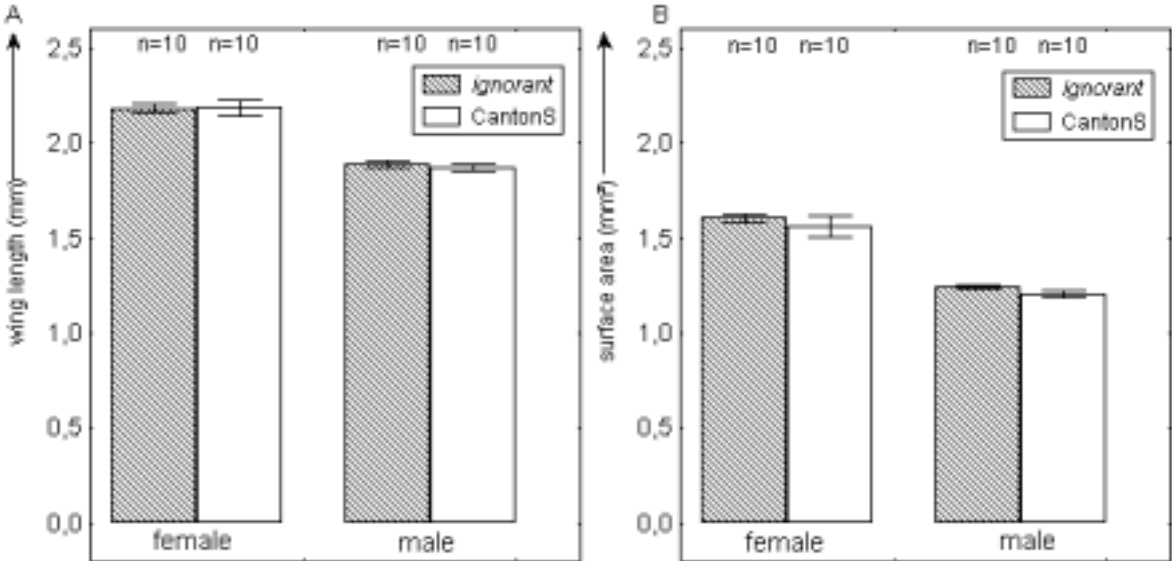
Figure 52: Wing length (A) and surface area of wings (B) of *ign^{P1}* flies compared to wild-type CantonS flies. 10 animals per group were investigated, including measurements of both wings of each animal.

4 DISCUSSION

4.1 Characterization of memories in *Drosophila* heat-box conditioning

4.1.1 Influence of age, sex, and larval density on test performance

A parametric study revealed that heat-box conditioning is a robust procedure which does not require the observance of strict rearing conditions. My experiments showed that it is neither required to severely control larval density (Fig. 14), nor necessary to restrict measurements of experimental flies to a certain age, as old flies perform as well as young flies. The ability of flies to learn to avoid a punished area was already observed at an age of 2 days and lasted for more than four weeks (Fig. 11). Thus, I expect this ability to be a natural relevant task. It might be vital for a fly to avoid sunny spots to evade drying out. I did not test performance of flies younger than 2 d, as just hatched flies are not very robust and handling of the flies in the experiment might lead to deformations, e.g. of the wings or legs, which might, then, influence walking behavior and as a consequence performance of the flies. Flies older than four weeks were not tested, as the effort to maintain them for such a long period of time makes it unlikely that a researcher will use flies of that age for behavioral experiments. I did not find a difference in test performance between CantonS males and females and, thus, conclude that wild-type measurements can be performed without considering sex. This improves experimental conditions in case only very few flies are available.

Data analysis revealed a weak negative correlation for walking activity and memory performance of flies (Fig. 13). Obvious effects, however, were restricted to certain groups of flies, e.g. 5 and 9 d old ones (Fig. 12). Those flies are characterized by severely increased activity levels together with low performance values. Walking activity was already increased in the pretest and remained elevated during the conditioning procedure. A possible explanation for the negative correlation is that hyper-active flies might pay less attention to the situation and thus do not learn an association between their behavior and the reinforcer. Other groups of flies did not show this effect. For instance, walking activity was increased in females compared to males, but there was no significant difference in performance between both sexes (Fig. 11). Inversely, in spite of similar walking activities of 2, 5, and 9 d old flies, statistically significant differences in test performance were found for 2 d old flies versus 5 and 9 d old ones. As the slope of the regression line of Figure 13 was flat and effects were only found in specific groups of flies, a general correction factor for the calculation of Performance Indices was not introduced. Nevertheless, I suggest consideration of severe hyper-activity for data interpretation. Further aspects of walking activity in heat-box learning are discussed in the following chapter.

Behavioral results of wild-type CantonS flies (Fig.11), as well as data from the P-element mutant screen (Appendix, Table 6) point out that, despite the robustness of the procedure, we get escapers from time to time. Repeated measurements of flies, e.g. of different generations, are, therefore, necessary to learn about the characteristics of the behavioral phenotype of the fly strain of interest and the consistency of the phenotype.

4.1.2 Memories in heat-box conditioning

Conditioning in the heat-box can be very effective. After a training of 20 min, flies stay on the previously heat-associated side for only about 10 % of the time (Fig. 15). This conditioned avoidance is about as strong as in odor discrimination learning (Tully and Quinn, 1985). At closer inspection, however, the two values are not really comparable, because in the heat-box avoidance during the test period is only partly due to the fly's preference for certain locations in the chamber. As the position of the flies cannot be 'randomized' between training and test the heat avoidance at the end of the training is carried over into the test phase. One may account for this effect in

the data evaluation procedure, but at the price of underestimating the associative memory score (Fig. 20). Part of the component one discards calculating the low estimate is due to the fact that most flies start the memory test on the non-punished side. Waiting until the first midline crossing for these flies means discarding a positive contribution to the memory score. The other part is due to flies showing no further midline crossing. In most cases, these flies show extremely low walking activity, that may be either because of a particularly strong conditioned side preference or due to mere heat avoidance.

The latter effect shows up not only in the locomotor activity data of Figures 18 and 19 where trained flies were compared with their yoked controls and training leads to a stonger reduction of locomotor activity than heating *per se*, but also in the number of flies evaluated in Fig. 20. For instance, after the intermittent training only 39 of the 145 flies could be included in the conservative estimate because only those flies crossed the midline within the first two minutes.

As expected, a training interrupted by rest periods is more effective than a continuous conditioning phase (Fig. 15). In many organisms and learning situations spaced training regimes with very different temporal patterns are known to improve memory (Hintzman, 1974). In *Drosophila*, extended memory spans after spaced training have been documented for odor (Tully et al., 1994) and visual pattern discrimination learning (Xia et al., 1997a). For operant conditioning, I showed that intermittent training mainly strengthens the *stay-where-you-are* effect. This was unexpected, as with intermittent presentation of the reinforcer the *stay-where-you-are* strategy should be more difficult to learn, whereas the conditioned side preference should become more robust against extinction training, as is indeed observed for the composite memory score (Fig. 16). The heterogeneous composition of the memory score must be taken into account in mapping experiments (Zars et al., 2000a) as well as future genetic and pharmacological analysis.

In the transfer experiments the avoidance at the end of the primary training is irrelevant for the final memory score after the transfer. Due to the high symmetry of the chamber, the fly has no cue as to its position in relation to the potentially heated side after the transfer. Only the 30-sec reminder training which immediately preceeds the test phase and provides the hot / cold polarity, may still affect it. The control experiments with naïve flies, however, showed that the *stay-where-you-are* effect from the reminder training is negligible. Moreover, after the transfer and the reminder training, locomotor activity was high for all groups (data not shown). I, therefore, conclude that the memory scores in the transfer experiments represent primarily the conditioned side preference.

In one of the control experiments with a 1-3 min retention interval in the food vial a new memory phenomenon was discovered indicating that a reminder training of 30 sec can be sufficient to induce a subsequent memory score provided that the fly is in the right disposition (Fig. 23). If in the first phase (what would be the training phase) the fly is kept in the chamber without the heating regime, the transfer back to a food vial and to a group of other flies between training and test is necessary to establish this dispositional state. A direct transfer from the exposure chamber to the test chamber does not (Fig. 21). In other words, after the first transfer from the regular food vial and group situation to the narrow dark chamber, the 'naïve' fly is not in the right disposition to build up a memory of the spatial distribution of heating periods during the following half minute. If, however, the same transfer occurs a second time, the fly is ready to attend to the contingency between the heat pulses and its own position in the chamber. Thus, the memory of the first transfer and exposure to the chamber disposes the fly favorably for the learning task after the second transfer. It is a well known phenomenon that pre-exposure to the training context without reinforcement can facilitate subsequent acquisition (Guo et al., 1996; Tolman and Honzik, 1930). Here, this is only part of the story. The transfer from the group of flies in the food vial to the chamber and the time in the chamber seem both to be relevant because omitting the rest phase in the food vial (direct transfer, Fig. 21) as well as shortening the first stay in the chamber (handling, Fig. 25) both abolish the effect. The length of the chamber is not critical (Fig. 27), whereas a plastic vial instead of a chamber does not fully serve as an adequate pre-exposure (Fig. 26), perhaps

because it is not dark. To fully understand what the fly is learning in the first phase to master the 30-sec learning task in the second phase requires more detailed investigations.

With the transfer experiments and yoked controls, I have finally demonstrated beyond doubt that the heat-box records an associative memory. The fly can remember, even two hours later (Fig. 23), that its position in the chamber controls temperature. Acquisition of this memory is an operant process. The fly's discovery that its behavior can modify temperature leads to a lasting modification of the fly's behavior. How the fly modifies its behavior to take advantage of its conditioned side preference remains to be determined. The fly may try to stay close to the 'cold' end of the alley and it may avoid long straight walks or even any locomotion. In any case, the side preference persists independently of the fly's actual position in the chamber. It must therefore be based on a 'percept' or 'cognitive map' of the chamber, simple as this representation may be. The map may consist of nothing but two antiparallel vectors for the safe and dangerous directions which the fly maintains irrespective of its own changing position and orientation.

In order to relate heat-box memories to the brain and to other forms of memory in *Drosophila*, mushroom body-less flies and their controls were included in this study. Flies store memories of odors in their mushroom bodies (Zars et al., 2000b). In many other forms of learning flies without mushroom bodies perform perfectly well. These include visual pattern recognition, colour discrimination learning, motor learning, conditioned courtship suppression in the light, and also learning in the heat-box (summarized in Wolf et al., 1998). I reinvestigated this problem here because Liu et al. (1999) discovered that the mushroom bodies render visual memories less sensitive to context changes. The transfer procedure necessitates a context change, the transfer from the chamber to the food vial and back. As it turns out, heat-box memories are sufficiently robust to sustain these context changes even in mushroom body-less flies (Fig. 28). Apparently, different neural circuits underlie the robustness of memories in the visual and ideothetic domains. This result, however, should not be surprising. 'Context' is a broad concept. Everything besides the

conditioned and unconditioned stimuli and the behavior in question might be regarded as the context. In visual pattern recognition at the flight simulator the part of the context that changes is the quality of illumination (Liu et al., 1999). All other aspects of the fly's precarious situation remain the same. In the present transfer experiments the situation of the fly dramatically changes from ample space, fresh food, light, and company to isolation, confinement, and darkness. The differences in these two types of context change could hardly be more profound. Nevertheless, one has to abandon the idea that the mushroom bodies might support a general mechanism protecting against all kinds of context changes in memory processes.

4.1.3 Defect of learning and memory mutants in operant conditioning

Drosophila mutants *amnesiac* (*amn[1]*), *dunce* (*dnc[ML]*), and *rutabaga* (*rut[2080]*) with mutations affecting the cAMP signaling cascade are known to be defective in classical conditioning (Tully and Quinn, 1985; Zars et al., 2000b). All three mutant lines also showed obvious defects in the standard experiment in the heat-box. They were characterized by reduced training and test performance with behavioral phenotypes being slightly different between the different mutations (Fig. 29). My results confirmed earlier data of Wustmann, who tested *rut[1]* and *dnc[1]* mutants in the heat-box and identified their defect for the first time (Wustmann et al., 1996). Furthermore, my data are consistent with results of Zars and colleagues who tested *rut[2080]* mutants in the heat-box (Zars et al., 2000a). He repeatedly showed that *rut[2080]* mutants are defective in both the training and the memory test. Performance scores of the mutants increased during the conditioning procedure, but did not reach the level of control flies. A cDNA rescue of the *Drosophila rutabaga* type I Ca^{2+} / CaM-dependent adenylyl cyclase (AC) gene restored the learning / memory phenotype in the heat-box. These results indeed proved a role for the cAMP signaling cascade in operant conditioning. In *dnc* and *amn* mutants, rescue experiments have not been performed. However, the fact that different alleles of *dnc*

(dnc^{ML} and dnc^{1}) and *amn* (amn^{1} and P-element lines 5054, 8570, and 9725) result in a defect in operant conditioning, support the idea that mutations in those genes and, thus, in the cAMP signaling cascade are responsible for the behavioral phenotype.

A comparison of described behavioral phenotypes of *dnc* and *rut* mutants in operant conditioning with results from classical odor avoidance conditioning shows that the phenotypes are similar. Following Tully´s classification of functionally distinct memory phases, *dnc* and *rut* flies belong to the class of STM mutants in olfactory learning with a reduction in initial learning and a rapid memory decay in the first 30 min after training (reviewed in Tully et al., 1996). Also in the heat-box both mutants are characterized by reduced learning and memory scores. The *radish* mutants are another example where no discrepancy between results of operant and classical conditioning was found. rad^{1} mutants are characterized by an abnormal rapid memory decay in the olfactory discrimination test, with a lack of the anesthesia-resistant memory (ARM), while STM is intact (Tully et al., 1994). After Tully, information that is acquired during learning is processed into consolidated memories (ARM and LTM) by passing sequentially through two earlier memory phases: short-term and middle-term memory (STM and MTM). Information flow then branches into the ARM and LTM parallel paths. ARM mutants are characterized by the entire decay of the memory within 8 hrs after training (Tully et al., 1996). In the standard experiment in the heat-box, rad^{1} mutants showed no behavioral defects (Fig. 29). Assuming that suggested memory classification for classical odor avoidance conditioning also accounts for operant learning processes in the heat-box, this result is not surprising, as a memory test directly following the conditioning procedure might uncover defects of early memory phases, but not the lack of the ARM.

However, as the nature of the two types of learning is quite different, it is unlikely that memory classifications can simply be transferred from classical to operant conditioning. Classical conditioning is a behavior-independent learning process, where animals learn about relations between stimuli.

It is often described as the transfer of the response-eliciting property of a biologically significant stimulus (US) to a new stimulus (CS) without that property (Pavlov, 1927; Hawkins et al., 1983; Kandel et al., 1983). The transfer is thought to occur only if the CS can serve as a predictor for the US (Rescorla and Wagner, 1972; Pearce, 1994). Classical conditioning, where the organism experiences and eventually memorizes contingencies in its environment (CS-US associations), is opposed to operant conditioning, a behavior-dependent learning (B-US associations), where an animal is constantly exploring the consequences of its own actions and is learning about them. Operant behavior requires a goal. In order to achieve it, a range of motor programs is activated (initiating activity). Efference copies of those motor programs are compared to the sensory input referring to the deviation from the desired state and in case of a significant coincidence the respective motor program is used to modify the sensory input in the direction towards the goal. The consistent control of a sensory stimulus by a behavior might result in a more permanent behavioral change (conditioning). Deviating concepts of classical and operant conditioning might also account for different cellular mechanisms underlying the two types of learning. Besides the different nature of operant and classical learning procedures, conditioning parameters, e.g. training duration, are different between heat-box and odor avoidance conditioning. Thus, it is not surprising that results for *amn* mutants deviate between the two types of conditioning. While the 3 min memory is intact in classical conditioning and the mutants, hence, are classified as MTM mutants, their performance in operant conditioning is already reduced in the training phase.

Also candidate structures where the memory is stored deviate between the two types of learning. *rutabaga* mutants are well studied concerning this question. Zars and colleagues showed that for classical odor avoidance learning *rutabaga* expression in the mushroom bodies is sufficient, whereas expression in the ventral ganglion, the antennal lobes, and the median bundle can rescue a behavioral defect in operant conditioning (Zars et al., 2000a, 2000b).

4.2 Behavioral mutant screen and the role of p90 ribosomal S6 kinase in operant conditioning

Are there other, yet unknown genes, signaling cascades, and cellular mechanisms which are involved in heat-box conditioning? With the isolation of 29 viable *Drosophila* X-chromosome P-element mutants from a behavioral screen and subsequent molecular characterization of their genetic defect, I indeed found new candidate genes which might have a role in operant conditioning, e.g. *S6KII*, *inaF*, *NetB*, *inx2*, *CG6340,* and *CG1420*. Among the selected mutants, 16 were disturbed in heat avoidance / learning and 13 lacked a memory. However, as I did not control for a uniform genetic background, the behavioral phenotypes could have resulted from second-site mutations and not from the P-element insertion itself. In my experiments, six of seven lines which were outcrossed to berlinised w^{G8} and w^{G11} flies kept their learning / memory deficit. Although the behavioral phenotype was, thus, likely to result from the P-element insertion or a genetic defect in close proximity to the P-element insertion site, there are still alternative explanations which might account for the behavioral defect, as three things stay constant in this outcrossing: the presence of the *white* mutation, the expression of the w^+ from p[lacW], and the P-element insertion in or next to a gene. Therefore, deficits in operant conditioning could result from the *white* mutation or misexpression of w^+. Recombination of w^+ onto the X-chromosome of several P-element candidates and subsequent outcrossing procedure against wild-type CantonS by Susanne Kramer, in fact, revealed that the behavioral phenotype disappeared after exchange of the genetic background in some lines (data not shown).

Instead of learning and memory defects, the lack of other abilities which are required for successful learning in heat-box conditioning might be responsible for the failure of a fly in that paradigm, e.g. reduced thermosensitivity or defective walking behavior. Defects in thermosensitivity were excluded and did not explain reduced heat-avoidance in the conditioning procedure (results for ign^{PI} in

Fig. 46). Another reason for low performance scores might be the inability of flies to orientate correctly in the chambers. Wustmann showed that flies do not use cues from outside the chamber for orientation, e.g. electromagnetic field. As my experiments were performed in complete darkness, flies also could not rely on visual cues. Thus, ideothetic orientation is required, because the animals can only gain information about space from their prehistory of movements (Wustmann and Heisenberg, 1997; Mittelstaedt and Mittelstaedt, 1973). Although there is no experiment applicable to test for intact ideothetic orientation, the ability to orientate in darkness is also needed in the thermosensitivity test. Thus, good performance scores of the selected lines in that assay are an indication that the flies do not lack this capability. Motor defects were expected to be evident in the analysis of time traces and walking activities. Among the investigated lines, such defects were not found.

The isolation of the *amn* mutants 5054, 8570 and 9725 in the P-element screen confirmed that the heat-box is a useful tool to search for genes involved in learning and memory. Surprisingly, other known learning / memory mutants like *dnc* and *rut* were not found in the screen. Possibly the *amn* locus is a hotspot for P[lacW] insertions, while the *dnc* and *rut* loci might belong to regions where P-element insertions happen with lower frequency. Alternatively, the screen might not have been saturating for this phenotype and chromosome, and therefore these mutations were not found.

In repeated measurements, ign^{PI} (8522) mutant flies were originally characterized as memory mutant candidates. A closer look at those data, however, shows that performance in the last minute of the training period varied between 0.452 and 0.606 in the standard experiment (Appendix, Table 6). Thus, although the ign^{PI} mutants did not historically fall in the category of heat avoidance candidates, because mutant criteria were chosen to be very strict to avoid focussing on lines with weak behavioral phenotypes, their performance was occasionally very low compared to typical performance levels of wild-type CantonS flies. However, the mutants were not in a uniform genetic background and, thus, direct comparison to an appropriate control line was not possible. After outcrossing the ign^{PI}

mutants to w^{G8} and w^{G11} flies, they showed a strong reduction of training performance compared to control flies. I focussed on sex specific differences for the first time when the ign^{P1} mutants were in the w^{G11} background and found a much stronger behavioral defect in males than females (Fig. 43). Results from conditioning experiments with increased training duration indicate that the defect in ign^{P1} males is rather due to a problem in learning a spatial preference for certain chamber locations, than due to a general inability to memorize associations (Fig. 44 and 45). The restriction of a severe phenotype to ign^{P1} males remained even after cantonisation of the flies (Fig. 49). Surprisingly, cantonized ign^{P1} males have a reduction in training performance, whereas they perform well in the memory test, which supports the idea that ign^{P1} males have a learning defect. The change of the behavioral phenotype might be due to the different genetic backgrounds of w^{G11} and CantonS flies or due to the exchange of the *white* mutation by w^+. Without separate analysis of sexes, the defect of males is masked by the performance of the females. This might explain the weak training defect of ign^{P1} mutants in the first measurements (Appendix, Table 6). The fact that behavioral results varied dependent on the genetic background of the flies, but also between different generations of the same genetic background, again points out that repeated measurements in the heat-box were indispensable to clearly characterize the behavioral phenotypes.

Although the reversion of the phenotype in cantonized precise jumpout lines $ign^{\Delta 1P1}$ and $ign^{\Delta 2P1}$ showed that the P-element is responsible for the learning defect, results of $Df(1)ign^{\Delta 58/1}$ and $Df(1)ign^{\Delta 67/1}$, with a complete loss of the *S6KII* coding region, indicate either that p90 ribosomal S6 kinase is not necessary for operant learning processes or that its function can be substituted by other genes or signaling cascades. Dufresne and colleagues suggest a compensatory mechanism for *RSK2* mouse mutants (Dufresne et al., 2001). They found that mice lacking a functional *RSK2* gene have a two-fold increase in ERK phoshorylation in skeletal muscle in response to insulin and exercise (two potent stimulators of the ERK cascade in skeletal muscle) compared to wild-type mice and claim a role in feedback inhibition of the ERK pathway. It could be

mediated by increased expression and / or activation of an ERK phosphatase, since the ERK pathway itself can increase the expression of certain ERK phosphatases (Brondello et al., 1997). In case ERK accomplishes this via RSK activation, KO mice are expected to have lower phosphatase expression levels and, thus, higher ERK phoshorylation which might compensate for the lack of RSK2 in muscle. Despite increased ERK phosphorylation learning is impaired in the *RSK2* mouse mutants and the lack of RSK2 is, thus, not completely compensated. To explain the revertion of the behavioral phenotype of *ignorant* null mutants, one would have to suppose a mechanism which also compensates the behavioral defect.

As there is as yet no proof for a mechanism which compensates for the lack of p90 ribosomal S6 kinase in *Drosophila*, it also has to be considered that the *ignorant (S6KII)* gene might have an indirect role in operant learning and memory processes. Several explanations might account for the behavioral results of ign^{P1} and investigated deletion lines. The ign^{P1} mutant and the deletion lines $Df(1)ign^{\Delta 24/3}$ and $Df(1)ign^{\Delta 30/2}$ might generate truncated proteins which interfere with a gene / protein necessary for heat-box learning. One possible mechanism for the synthesis of peptides with a dominant negative effect is based on the mutation affecting promotors. To account for the sex specific behavioral phenotype in ign^{P1}, different promotors have to be proposed for males and females. While the promotor of females might be intact, the defective male promotor might result in a transcript that contains a non-optimal translation start site inhibiting the first ATG translation. Translation could then start at a cryptic ATG, producing the truncated peptide. The dominant effect of this protein interacting with genes / proteins involved in learning and memory processes, might be responsible for the learning defects. In deletion lines $Df(1)ign^{\Delta 24/3}$ and $Df(1)ign^{\Delta 30/2}$, lacking 1 to 2 kb of the *S6KII* coding region, the promotor might be removed in both sexes, with an alternative promoter overtaking its function and resulting in a transcript which also leads to a truncated peptide. The fact that the genomic region encoding the N-terminal kinase domain (which is responsible for substrate phosphorylation) is at least partly deleted in those lines and that transcript size is reduced in deletion lines

$Df(1)ign^{\Delta24/3}$ and $Df(1)ign^{\Delta30/2}$ would support this idea (Table 14). Despite the fact that the proposed mechanism can describe all observed phenotypes of the investigated *ignorant* mutations, there might be different mechanisms responsible for the behavioral defect in ign^{P1} and deletion lines.

Instead of differential promotor use, defective dosage compensation in males might explain the sex specific behavioral phenotype of ign^{P1} mutants. In *Drosophila,* gene dosage is regulated by hyper-transcription of the X-chromosome in males. In case the *ignorant* gene underlies the gene dosage compensation mechanism and that mechanism is defective in ign^{P1} males due to the P-element insertion, males will only generate 50 % of the amount of protein of wild-type males and, thus, might show a mutant phenotype. Experiments which confirmed transcript production for the ign^{P1} mutants (Fig. 48) were performed with the cDNAs of mixed populations of males and females. It remains to be investigated whether the amount of male transcript or protein is reduced or whether they produce no peptide at all. Deviating results in ign^{P1} males and females might also be due to the loss of a male specific splice product which might account for the more severe behavioral defect. 5´RACE and cDNA sequencing revealed different 5´ ends which support the idea that splice variants are generated. Both hypotheses, however, can only explain the behavioral phenotype of ign^{P1} mutants and require the consideration of other mechanisms for the deletion lines.

Besides a direct effect of *ignorant* on learning processes or indirect effects including the interference of truncated proteins on other proteins, mutations in the *ignorant* gene might influence the regulation of genes required for successful operant learning. The *S6KII* coding region might include a regulatory element, e.g. silencer or enhancer, of a neighbouring gene, which is destroyed in the mutant by the insertion of p[lacW] and mutated / removed in the deletion lines. In this case, however, the null mutants would be expected to have a defect in operant learning, except a compensatory mechanism for *ignorant* is supposed. The behavioral defects of *ignorant* mutants could also be explained by the effect of an undetected P-element fragment which is located in the *ignorant* gene or in close proximity to it (otherwise outcrossing procedures would have removed it) affecting a neighbouring gene. In both hypothesis, however, the sex specific phenotype of males is hard to explain. The different hypothesis remain to be tested.

The potential role of the *Drosophila S6KII* gene in phosphorylation of CREB and LTM has to be tested in a learning paradigm other than the heat-box, as a robust training procedure for long-term memory is not yet establised for that paradigm. The olfactory discrimination task has been chosen to address this question (Tully and Quinn, 1985). First results of ign^{P1}, deletion line $Df(1)ign^{\Delta58/1}$, and precise jumpout line $ign^{\Delta1P1}$ in the olfactory discrimination task show that p90 ribosomal S6 kinase is indeed involved in classical conditioning (Bertolucci, 2002; in prep). The phenotypes deviate from those observed in the heat-box. While performance of the P-insertion line was not significantly reduced in 3 min, 30 min, or 3 hr memory, the complete loss of the *S6KII*-coding region led to an impaired memory for all mentioned retention intervals. No significant difference was found between males and females. With the precise jumpouts performing like wild-type flies, Bertolucci showed that the behavioral phenotype was caused by the loss of *S6KII*. Preliminary results indicate that LTM is not defective in the null mutants and, consequently, no role of S6KII in CREB phosphorylation is expected. The behavioral phenotype of the *ignorant* null mutant in odor avoidance learning can be explained either by a defect in STM or learning. It has to be tested whether aquisition is normal. Deviating results from operant and classical conditioning support the idea that two independent cellular mechanisms are underlying those conditioning processes as proposed by Brembs and Heisenberg for visual pattern discrimination at the flight simulator (Brembs, 1996; Brembs and Heisenberg, 2000).

Results from classical and operant conditioning indicate a role of the *ignorant* gene at an early stage of memory formation. *Ignorant*, however, might have different functions in the two learning processes. It remains to be investigated whether *ignorant* is involved in the MAPK cascade and, if any, what role it plays in the cascade. Studies on the olfactory learning mutant *leonardo* (encodes 14-3-3; Skoulakis and Davis, 1996) already

showed a role for MAPK signaling in memory formation in *Drosophila.* Excision line *leo*[2.3], which is lacking portions of the genomic sequence of *leonardo*, exhibits a 30 % reduction in the 3 min memory in odor avoidance conditioning. Remarkably, in *leo*[2.3] mutants as well as in the *ignorant* null mutants, the behavioral defect remains over time through 3 hrs. *leo*[2.3] mutants were still defective after 4 hrs, while 4 hr memory was not tested in *ignorant* mutants. Assuming a role for both genes in MAPK signaling, one might expect mutations in these two genes to result in similar phenotypes. Originally, in vertebrates, short-term effects were excluded for the MAP kinase signaling cascade (Martin et al., 1997; Blum et al., 1999; Berman et al., 1998). Recently, however, studies in rats revealed impaired short-term memory in inhibitory avoidance learning and defective memory aquisition in spatial learning when the cascade was blocked by MEK inhibitor PD98059 or SL327 (Selcher et al., 1999; Vianna et al., 2000; Walz et al., 1999). The

idea that the *Drosophila S6KII* gene has a role in learning processes of a spatial orientation task is consistent with the finding that spatial learning is significantly attenuated in mice lacking a functional *RSK2* gene (Dufresne et al., 2001). Thus, besides CREB that is involved in long-term memory, there might be other substrates, e.g. kinases, phosphorylated by p90 ribosomal S6 kinase during memory acquisition. However, it is also possible that the kinase acts independently of the MAPK cascade in the fly. Studying the behavior of *Drosophila* mutants with a disturbed MAPK cascade in the heat-box, e.g. *rol*[1], will provide evidence for MAPK signaling in operant conditioning. *In vitro* assays can then be used to investigate the role of p90 ribosomal S6 kinase in that signaling cascade. The learning phenotype of *ign*[P1] mutants is most likely not attributed to developmental defects, as brain structure as well as body size did not show obvious deviations. Nevertheless, further work will attempt to rescue the phenotype using the *ignorant (S6KII)* transgene.

5 ZUSAMMENFASSUNG

Es wurden die Lern- und Gedächtnisprozesse bei der operanten Konditionierung in der Hitzekammer untersucht. Alter, Geschlecht und Larvendichte waren keine kritischen Parameter, die das Gedächtnis beeinflussten, während sowohl niedrige als auch hohe Laufaktivität der Fliegen mit deren Performance negativ korreliert war. Auf der Suche nach Konditionierungsparametern, die zu hohen Gedächtniswerten führen, lieferte ein Training mit mehreren Training/Test-Intervallen bessere Ergebnisse als ein kontinuierliches Training. Da der Gedächtnistest, bei dem die Hitze abgestellt wird, direkt im Anschluß an die Konditionierungsphase erfolgt, erhalten wir einen Gedächtniswert, der zwei Komponenten beinhaltet: eine räumliche Präferenz für eine Kammerhälfte und einem *"bleib-wo-du-bist* Effekt", der sich aus Seitenpräferenz und langanhaltender Hitzevermeidung *per se* zusammensetzt. Ein Training mit mehreren Training/Test-Intervallen verstärkt letzteren Effekt.

Im nächsten Teil meiner Arbeit wurde der Gedächtnisabfall untersucht. Fliegen wurden in einer Kammer trainiert und nach einem kurzen Erinnerungstraining in einer zweiten Kammer getestet. In diesem direkten Transfer spiegeln die Gedächtniswerte einen assoziativen Lernprozeß wieder, der in der ersten Kammer stattfindet. Um den Gedächtnisabfall nach längeren Zeitintervallen untersuchen zu können, wurden indirekte Transferexperimente durchgeführt. Die Fliege wurde dazu zwischen Trainings- und Testphasen in eine andere Umgebung gebracht. Mit Hilfe dieser Methode konnte ein Nacheffekt noch zwei Stunden nach dem Training beobachtet werden. Überraschenderweise führt im indirekten Transferexperiment ein Aufenthalt in der Kammer auch ohne Konditionierung zu einem Gedächtniseffekt. Dieser "Aufenthaltseffekt" spiegelt eine dispositionelle Veränderung wieder, die das operante Lernen während des Erinnerungstrainings begünstigt. Die verschiedenen Gedächtniseffekte sind pilzkörperunabhängig. Transferexperimente und Yoked-Kontrollen zeigten, dass in der Hitzekammer assoziatives Gedächtnis gemessen wird. Selbst zwei Stunden nach der operanten Konditionierung, erinnert sich die Fliege daran, dass ihre Position in der Kammer die dortige Temperatur kontrolliert.

Die cAMP Signaltransduktionskaskade ist an den Lernprozessen der Fliegen in der Hitzekammer beteiligt. *amnesiac*, *rutabaga* und *dunce* Mutanten haben daher eine verminderte Lern- / Gedächtnisleistung. Um nach bisher unbekannten Genen und Signalkaskaden zu suchen, die in der operanten Konditionierung eine Rolle spielen, wurde ein *Drosophila melanogaster* Mutanten Screen mit 1221 lebensfähigen X-chromosomalen P-element Linien durchgeführt. 29 Linien mit konsistet reduzierten Lern- oder Gedächtniswerten wurden isoliert. Darunter befanden sich drei Linien mit einer p[lacW] Insertion im *amnesiac* ORF. Dieses Ergebnis bestätigt, dass die Hitzekammer mit den gewählten Kriterien ein hilfreiches Werkzeug bei der Suche nach Lern- und / oder Gedächtnismutanten ist. Die Mutante ign^{P1} (8522), die im Gen für p90 ribosomale S6 kinase (*S6KII*) einen Defekt besitzt, wurde untersucht. Die P-Insertion des ign^{P1} Stammes ist die erste Mutation im *ignorant* (*S6KII*) Gen. Das Transposons ist im ersten Exon inseriert. Männliche Mutanten sind durch eine niedrige Trainingsperformance gekennzeichnet, während sich Weibchen im Standardexperiment wildtypisch verhalten. Mehrere Deletionsmutanten im *ignorant* Gen wurden hergestellt. In präzisen Exzisionslinien war der Phänotyp revertiert, während impräzise Exzisionslinien mit teilweisem Verlust der kodierenden Region in der operanten Konditionierung einen Defekt zeigten defekt. Überraschenderweise wurde bei Nullmutanten wildtypisches Verhalten beobachtet. Dies könnte auf einen indirekten Effekt des mutierten *ignorant* Gens auf Lernprozesse hindeuten. Bei der klassischen Duftkonditionierung zeigten *ignorant* Nullmutanten einen Defekt im 3-min, 30-min und 3-Stunden Gedächtnis, während präzise Exzisionen des Transposons zu einer Reversion des Verhaltensphänotyps führten. Voneinander abweichende Ergebnisse bei der operanten und klassischen Konditionierung weisen darauf hin, dass *S6KII* unterschiedliche Rollen in diesen Formen des Lernens spielt.

6 SUMMARY

Learning and memory processes of operant conditioning in the heat-box were analysed. Age, sex, and larval desity were not critical parameters influencing memory, while low or high activity levels of flies were negatively correlated with their performance. In a search for conditioning parameters leading to high retention scores, intermittent training was shown to give better results than continuous training. As the memory test is the immediate continuation of the conditioning phase just omitting reinforcement, we obtain a memory which consists of two components: a spatial preference for one side of the chamber and a *stay-where-you-are* effect in which the side preference is contaminated by the persistence of heat avoidance. Intermittent training strengthens the latter.

In the next part, memory retention was investigated. Flies were trained in one chamber and tested in a second one after a brief reminder training. With this direct transfer, memory scores reflect an associative learning process in the first chamber. To investigate memory retention after extended time periods, indirect transfer experiments were performed. The fly was transferred to a different environment between training and test phases. With this procedure an after-effect of the training was still observed two hours later. Surprisingly, exposure to the chamber without conditioning also lead to a memory effect in the indirect transfer experiment. This exposure effect revealed a dispositional change that facilitates operant learning during the reminder training. The various memory effects are independent of the mushroom bodies. The transfer experiments and yoked controls proved that the heat-box records an associative memory. Even two hours after the operant conditioning procedure, the fly remembers that its position in the chamber controls temperature.

The cAMP signaling cascade is involved in heat-box learning. Thus, *amnesiac*, *rutabaga*, and *dunce* mutants have an impaired learning / memory. Searching for, yet unknown, genes and signaling cascades involved in operant conditioning, a *Drosophila melanogaster* mutant screen with 1221 viable X-chromosome P-element lines was performed. 29 lines with consistently reduced heat avoidance/ learning or memory scores were isolated. Among those, three lines have the p[lacW] located in the *amnesiac* ORF, confirming that with the chosen candidate criteria the heat-box is a useful tool to screen for learning and /or memory mutants. The mutant line ign^{P1} (8522), which is defective in the gene encoding p90 ribosomal S6 kinase (*S6KII)*, was investigated. The P-insertion of line ign^{P1} is the first *Drosophila* mutation in the *ignorant (S6KII)* gene. It has the transposon inserted in the first exon. Mutant males are characterized by low training performance, while females perform well in the standard experiment. Several deletion mutants of the *ignorant* gene have been generated. In precise jumpouts the phenotype was reverted. Imprecise jumpouts with a partial loss of the coding region were defective in operant conditioning. Surprisingly, null mutants showed wild-type behavior. This might indicate an indirect effect of the mutated *ignorant* gene on learning processes. In classical odor avoidance conditioning, *ignorant* null mutants showed a defect in the 3-min, 30-min, and 3-hr memory, while the precise jumpout of the transposon resulted in a reversion of the behavioral phenotype. Deviating results from operant and classical conditioning indicate different roles for *S6KII* in the two types of learning.

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8 APPENDIX

8.1 Figures

Figure 1: Section of genomic fragment. Figure shows nucleotides 22021-28020 of genomic scaffold including the genomic region of *S6KII*.

```
22021 cagtttttat catagtaggc cactttttgta tacatgagtt ttaatctaat cgctggatta
22081 gatttctcat cagcccgtgg actaaaattc tttttctgcc tgtctcaact tcgtttttata
22141 atttgtgtat gaatctgtta aaaaacgaag cagggaaacg acgtatccct tcaatttggt
22201 tgtcgtcatt tcaaaacaag gtaattaagt ataaatcatt gctgtagacg caacatattt
22261 tgcaaaagtt tatcgtgaca actattaggc gaaatgttca tctcaagatc cttcttaaat
22321 attttctata ttatatgtca atgtgtacac attgtttttcc ctccgcagct agctaattat
22381 atatgtactt taggataatc ataccatttt ccccctccaa ctccttttaa taacaccacg
22441 aaaatacagt tttattcaag ggatgttatc ctttttttaaa aaatttctaa gttttactaa
22501 ccttgtttag gtggtgtaaa tatgtatatg aagttattgt tttattttttg gtagcagtaa
22561 gttcagtagt ggcacagcac aataatcctt tagcacacac acacatgcac ttacaagaag
22621 cacacacaca gcctcttatg cacacacaca agctcgcaaa caagaaactt ctgcttttca
22681 agtggcatac atagcttcta aggattcaca tggctcttct acataattta ttcaatttcg
22741 ttccactttg cttgtatttt ctttttttttt ttttgtatat ttccctctag cataattttta
22801 acaagttttc cttggcaaaa atacaacaaa attggaattg tcttcgaaga gtcgaaacaa
22861 gttgttggga actgatgatc gtgcagtttt tagagaaact agcacaaaca aatggaattc
22921 tctgcattaa cccaagtcgt ttatacgacg ctatcctatt tgtattcagt acgagtatga
22981 tggaatatat cgcggttttcg gttctaaatc atccaatggt aagtcggaac tttctatatc
23041 tggttacagc gcaaaaaggt atattgtttg atataatttc tgttggaaga cgttagcaaa
23101 caaaatggaa acccaatttg atgttgtcac aactgtttcc ccggcgttat tacaactaaa
23161 ctgtgcgttt cgcgactgta agacaagtta gcaggattta cgtttcttaa ttacaattaa
23221 ataacaatat gcacccttgt gcagtgttgg aaagaacgtc ggtttttatt gtttagggaa
23281 ttattaataa aatactcttt tctaatatcc tttgccttat aaacccgaaa agcacagttt
23341 ccgctttatg tacacattct gtataatatg gtagttatgt ttatctttttg cttagtttca
23401 tatttcattg tgcaaattga cattccaagt aagaattgtg gaaactcttg tggtttgatt
23461 ttcttatctt tctacacagt tttttttggg ttctttgttc ttcctttata gtttggcgaa
23521 aaaagttatt attatataaa aggattgcgg cttttattct taattttttgt ttgtttttgct
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23641 tgatgttgaa aaatttagct acgattaatt taattaaaaa tatgtaaact tgggcgagag
23701 gctttttttt aatttccagt ttgggataaa taatgtggtt tcaggaatca tctgggatga
23761 tcctggcgcc acattcacac gcgctctcta ggcccttgct ttaggattcg ttatataaat
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23881 cgcacagtgc gttatggcct tggcatattg gcatgctttc atataaaata aataattaac
23941 acttgtcaaa tactttaggt taggccgatt cccccaaaac ctaaaccaca cgcagcttgg
24001 cgctgcggca gcgttggcga ggattgccca gcgtttgtgg atgtgaacca agcttctgcg
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24841 ttgcagaaat aaatgctttc tgaatttaaa tattatcaaa tgatttcaat tgcaatagga
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25561 ttgcagtaga ttatattccg catggaagtt tccgggaaga acaccaggta atgttcgagg
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25741 gcggaagatc tcatgggcag atgcggagat cgggccaccc ggagaatccc tgggggactt
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26161 tcggttcacg atctccggcg ccatgtattc tacggttcca caaaagctat atgtttttga
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27061 atcgtcctcg gtgggcgtgt gctccatggg cgtgaccgcc aggctgctgc agccggacga
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27841 atagtttttat ttgagaatga tttggcccgt gtgaccgtgt ctagcaatat accaaacgcg
27901 aaaaatacca attcattacg aaaacgtcgt cacgaaacaa gttatcttgt gtaaaccgtc
27961 taaaaaccgt tattataaat ttctatatcc cttttatatt gatttccgga aactttgcta
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Figure 2: Sequence of clone SD05277 with 3763 bp length corresponding to clot 1711. Tissue source were *Drosophila melanogaster* Schneider L2 cell culture. cDNA was cloned in vector pOT2a. ORF is indicated in red.

```
AAAAAACTTTATTGTGAATGTGTAAATAATAGTGATAAGAAGCAACACAG
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GAGAAATCCGGAGAGGCAACAGCGCAGTCGACTGCGACGCCCACGCAGCG
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<span style="color:red">CCAGCAGCAGCAACAATGCGGAGCAGCAATGCAGCAGCAGCGGATTGGGT</span>
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<span style="color:red">GAGGCAACAGCGGTGTCACTTCCTCGGTGACCACTGTGACATCATCGCAA</span>
```

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CCTCGAGCAGCATCACATCTCACCAACAGCAGACTTGGATTCCGCTAGGA
AAAGGCCGACGCATCGAACCTTGTGCCCGCCGCCAGAACTTATGGAGCTG
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TTTCATCGTACGTCTTCACTATGCCTTCCAAACTCCCGGAAAACTCTACT
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GCTAGCTATGAATCACCTACACACATTGGGCATTATCTACAGGGATCTGA
AACCGGAAAATATTCTACTGGACGAGCATGGCCATATAGCCTTGACGGAC
TTTGGTCTATCCAAGCAGCCTTTGGATGGCTCAAAAACATATAGCTTTTG
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ATTTTGCCGCTGATTGGTGGAGTTTCGGGGTGCTCATGTACGAAATGTTA
ACGGGGAATTTACCCTTTCATGGCCAAACCCGCCAAGAGACTATGAATCA
GATCCTTAGAAGTAAGCTGGGCATGCCGGAGAATTTGTCGCCAGAGGCGC
AATCCCTGCTACGTGCTCTCTTCAAAAGAAACCCCCAGAATCGTTTGGGT
GCGGGTGCCCAAGGAATTCTGGACATCAAGGCGCACTGCTTTTTTGCCAC
CATCGACTGGGTGAGATTAGAACGAAAGCAGGTGCGTCCGCCTTTCATAC
CGGCGGTTAGCCGTGACGATGCCTTTTACTTTGATGTGGAGTACACCTCA
AAGTCCCCCAGGGATTCTCCGGGTGGCCCGATCTCCGCATCTGCCCATGA
GATCTTCCGCGGGTTCAGCTTTGTGGCTCCTGTCCTTCTGGAAGGTCAGT
GTGCTGGCTCGAATATGTGCTCCACCAGCGCCAGTCCTCTGCATAGTATA
GCTCCTATTCCTGCTGCTCCAGTGGGAGCCCCTCGAACATTACCTGGTGT
TCTTCCCGGAAACTTCCATGCGGAATATAATCTACTGCAAGAACTGGGAC
GTGGAACCTTTTCAGTTTGTCGGTTGTGCGAGCATCGAGCCTCCAAGAAA
CATTACGCAGTAAAAGTAATCGAAAAGGCAGCTGTGGCCGCAGCATCCAC
ATCCACTTCTGCCGATTGTTGGGAGGAGGTGGAGATTATGCTGAGGTACG
GCAACCACCCAAATATCGTCACTCTGTACTCTGTTTACGAGGATGCGGGG
TCCGCATATCTTGTGATGGAGCTGCTTAAGGGTGGCGAGCTTCTCGATCG
GATACTTGCCGTGGGCCAGATGTGCGAGAGTGAGGCCAGCGCGGTGTTAA
GGACAATTGCATCTGCGGTAGCATATCTCCATGAACATGGCGTGGTCCAT
CGAGATCTTAAGCCTTCAAATATGATATATGCCAGTATGCGGCAAACTCC
CGAGACCCTAAAGCTCTGCGATTTGGGTTTCGCGAAGCAGCTGCGCGCGG
ACAACGGCCTCCTGATGACGCCATGCTACACAGCCAATTTTGTGGCTCCC
GAGGTTCTAAAGAGACAGGGCTATGACCTGGCTTGCGACATCTGGTCGCT
CGGTGTGCTGCTTTACATCATGTTATCCGGCCGGACGCCTTTCGCCAGCA
CTCCAAATGATTCACCGGACGTAATACTGAAGCGCATCGGATCAGGACAA
ATTGACTTCACAAGCAGTCGCTGGGCACTGATCAGTGTGCCGGCCAAAGA
ACTTTTGCGTCAGATGCTACACATAGTACCGGAGAATCGGCCGACGGCGG
CGCGAATACTTGAGCACGACTGGCTGCGGGAGCAATTCGCCGGCGGCGTA
CAGCTTACAGAGTATGCGGTGGCGCCCGGATCCCAACTTTCGCTGGGCGC
CCAGCAGCAGCAGCAGAATCACATCTCCATGGCCTTAAGAGGCGCTGTTG
ATGCCACTTTCCGGGCTATTGCCATACCCCAGGCGGCGAATGTGGGACCC
GTAGAACTTTCCATGCTCGCCAAGAGGCGGGCCAAAGATCGAGCCAACCT
GCACTCCTAATCCTGGGCGGCTGCATGGTGTCCGCGGCGCCAGGCCAAGC
GGCAGATAGTCCGACTACTTTTCCGAATCTATAGTATTCTAATCCAATGC
TGCTGTGCCAATCCGCGTCGTCTTGTCAAGGACAATGCGCAGAAGCTTGG

TTCACATCCACAAACGCTGGGCAATCCTCGCCAACGCTGCCGCAGCGCCA
AGCTGCGTGTGGTTTAGGTTTTGGGGGAATCGGCCTAACCTAAAGTATTT
GACAAGTGTTAATTATTTATTTTATATGAAAGCATGCCAATATGCCAAGG
CCATAACGCACTGTGCGGGGCATCTATATACACATATATATAACTATACA
AATACTGATATACATCTATATATAGCTATTTATATAACGAATCCTAAAGC
AAGGGCCTAGAGAGCGCGTGTGAATGTGGCGCCAGGATCATCCCAGATGA
TTCCTGAAACCACATTATTTATCCCAAACTGGAAATTAAAAAAAGCCTC
TCGCCCAAGTTTACATATTTTTAATTAAATTAATCGTAGCTAAATTTTTC
AACATCACCGTGTTATTGGTGCTTAATTAAATTAATTAAAATGAAAAAAA
AAATGGAAAAGTATTAGCAAAACAAACAAAAATTAAGAATAAAAGCCGC
 AATCCTTTTATAT

Figure 3: Sequence of clone GH08264 with 3635 bp length corresponding to clot 1711. Tissue source was *Drosophila melanogaster* head pOT2a. cDNA was cloned in vector pOT2. ORF is indicated in red.

GGATTGCGAAGCGGATAGTAAAGCAGACGGCGTACGAAACGTGGGCGCAT
TAGGAAAGCGCTGCTTGTTGGCCGTAGGAGCAGCAGGGAAACCAGAGAAA
TCCGGAGAGGCAACAGCGCAGTCGACTGCGACGCCCACGCAGCGGAAGTA
ATAAAAAGAAAAGTGAAATCGGAAGTAGGGAGAGTTCGGTGGAGGAGGA
GCAGCAGGAGGAGTTGGAAACGCAGGGGGAACTCGGGCTCTAGCATTGTG
CATCAGGATCTGGAAACAAAGGAACCGCTAGGAGCAGTTGCCTCACAACG
CTAGCAGCCACCGAAGTCATGCCGCTGGCCGATTCGCAAAAGGATCTCCG
CCAGCAGCCTCAGCAGCAGCAACAGCAGCAGCATGTGTCTCCCACCAGCA
GCAGCAACAATGCGGAGCAGCAATGCAGCAGCAGCGGATTGGGTCTGCAG
CTGCGCCAGCGCATGCAAATTACCTCGTCCGGCTGCAGCAGCCTGGCGGT
CACGCCCATGGAGCACACGCCCACCGAGGACGATGAGAGTGGCGGAGGCA
ACAGCGGTGTCACTTCCTCGGTGACCACTGTGACATCATCGCAACGCCGC
CAGCAGCTGCAACAGGTGCAACAGCAATCTGCTCTGCAAGCGGCCCTCGA
GCAGCATCACATCTCACCAACAGCAGACTTGGATTCCGCTAGGAAAAGGC
CGACGCATCGAACCTTGTGCCCGCCGCCAGAACTTATGGAGCTGAGTGAT
TCCGAGTCCCAGGGAGGCGTGGAAACTGGCGGTAGGAGAGAAGGGGCTAC
TGGGCGAAGTGCACCTGATTTAGAAGACACCGAGCCCCTATATGAAACAG
AAAACGAATTCGAGCTTAAGGAAGTCATCAAGGAGGGTCACGACAAGGCC
GATCCTTCCCAGTTCGAGCTCCTACGGGTTCTGGGCGAAGGTAGCTTTGG
AAAGGTGTTTCTAGTGCGAAAGATCATAGGCAAAGATGCAGGAACACTCT
ATGCCATGAAGGTGCTCAAAAAGGCCACCCTTAAAGTAAAAGATCGCGTA
AGGAGCACAAATGAACGAAAATACTAGCGGACGTGGGTCATGCTTTCAT
CGTACGTCTTCACTATGCCTTCCAAACTCCCGGAAAACTCTACTTGATAC
TGGATTTTCTTCGTGGCGGTGATCTGTTTACCCGTCTATCCAAAGAAGTA
ATGTTTACGGAAGAAGATGTCAAGTTCTATTTAGCGGAACTGGCGCTAGC
TATGAATCACCTACACACATTGGGCATTATCTACAGGGATCTGAAACCGG
AAAATATTCTACTGGACGAGCATGGCCATATAGCCTTGACGGACTTTGGT
CTATCCAAGCAGCCTTTGGATGGCTCAAAAACATATAGCTTTTGTGGAAC
CGTAGAATACATGGCGCCGGAGATCGTGAACCGAAAGGGACACGATTTTG
CCGCTGATTGGTGGAGTTTCGGGGTGCTCATGTACGAAATGTTAACGGGG
AATTTACCCTTTCATGGCCAAACCCGCCAAGAGACTATGAATCAGATCCT
TAGAAGTAAGCTGGGCATGCCGGAGAATTTGTCGCCAGAGGCGCAATCCC
TGCTACGTGCTCTCTTCAAAAGAAACCCCCAGAATCGTTTGGGTGCGGGT
GCCCAAGGAATTCTGGACATCAAGGCGCACTGCTTTTTTGCCACCATCGA
CTGGGTGAGATTAGAACGAAAGCAGGTGCGTCCGCCTTTCATACCGGCGG

TTAGCCGTGACGATGCCTTTTACTTTGATGTGGAGTACACCTCAAAGTCC
CCCAGGGATTCTCCGGGTGGCCCGATCTCCGCATCTGCCCATGAGATCTT
CCGCGGGTTCAGCTTTGTGGCTCCTGTCCTTCTGGAAGGTCAGTGTGCTG
GCTCGAATATGTGCTCCACCAGCGCCAGTCCTCTGCATAGTATAGCTCCT
ATTCCTGCTGCTCCAGTGGGAGCCCCTCGAACATTACCTGGTGTTCTTCC
CGGAAACTTCCATGCGGAATATAATCTACTGCAAGAACTGGGACGTGGAA
CCTTTTCAGTTTGTCGGTTGTGCGAGCATCGAGCCTCCAAGAAACATTAC
GCAGTAAAAGTAATCGAAAAGGCAGCTGTGGCCGCAGCATCCACATCCAC
TTCTGCCGATTGTTGGGAGGAGGTGGAGATTATGCTGAGGTACGGCAACC
ACCCAAATATCGTCACTCTGTACTCTGTTTACGAGGATGCGGGGTCCGCA
TATCTTGTGATGGAGCTGCTTAAGGGTGGCGAGCTTCTCGATCGGATACT
TGCCGTGGGCCAGATGTGCGAGAGTGAGGCCAGCGCGGTGTTAAGGACAA
TTGCATCTGCGGTAGCATATCTCCATGAACATGGCGTGGTCCATCGAGAT
CTTAAGCCTTCAAATATGATATATGCCAGTATGCGGCAAACTCCCGAGAC
CCTAAAGCTCTGCGATTTGGGTTTCGCGAAGCAGCTGCGCGCGGACAACG
GCCTCCTGATGACGCCATGCTACACAGCCAATTTTGTGGCTCCCGAGGTT
CTAAAGAGACAGGGCTATGACCTGGCTTGCGACATCTGGTCGCTCGGTGT
GCTGCTTTACATCATGTTATCCGGCCGGACGCCTTTCGCCAGCACTCCAA
ATGATTCACCGGACGTAATACTGAAGCGCATCGGATCAGGACAAATTGAC
TTCACAAGCAGTCGCTGGGCACTGATCAGTGTGCCGGCCAAAGAACTTTT
GCGTCAGATGCTACACATAGTACCGGAGAATCGGCCGACGGCGGCGCGAA
TACTTGAGCACGACTGGCTGCGGGAGCAATTCGCCGGCGGCGTACAGCTT
ACAGAGTATGCGGTGGCGCCCGGATCCCAACTTTCGCTGGGCGCCCAGCA
GCAGCAGCAGAATCACATCTCCATGGCCTTAAGAGGCGCTGTTGATGCCA
CTTTCCGGGCTATTGCCATACCCCAGGCGGCGAATGTGGGACCCGTAGAA
CTTTCCATGCTCGCCAAGAGGCGGGCCAAAGATCGAGCCAACCTGCACTC
CTAATCCTGGGCGGCTGCATGGTGTCCGCGGCGCCAGGCCAAGCGGCAGA
TAGTCCGACTACTTTTCCGAATCTATAGTATTCTAATCCAATGCTGCTGT
GCCAATCCGCGTCGTCTTGTCAAGGACAATGCGCAGAAGCTTGGTTCACA
TCCACAAACGCTGGGCAATCCTCGCCAACGCTGCCGCAGCGCCAAGCTGC
GTGTGGTTTAGGTTTTGGGGGAATCGGCCTAACCTAAAGTATTTGACAAG
TGTTAATTATTTATTTTATATGAAAGCATGCCAATATGCCAAGGCCATAA
CGCACTGTGCGGGGCATCTATATACACATATATATAACTATACAAATACT
GATATACATCTATATATAGCTATTTATATAACGAATCCTAAAGCAAGGGC
CTAGAGAGCGCGTGTGAATGTGGCGCCAGGATCATCCCAGATGATTCCTG
AAACCACATTATTTATCCCAAACTGGAAATTAAAAAAAAGCCTCTCGCCC
AAGTTTACATATTTTTAATTAAATTAATCGTAGCTAATTTTTCAACATCA
 CCGTGTTATTGTTGCTTAATTAAATTAATTAAAAA

Figure 4: Sequence of clone GH21818 with 3633 bp length corresponding to clot 1711. Tissue source was *Drosophila* head pOT2a. cDNA was cloned in vector pOT2a. ORF is indicated in red.

GGATTGCGAAGCGGATAGTAAAGCCAGACGGCGTACGAAACGTGGGCGCA
TTAGGAAAGCGCTGCTTGTTGGCCGTAGGAGCAGCAGGGAAACCAGAGAA
ATCCGGAGAGGCAACAGCGCAGTCGACTGCGACGGCCCACGCAGCGGAAG
TAATAAAAAGAAAAGTGAAATCGGAAGTAGGGAGAGTTCGGTGGAGGAG
GAGCAGCAGGAGGAGTTGGAAACGCAGGGGGAACTCGGGCTCTAGCATTG
TGCATCAGGATCTGGAAACAAAGGAACCGCTAGGAGCAGTTGCCTCACAA
CGCTAGCAGCCACCGAAGTCATGCCGCTGGCCGATTCGCAAAAGGATCTC
CGCCAGCAGCCTCAGCAGCAGCAGCAGCAGCAGCATGTGTCCTCCACCAG
CAGCAGCAACAATGCGGAGCAGCAATGCAGCAGCAGCGGATTGGGTCTGC

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AGCTGCGCCAGCGCATGCAAATTACCTCGTCCGGCTGCAGCAGCCTGGCG
GTCACGCCCATGGAGCACACGCCCACCGAGGACGATGAGAGTGGCGGAGG
CAACAGCGGTGTCACTTCCTCGGTGACCACTGTGACATCATCGCAACGCC
GCCAGCAGCTGCAACAGGTGCAACAGCAATCTGCTCTGCAAGCGGCCCTC
GAGCAGCATCACATCTCACCAACAGCAGACTTGGATTCCGCTAGGAAAAG
GCCGACGCATCGAACCTTGTGCCCGCCGCCAGAACTTATGGAGCTGAGTG
ATTCCGAGTCCCAGGGAGGCGTGGAAACTGGCGGTAGGAGAGAAGGGGCT
ACTGGGCGAAGTGCACCTGATTTAGAAGACACCGAGCCCCTATATGAAAC
AGAAAACGAATTCGAGCTTAAGGAAGTCATCAAGGAGGGTCACGACAAGG
CCGATCCTTCCCAGTTCGAGCTCCTACGGGTTCTGGGCGAAGGTAGCTTT
GGAAAGGTGTTTCTAGTGCGAAAGATCATAGGCAAAGATGCAGGAACACT
CTATGCCATGAAGGTGCTCAAAAAGGCCACCCTAAAAGTAAAAGATCGCG
TAAGGAGCACAAATGAACGAAAAATACTAGCGGACGTGGGTCATGCTTTC
ATCGTACGTCTTCACTATGCCTTCCAAACTCCCGGAAAACTCTACTTGAT
ACTGGATTTTCTTCGTGGCGGTGATCTGTTTACCCGTCTATCCAAAGAAG
TAATGTTTACGGAAGAAGATGTCAAGTTCTATTTAGCGGAACTGGCGCTA
GCTATGAATCACCTACACACATTGGGCATTATCTACAGGGATCTGAAACC
GGAAAATATTCTACTGGACGAGCATGGCCATATAGCCTTGACGGACTTTG
GTCTATCCAAGCAGCCTTTGGATGGCTCAAAAACATATAGCTTTTGTGGA
ACCGTAGAATACATGGCGCCGGAGATCGTGAACCGAAAGGGACACGATTT
TGCCGCTGATTGGTGGAGTTTCGGGGTGCTCATGTACGAAATGTTAACGG
GGAATTTACCCTTTCATGGCCAAACCCGCCAAGAGACTATGAATCAGATC
CTTAGAAGTAAGCTGGGCATGCCGGAGAATTTGTCGCCAGAGGCGCAATC
CCTGCTACGTGCTCTCTTCAAAAGAAACCCCCAGAATCGTTTGGGTGCGG
GTGCCCAAGGAATTCTGGACATCAAGGCGCACTGCTTTTTTGCCACCATC
GACTGGGTGAGATTAGAACGAAAGCAGGTGCGTCCGCCTTTCATACCGGC
GGTTAGCCGTGACGATGCCTTTTACTTTGATGTGGAGTACACCTCAAAGT
CCCCCAGGGATTCTCCGGGTGGCCCGATCTCCGCATCTGCCCATGAGATC
TTCCGCGGGTTCAGCTTTGTGGCTCCTGTCCTTCTGGAAGGTCAGTGTGC
TGGCTCGAATATGTGCTCCACCAGCGCCAGTCCTCTGCATAGTATAGCTC
CTATTCCTGCTGCTCCAGTGGGAGCCCCTCGAACATTACCTGGTGTTCTT
CCCGGAAACTTCCATGCGGAATATAATCTACTGCAAGAACTGGGACGTGG
AACCTTTTCAGTTTGTCGGTTGTGCGAGCATCGAGCCTCCAAGAAACATT
ACGCAGTAAAAGTAATCGAAAAGGCAGCTGTGGCCGCAGCATCCACATCC
ACTTCTGCCGATTGTTGGGAGGAGGTGGAGATTATGCTGAGGTACGGCAA
CCACCCAAATATCGTCACTCTGTACTCTGTTTACGAGGATGCGGGGTCCG
CATATCTTGTGATGGAGCTGCTTAAGGGTGGCGAGCTTCTCGATCGGATA
CTTGCCGTGGGCCAGATGTGCGAGAGTGAGGCCAGCGCGGTGTTAAGGAC
AATTGCATCTGCGGTAGCATATCTCCATGAACATGGCGTGGTCCATCGAG
ATCTTAAGCCTTCAAATATGATATATGCCAGTATGCGGCAAACTCCCGAG
ACCCTAAAGCTCTGCGATTTGGGTTTCGCGAAGCAGCTGCGCGCGGACAA
CGGCCTCCTGATGACGCCATGCTACACAGCCAATTTTGTGGCTCCCGAGG
TTCTAAAGAGACAGGGCTATGACCTGGCTTGCGACATCTGGTCGCTCGGT
GTGCTGCTTTACATCATGTTATCCGGCCGGACGCCTTTCGCCAGCACTCC
AAATGATTCACCGGACGTAATACTGAAGCGCATCGGATCAGGACAAATTG
ACTTCACAAGCAGTCGCTGGGCACTGATCAGTGTGCCGGCCAAAGAACTT
TTGCGTCAGATGCTACACATAGTACCGGAGAATCGGCCGACGGCGGCGCG
AATACTTGAGCACGACTGGCTGCGGGAGCAATTCGCCGGCGGCGTACAGC
TTACAGAGTATGCGGTGGCGCCCGGATCCCAACTTTCGCTGGGCGCCCAG
CAGCAGCAGCAGAATCACATCTCCATGGCCTTAAGAGGCGCTGTTGATGC
CACTTTCCGGGCTATTGCCATACCCCAGGCGGCGAATGTGGGACCCGTAG
AACTTTCCATGCTCGCCAAGAGGCGGGCCAAAGATCGAGCCAACCTGCAC
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TCCTAATCCTGGGCGGCTGCATGGTGTCCGCGGCGCCCAGGCCAAGCGGCA
GATAGTCCGACTACTTTTCCGAATCTATAGTATTCTAATCCAATGCTGCT
GTGCCAATCCGCGTCGTCTTGTCAAGGACAATGCGCAGAAGCTTGGTTCA
CATCCACAAACGCTGGGCAATCCTCGCCAACGCTGCCGCAGCGCCAAGCT
GCGTGTGGTTTAGGTTTTGGGGGAATCGGCCTAACCTAAAGTATTTGACA
GGTGTTAATTATTTATTTTATATGAAAGCATGCCAATATGCCAAGGCCAT
AACGCACTGTGCGGGGCATCTATATACACATATATATAACTATACAAATA
CTGATATACATCTATATATAGCTATTTATATAACGAATCCTAAAGCAAGG
GCCTAGAGAGCGCGTGTGAATGTGGCGCCAGGATCATCCCAGATGATTCC
TGAAACCACATTATTTATCCCAAACTGGAAATTAAAAAAAAGCCTCTCGC
CCAAGTTTACATATTTTTAATTAAATTAATCGTAGCTAAATTTTTCAACA
 TCACCGTGTTATTGTTGCTTAATTAAATTAATT
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Figure 5: ORF corresponding to sequence of clone SD05277 with 911 aa length and reading frame +3. The N-terminal kinase domain is indicated in blue, the C-terminal kinase domain in green.

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375  atgccgctggccgattcgcaaaaggatctccgccagcagcctcag
     M   P   L   A   D   S   Q   K   D   L   R   Q   Q   P   Q
420  cagcagcagcagcagcagcatgtgtcctccaccagcagcagcaac
     Q   Q   Q   Q   Q   Q   H   V   S   S   T   S   S   S   N
465  aatgcggagcagcaatgcagcagcagcggattgggtctgcagctg
     N   A   E   Q   Q   C   S   S   S   G   L   G   L   Q   L
510  cgccagcgcatgcaaattacctcgtccggctgcagcagcctggcg
     R   Q   R   M   Q   I   T   S   S   G   C   S   S   L   A
555  gtcacgcccatggagcacacgcccaccgaggacgatgagagtggc
     V   T   P   M   E   H   T   P   T   E   D   D   E   S   G
600  ggaggcaacagcggtgtcacttcctcggtgaccactgtgacatca
     G   G   N   S   G   V   T   S   S   V   T   T   V   T   S
645  tcgcaacgccgccagcagctgcaacaggtgcaacagcaatctgct
     S   Q   R   R   Q   Q   L   Q   Q   V   Q   Q   Q   S   A
690  ctgcaagcggccctcgagcagcatcacatctcaccaacagcagac
     L   Q   A   A   L   E   Q   H   H   I   S   P   T   A   D
735  ttggattccgctaggaaaaggccgacgcatcgaaccttgtgcccg
     L   D   S   A   R   K   R   P   T   H   R   T   L   C   P
780  ccgccagaacttatggagctgagtgattccgagtcccagggaggc
     P   P   E   L   M   E   L   S   D   S   E   S   Q   G   G
825  gtggaaactggcggtaggagagaaggggctactgggcgaagtgca
     V   E   T   G   G   R   R   E   G   A   T   G   R   S   A
870  cctgatttagaagacaccgagcccctatatgaaacagaaacgaa
     P   D   L   E   D   T   E   P   L   Y   E   T   E   N   E
915  ttcgagcttaaggaagtcatcaaggagggtcacgacaaggccgat
     F   E   L   K   E   V   I   K   E   G   H   D   K   A   D
960  ccttcccagttcgagctcctacgggttctgggcgaaggtagcttt
     P   S   Q   F   E   L   L   R   V   L   G   E   G   S   F
1005 ggaaaggtgtttctagtgcgaaagatcataggcaaagatgcagga
     G   K   V   F   L   V   R   K   I   I   G   K   D   A   G
1050 acactctatgccatgaaggtgctcaaaaaggccaccctaaaagta
     T   L   Y   A   M   K   V   L   K   K   A   T   L   K   V
1095 aaagatcgcgtaaggagcacaaatgaacgaaaaatactagcggac
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             K   D   R   V   R   S   T   N   E   R   K   I   L   A   D
     1140  gtgggtcatgctttcatcgtacgtcttcactatgccttccaaact
             V   G   H   A   F   I   V   R   L   H   Y   A   F   Q   T
     1185  cccggaaaactctacttgatactggattttcttcgtggcggtgat
             P   G   K   L   Y   L   I   L   D   F   L   R   G   G   D
     1230  ctgtttacccgtctatccaaagaagtaatgtttacggaagaagat
             L   F   T   R   L   S   K   E   V   M   F   T   E   E   D
     1275  gtcaagttctatttagcggaactggcgctagctatgaatcaccta
             V   K   F   Y   L   A   E   L   A   L   A   M   N   H   L
     1320  cacacattgggcattatctacagggatctgaaaccggaaaatatt
             H   T   L   G   I   I   Y   R   D   L   K   P   E   N   I
     1365  ctactggacgagcatggccatatagccttgacggactttggtcta
             L   L   D   E   H   G   H   I   A   L   T   D   F   G   L
     1410  tccaagcagcctttggatggctcaaaaacatatagcttttgtgga
             S   K   Q   P   L   D   G   S   K   T   Y   S   F   C   G
     1455  accgtagaatacatggcgccggagatcgtgaaccgaaagggacac
             T   V   E   Y   M   A   P   E   I   V   N   R   K   G   H
     1500  gattttgccgctgattggtggagtttcggggtgctcatgtacgaa
             D   F   A   A   D   W   W   S   F   G   V   L   M   Y   E
     1545  atgttaacggggaatttaccctttcatggccaaacccgccaagag
             M   L   T   G   N   L   P   F   H   G   Q   T   R   Q   E
     1590  actatgaatcagatccttagaagtaagctgggcatgccggagaat
             T   M   N   Q   I   L   R   S   K   L   G   M   P   E   N
     1635  ttgtcgccagaggcgcaatccctgctacgtgctctcttcaaaaga
             L   S   P   E   A   Q   S   L   L   R   A   L   F   K   R
     1680  aacccccagaatcgtttgggtgcgggtgcccaaggaattctggac
             N   P   Q   N   R   L   G   A   G   A   Q   G   I   L   D
     1725  atcaaggcgcactgctttttttgccaccatcgactgggtgagatta
             I   K   A   H   C   F   F   A   T   I   D   W   V   R   L
     1770  gaacgaaagcaggtgcgtccgcctttcataccggcggttagccgt
             E   R   K   Q   V   R   P   P   F   I   P   A   V   S   R
     1815  gacgatgccttttactttgatgtggagtacacctcaaagtccccc
             D   D   A   F   Y   F   D   V   E   Y   T   S   K   S   P
     1860  agggattctccgggtggcccgatctccgcatctgcccatgagatc
             R   D   S   P   G   G   P   I   S   A   S   A   H   E   I
     1905  ttccgcgggttcagctttgtggctcctgtccttctggaaggtcag
             F   R   G   F   S   F   V   A   P   V   L   L   E   G   Q
     1950  tgtgctggctcgaatatgtgctccaccagcgccagtcctctgcat
             C   A   G   S   N   M   C   S   T   S   A   S   P   L   H
     1995  agtatagctcctattcctgctgctccagtgggagcccctcgaaca
             S   I   A   P   I   P   A   A   P   V   G   A   P   R   T
     2040  ttacctggtgttctt cccggaaacttccatgcggaatataatcta
             L   P   G   V   L   P   G   N   F   H   A   E   Y   N   L
     2085  ctgcaagaactgggacgtggaacctttttcagtttgtcggttgtgc
             L   Q   E   L   G   R   G   T   F   S   V   C   R   L   C
     2130  gagcatcgagcctccaagaaacattacgcagtaaaagtaatcgaa
             E   H   R   A   S   K   K   H   Y   A   V   K   V   I   E
     2175  aaggcagctgtggccgcagcatccacatccacttctgccgattgt
             K   A   A   V   A   A   A   S   T   S   T   S   A   D   C
     2220  tgggaggaggtggagattatgctgaggtacggcaaccacccaaat
             W   E   E   V   E   I   M   L   R   Y   G   N   H   P   N
     2265  atcgtcactctgtactctgtttacgaggatgcggggtccgcatat
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            I   V   T   L   Y   S   V   Y   E   D   A   G   S   A   Y
2310  cttgtgatggagctgcttaagggtggcgagcttctcgatcggata
            L   V   M   E   L   L   K   G   G   E   L   L   D   R   I
2355  cttgccgtgggccagatgtgcgagagtgaggccagcgcggtgtta
            L   A   V   G   Q   M   C   E   S   E   A   S   A   V   L
2400  aggacaattgcatctgcggtagcatatctccatgaacatggcgtg
            R   T   I   A   S   A   V   A   Y   L   H   E   H   G   V
2445  gtccatcgagatcttaagccttcaaatatgatatatgccagtatg
            V   H   R   D   L   K   P   S   N   M   I   Y   A   S   M
2490  cggcaaactcccgagaccctaaagctctgcgatttgggtttcgcg
            R   Q   T   P   E   T   L   K   L   C   D   L   G   F   A
2535  aagcagctgcgcgcggacaacggcctcctgatgacgccatgctac
            K   Q   L   R   A   D   N   G   L   L   M   T   P   C   Y
2580  acagccaattttgtggctcccgaggttctaaagagacagggctat
            T   A   N   F   V   A   P   E   V   L   K   R   Q   G   Y
2625  gacctggcttgcgacatctggtcgctcggtgtgctgctttacatc
            D   L   A   C   D   I   W   S   L   G   V   L   L   Y   I
2670  atgttatccggccggacgcctttcgccagcactccaaatgattca
            M   L   S   G   R   T   P   F   A   S   T   P   N   D   S
2715  ccggacgtaatactgaagcgcatcggatcaggacaaattgacttc
            P   D   V   I   L   K   R   I   G   S   G   Q   I   D   F
2760  acaagcagtcgctgggcactgatcagtgtgccggccaaagaactt
            T   S   S   R   W   A   L   I   S   V   P   A   K   E   L
2805  ttgcgtcagatgctacacatagtaccggagaatcggccgacggcg
            L   R   Q   M   L   H   I   V   P   E   N   R   P   T   A
2850  gcgcgaatacttgagcacgactggctgcgggagcaattcgccggc
            A   R   I   L   E   H   D   W   L   R   E   Q   F   A   G
2895  ggcgtacagcttacagagtatgcggtggcgcccggatcccaactt
            G   V   Q   L   T   E   Y   A   V   A   P   G   S   Q   L
2940  tcgctgggcgcccagcagcagcagcagaatcacatctccatggcc
            S   L   G   A   Q   Q   Q   Q   Q   N   H   I   S   M   A
2985  ttaagaggcgctgttgatgccactttccgggctattgccataccc
            L   R   G   A   V   D   A   T   F   R   A   I   A   I   P
3030  caggcggcgaatgtgggacccgtagaactttccatgctcgccaag
            Q   A   A   N   V   G   P   V   E   L   S   M   L   A   K
3075  aggcgggccaaagatcgagccaacctgcactcctaa 3110
            R   R   A   K   D   R   A   N   L   H   S   *
```

Figure 6: ORF corresponding to sequence of clone GH08264 with 911 aa length and reading frame +1.

```
319  atgccgctggccgattcgcaaaaggatctccgccagcagcctcag
           M   P   L   A   D   S   Q   K   D   L   R   Q   Q   P   Q
364  cagcagcaacagcagcagcatgtgtctcccaccagcagcagcaac
           Q   Q   Q   Q   Q   Q   H   V   S   P   T   S   S   S   N
409  aatgcggagcagcaatgcagcagcagcggattgggtctgcagctg
           N   A   E   Q   Q   C   S   S   S   G   L   G   L   Q   L
454  cgccagcgcatgcaaattacctcgtccggctgcagcagcctggcg
           R   Q   R   M   Q   I   T   S   S   G   C   S   S   L   A
499  gtcacgcccatggagcacacgcccaccgaggacgatgagagtggc
           V   T   P   M   E   H   T   P   T   E   D   D   E   S   G
```

```
 544  ggaggcaacagcggtgtcacttcctcggtgaccactgtgacatca
       G  G  N  S  G  V  T  S  S  V  T  T  V  T  S
 589  tcgcaacgccgccagcagctgcaacaggtgcaacagcaatctgct
       S  Q  R  R  Q  Q  L  Q  Q  V  Q  Q  Q  S  A
 634  ctgcaagcggccctcgagcagcatcacatctcaccaacagcagac
       L  Q  A  A  L  E  Q  H  H  I  S  P  T  A  D
 679  ttggattccgctaggaaaaggccgacgcatcgaaccttgtgcccg
       L  D  S  A  R  K  R  P  T  H  R  T  L  C  P
 724  ccgccagaacttatggagctgagtgattccgagtcccagggaggc
       P  P  E  L  M  E  L  S  D  S  E  S  Q  G  G
 769  gtggaaactggcggtaggagagaaggggctactgggcgaagtgca
       V  E  T  G  G  R  R  E  G  A  T  G  R  S  A
 814  cctgatttagaagacaccgagcccctatatgaaacagaaaacgaa
       P  D  L  E  D  T  E  P  L  Y  E  T  E  N  E
 859  ttcgagcttaaggaagtcatcaaggagggtcacgacaaggccgat
       F  E  L  K  E  V  I  K  E  G  H  D  K  A  D
 904  ccttcccagttcgagctcctacgggttctgggcgaaggtagcttt
       P  S  Q  F  E  L  L  R  V  L  G  E  G  S  F
 949  ggaaaggtgtttctagtgcgaaagatcataggcaaagatgcagga
       G  K  V  F  L  V  R  K  I  I  G  K  D  A  G
 994  acactctatgccatgaaggtgctcaaaaaggccacccttaaagta
       T  L  Y  A  M  K  V  L  K  K  A  T  L  K  V
1039  aaagatcgcgtaaggagcacaaatgaacgaaaaatactagcggac
       K  D  R  V  R  S  T  N  E  R  K  I  L  A  D
1084  gtgggtcatgctttcatcgtacgtcttcactatgccttccaaact
       V  G  H  A  F  I  V  R  L  H  Y  A  F  Q  T
1129  cccggaaaactctacttgatactggattttcttcgtggcggtgat
       P  G  K  L  Y  L  I  L  D  F  L  R  G  G  D
1174  ctgtttacccgtctatccaaagaagtaatgtttacggaagaagat
       L  F  T  R  L  S  K  E  V  M  F  T  E  E  D
1219  gtcaagttctatttagcggaactggcgctagctatgaatcaccta
       V  K  F  Y  L  A  E  L  A  L  A  M  N  H  L
1264  cacacattgggcattatctacagggatctgaaaccggaaaatatt
       H  T  L  G  I  I  Y  R  D  L  K  P  E  N  I
1309  ctactggacgagcatggccatatagccttgacggactttggtcta
       L  L  D  E  H  G  H  I  A  L  T  D  F  G  L
1354  tccaagcagcctttggatggctcaaaaacatatagcttttgtgga
       S  K  Q  P  L  D  G  S  K  T  Y  S  F  C  G
1399  accgtagaatacatggcgccggagatcgtgaaccgaaagggacac
       T  V  E  Y  M  A  P  E  I  V  N  R  K  G  H
1444  gattttgccgctgattggtggagtttcggggtgctcatgtacgaa
       D  F  A  A  D  W  W  S  F  G  V  L  M  Y  E
1489  atgttaacggggaatttaccctttcatggccaaacccgccaagag
       M  L  T  G  N  L  P  F  H  G  Q  T  R  Q  E
1534  actatgaatcagatccttagaagtaagctgggcatgccggagaat
       T  M  N  Q  I  L  R  S  K  L  G  M  P  E  N
1579  ttgtcgccagaggcgcaatccctgctacgtgctctcttcaaaaga
       L  S  P  E  A  Q  S  L  L  R  A  L  F  K  R
1624  aaccccagaatcgtttgggtgcgggtgcccaaggaattctggac
       N  P  Q  N  R  L  G  A  G  A  Q  G  I  L  D
1669  atcaaggcgcactgcttttttgccaccatcgactgggtgagatta
       I  K  A  H  C  F  F  A  T  I  D  W  V  R  L
```

```
1714  gaacgaaagcaggtgcgtccgcctttcataccggcggttagccgt
      E   R   K   Q   V   R   P   P   F   I   P   A   V   S   R
1759  gacgatgccttttactttgatgtggagtacacctcaaagtccccc
      D   D   A   F   Y   F   D   V   E   Y   T   S   K   S   P
1804  agggattctccgggtggcccgatctccgcatctgcccatgagatc
      R   D   S   P   G   G   P   I   S   A   S   A   H   E   I
1849  ttccgcgggttcagctttgtggctcctgtccttctggaaggtcag
      F   R   G   F   S   F   V   A   P   V   L   L   E   G   Q
1894  tgtgctggctcgaatatgtgctccaccagcgccagtcctctgcat
      C   A   G   S   N   M   C   S   T   S   A   S   P   L   H
1939  agtatagctcctattcctgctgctccagtgggagcccctcgaaca
      S   I   A   P   I   P   A   A   P   V   G   A   P   R   T
1984  ttacctggtgttcttcccggaaacttccatgcggaatataatcta
      L   P   G   V   L   P   G   N   F   H   A   E   Y   N   L
2029  ctgcaagaactgggacgtggaaccttttcagtttgtcggttgtgc
      L   Q   E   L   G   R   G   T   F   S   V   C   R   L   C
2074  gagcatcgagcctccaagaaacattacgcagtaaaagtaatcgaa
      E   H   R   A   S   K   K   H   Y   A   V   K   V   I   E
2119  aaggcagctgtggccgcagcatccacatccacttctgccgattgt
      K   A   A   V   A   A   A   S   T   S   T   S   A   D   C
2164  tgggaggaggtggagattatgctgaggtacggcaaccacccaaat
      W   E   E   V   E   I   M   L   R   Y   G   N   H   P   N
2209  atcgtcactctgtactctgtttacgaggatgcggggtccgcatat
      I   V   T   L   Y   S   V   Y   E   D   A   G   S   A   Y
2254  cttgtgatggagctgcttaagggtggcgagcttctcgatcggata
      L   V   M   E   L   L   K   G   G   E   L   L   D   R   I
2299  cttgccgtgggccagatgtgcgagagtgaggccagcgcggtgtta
      L   A   V   G   Q   M   C   E   S   E   A   S   A   V   L
2344  aggacaattgcatctgcggtagcatatctccatgaacatggcgtg
      R   T   I   A   S   A   V   A   Y   L   H   E   H   G   V
2389  gtccatcgagatcttaagccttcaaatatgatatatgccagtatg
      V   H   R   D   L   K   P   S   N   M   I   Y   A   S   M
2434  cggcaaactcccgagaccctaaagctctgcgatttgggtttcgcg
      R   Q   T   P   E   T   L   K   L   C   D   L   G   F   A
2479  aagcagctgcgcgcggacaacggcctcctgatgacgccatgctac
      K   Q   L   R   A   D   N   G   L   L   M   T   P   C   Y
2524  acagccaattttgtggctcccgaggttctaaagagacagggctat
      T   A   N   F   V   A   P   E   V   L   K   R   Q   G   Y
2569  gacctggcttgcgacatctggtcgctcggtgtgctgctttacatc
      D   L   A   C   D   I   W   S   L   G   V   L   L   Y   I
2614  atgttatccggccggacgcctttcgccagcactccaaatgattca
      M   L   S   G   R   T   P   F   A   S   T   P   N   D   S
2659  ccggacgtaatactgaagcgcatcggatcaggacaaattgacttc
      P   D   V   I   L   K   R   I   G   S   G   Q   I   D   F
2704  acaagcagtcgctgggcactgatcagtgtgccggccaaagaactt
      T   S   S   R   W   A   L   I   S   V   P   A   K   E   L
2749  ttgcgtcagatgctacacatagtaccggagaatcggccgacggcg
      L   R   Q   M   L   H   I   V   P   E   N   R   P   T   A
2794  gcgcgaatacttgagcacgactggctgcgggagcaattcgccggc
      A   R   I   L   E   H   D   W   L   R   E   Q   F   A   G
2839  ggcgtacagcttacagagtatgcggtggcgcccggatcccaactt
      G   V   Q   L   T   E   Y   A   V   A   P   G   S   Q   L
```

```
2884  tcgctgggcgcccagcagcagcagcagaatcacatctccatggcc
       S   L   G   A   Q   Q   Q   Q   Q   N   H   I   S   M   A
2929  ttaagaggcgctgttgatgccactttccgggctattgccataccc
       L   R   G   A   V   D   A   T   F   R   A   I   A   I   P
2974  caggcggcgaatgtgggacccgtagaactttccatgctcgccaag
       Q   A   A   N   V   G   P   V   E   L   S   M   L   A   K
3019  aggcgggccaaagatcgagccaacctgcactcctaa 3054
       R   R   A   K   D   R   A   N   L   H   S   *
```

Figure 7: ORF corresponding to sequence of clone GH21818 with 911 aa length and reading frame +3.

```
 321  atgccgctggccgattcgcaaaaggatctccgccagcagcctcag
       M   P   L   A   D   S   Q   K   D   L   R   Q   Q   P   Q
 366  cagcagcagcagcagcagcatgtgtcctccaccagcagcagcaac
       Q   Q   Q   Q   Q   Q   H   V   S   S   T   S   S   S   N
 411  aatgcggagcagcaatgcagcagcagcggattgggtctgcagctg
       N   A   E   Q   Q   C   S   S   S   G   L   G   L   Q   L
 456  cgccagcgcatgcaaattacctcgtccggctgcagcagcctggcg
       R   Q   R   M   Q   I   T   S   S   G   C   S   S   L   A
 501  gtcacgcccatggagcacacgcccaccgaggacgatgagagtggc
       V   T   P   M   E   H   T   P   T   E   D   D   E   S   G
 546  ggaggcaacagcggtgtcacttcctcggtgaccactgtgacatca
       G   G   N   S   G   V   T   S   S   V   T   T   V   T   S
 591  tcgcaacgccgccagcagctgcaacaggtgcaacagcaatctgct
       S   Q   R   R   Q   Q   L   Q   Q   V   Q   Q   Q   S   A
 636  ctgcaagcggccctcgagcagcatcacatctcaccaacagcagac
       L   Q   A   A   L   E   Q   H   H   I   S   P   T   A   D
 681  ttggattccgctaggaaaaggccgacgcatcgaaccttgtgcccg
       L   D   S   A   R   K   R   P   T   H   R   T   L   C   P
 726  ccgccagaacttatggagctgagtgattccgagtcccagggaggc
       P   P   E   L   M   E   L   S   D   S   E   S   Q   G   G
 771  gtggaaactggcggtaggagagaaggggctactgggcgaagtgca
       V   E   T   G   G   R   R   E   G   A   T   G   R   S   A
 816  cctgatttagaagacaccgagcccctatatgaaacagaaacgaa
       P   D   L   E   D   T   E   P   L   Y   E   T   E   N   E
 861  ttcgagcttaaggaagtcatcaaggagggtcacgacaaggccgat
       F   E   L   K   E   V   I   K   E   G   H   D   K   A   D
 906  ccttcccagttcgagctcctacgggttctgggcgaaggtagcttt
       P   S   Q   F   E   L   L   R   V   L   G   E   G   S   F
 951  ggaaaggtgtttctagtgcgaaagatcataggcaaagatgcagga
       G   K   V   F   L   V   R   K   I   I   G   K   D   A   G
 996  acactctatgccatgaaggtgctcaaaaaggccaccctaaaagta
       T   L   Y   A   M   K   V   L   K   K   A   T   L   K   V
1041  aaagatcgcgtaaggagcacaaatgaacgaaaaatactagcggac
       K   D   R   V   R   S   T   N   E   R   K   I   L   A   D
1086  gtgggtcatgctttcatcgtacgtcttcactatgccttccaaact
       V   G   H   A   F   I   V   R   L   H   Y   A   F   Q   T
1131  cccggaaaactctacttgatactggattttcttcgtggcggtgat
       P   G   K   L   Y   L   I   L   D   F   L   R   G   G   D
1176  ctgtttacccgtctatccaaagaagtaatgtttacggaagaagat
```

```
          L   F   T   R   L   S   K   E   V   M   F   T   E   E   D
1221 gtcaagttctatttagcggaactggcgctagctatgaatcaccta
          V   K   F   Y   L   A   E   L   A   L   A   M   N   H   L
1266 cacacattgggcattatctacagggatctgaaaccggaaaatatt
          H   T   L   G   I   I   Y   R   D   L   K   P   E   N   I
1311 ctactggacgagcatggccatatagccttgacggactttggtcta
          L   L   D   E   H   G   H   I   A   L   T   D   F   G   L
1356 tccaagcagcctttggatggctcaaaaacatatagcttttgtgga
          S   K   Q   P   L   D   G   S   K   T   Y   S   F   C   G
1401 accgtagaatacatggcgccggagatcgtgaaccgaaagggacac
          T   V   E   Y   M   A   P   E   I   V   N   R   K   G   H
1446 gattttgccgctgattggtggagtttcggggtgctcatgtacgaa
          D   F   A   A   D   W   W   S   F   G   V   L   M   Y   E
1491 atgttaacggggaatttaccctttcatggccaaacccgccaagag
          M   L   T   G   N   L   P   F   H   G   Q   T   R   Q   E
1536 actatgaatcagatccttagaagtaagctgggcatgccggagaat
          T   M   N   Q   I   L   R   S   K   L   G   M   P   E   N
1581 ttgtcgccagaggcgcaatccctgctacgtgctctcttcaaaaga
          L   S   P   E   A   Q   S   L   L   R   A   L   F   K   R
1626 aacccccagaatcgtttgggtgcgggtgcccaaggaattctggac
          N   P   Q   N   R   L   G   A   G   A   Q   G   I   L   D
1671 atcaaggcgcactgcttttttgccaccatcgactgggtgagatta
          I   K   A   H   C   F   F   A   T   I   D   W   V   R   L
1716 gaacgaaagcaggtgcgtccgcctttcataccggcggttagccgt
          E   R   K   Q   V   R   P   P   F   I   P   A   V   S   R
1761 gacgatgccttttactttgatgtggagtacacctcaaagtccccc
          D   D   A   F   Y   F   D   V   E   Y   T   S   K   S   P
1806 agggattctccggggtggcccgatctccgcatctgcccatgagatc
          R   D   S   P   G   G   P   I   S   A   S   A   H   E   I
1851 ttccgcgggttcagctttgtggctcctgtccttctggaaggtcag
          F   R   G   F   S   F   V   A   P   V   L   L   E   G   Q
1896 tgtgctggctcgaatatgtgctccaccagcgccagtcctctgcat
          C   A   G   S   N   M   C   S   T   S   A   S   P   L   H
1941 agtatagctcctattcctgctgctccagtgggagcccctcgaaca
          S   I   A   P   I   P   A   A   P   V   G   A   P   R   T
1986 ttacctggtgttcttcccggaaacttccatgcggaatataatcta
          L   P   G   V   L   P   G   N   F   H   A   E   Y   N   L
2031 ctgcaagaactgggacgtggaaccttttcagtttgtcggttgtgc
          L   Q   E   L   G   R   G   T   F   S   V   C   R   L   C
2076 gagcatcgagcctccaagaaacattacgcagtaaaagtaatcgaa
          E   H   R   A   S   K   K   H   Y   A   V   K   V   I   E
2121 aaggcagctgtggccgcagcatccacatccacttctgccgattgt
          K   A   A   V   A   A   A   S   T   S   T   S   A   D   C
2166 tgggaggaggtggagattatgctgaggtacggcaaccacccaaat
          W   E   E   V   E   I   M   L   R   Y   G   N   H   P   N
2211 atcgtcactctgtactctgtttacgaggatgcggggtccgcatat
          I   V   T   L   Y   S   V   Y   E   D   A   G   S   A   Y
2256 cttgtgatggagctgcttaagggtggcgagcttctcgatcggata
          L   V   M   E   L   L   K   G   G   E   L   L   D   R   I
2301 cttgccgtgggccagatgtgcgagagtgaggccagcgcggtgtta
          L   A   V   G   Q   M   C   E   S   E   A   S   A   V   L
2346 aggacaattgcatctgcggtagcatatctccatgaacatggcgtg
```

```
           R   T   I   A   S   A   V   A   Y   L   H   E   H   G   V
     2391  gtccatcgagatcttaagccttcaaatatgatatatgccagtatg
           V   H   R   D   L   K   P   S   N   M   I   Y   A   S   M
     2436  cggcaaactcccgagaccctaaagctctgcgatttgggtttcgcg
           R   Q   T   P   E   T   L   K   L   C   D   L   G   F   A
     2481  aagcagctgcgcgcggacaacggcctcctgatgacgccatgctac
           K   Q   L   R   A   D   N   G   L   L   M   T   P   C   Y
     2526  acagccaattttgtggctcccgaggttctaaagagacagggctat
           T   A   N   F   V   A   P   E   V   L   K   R   Q   G   Y
     2571  gacctggcttgcgacatctggtcgctcggtgtgctgctttacatc
           D   L   A   C   D   I   W   S   L   G   V   L   L   Y   I
     2616  atgttatccggccggacgcctttcgccagcactccaaatgattca
           M   L   S   G   R   T   P   F   A   S   T   P   N   D   S
     2661  ccggacgtaatactgaagcgcatcggatcaggacaaattgacttc
           P   D   V   I   L   K   R   I   G   S   G   Q   I   D   F
     2706  acaagcagtcgctgggcactgatcagtgtgccggccaaagaactt
           T   S   S   R   W   A   L   I   S   V   P   A   K   E   L
     2751  ttgcgtcagatgctacacatagtaccggagaatcggccgacggcg
           L   R   Q   M   L   H   I   V   P   E   N   R   P   T   A
     2796  gcgcgaatacttgagcacgactggctgcgggagcaattcgccggc
           A   R   I   L   E   H   D   W   L   R   E   Q   F   A   G
     2841  ggcgtacagcttacagagtatgcggtggcgcccggatcccaactt
           G   V   Q   L   T   E   Y   A   V   A   P   G   S   Q   L
     2886  tcgctgggcgcccagcagcagcagcagaatcacatctccatggcc
           S   L   G   A   Q   Q   Q   Q   Q   N   H   I   S   M   A
     2931  ttaagaggcgctgttgatgccactttccgggctattgccataccc
           L   R   G   A   V   D   A   T   F   R   A   I   A   I   P
     2976  caggcggcgaatgtgggacccgtagaactttccatgctcgccaag
           Q   A   A   N   V   G   P   V   E   L   S   M   L   A   K
     3021  aggcgggccaaagatcgagccaacctgcactcctaa 3056
           R   R   A   K   D   R   A   N   L   H   S   *
```

Figure 8: Matchbox result from MATCH-BOX Server 1.3. Figure shows optimal multipe alignment of mRNA of *S6KII*, SD05277, GH08264, and GH21818 with indices of reliability. A score from 1 to 9 is written below each position in the boxes. It is related to the statistical significance of the alignment at this position. A score of 5 corresponds to a similarity of equal occurence in related and unrelated sequences. Lower the score is, higher the reliability of the alignment. Red letters indicate regions where amino acids deviate from each other comparing sequences 1 to 4. Green letters show 8 bp duplicated by the insertion of the P-element. ORF starts at base number 375 with Methionin indicated in blue. ORF corresponds to mRNA of *S6KII* published in flybase and was added by hand.

1   mRNA of *S6KII* (3137 bp)
2   SD05277 (3763 bp)
3   GH08264 (3635 bp)
4   GH21818 (3633 bp)

```
          10        20        30        40        50        60        70
           +         +         +         +         +         +         +
    1  ----------------------------------------------------------------
    2  AAAAAACTTTATTGTGAATGTGTAAATAATAGTGATAAGAAGCAACACAGCTGTGCGGATTGCGAAGCGG
    3  -----------------------------------------------------GGATTGCGAAGCGG
    4  -----------------------------------------------------GGATTGCGAAGCGGAT
```

```
              80        90       100       110       120       130       140
               +         +         +         +         +         +         +
1   ------------------------------------------------------------------
2   ATAGTAAAGCAGACGGCGTACGAAACGTGGGCGCATTAGGAAAGCGCTGCTTGTTGGCCGTAGGAGCAGC
3   ATAGTAAAGCAGACGGCGTACGAAACGTGGGCGCATTAGGAAAGCGCTGCTTGTTGGCCGTAGGAGCAGC
4   AGTAAAGCCAGACGGCGTACGAAACGTGGGCGCATTAGGAAAGCGCTGCTTGTTGGCCGTAGGAGCAGCA


              150       160       170       180       190       200       210
               +         +         +         +         +         +         +
1   -------------------------------------------------gaattcccggaagtaataa
2   AGGGAAACCAGAGAAATCCGGAGAGGCAACAGCGCAGTCGACTGCGACGCCcacgcagcggaagtaataa
3   AGGGAAACCAGAGAAATCCGGAGAGGCAACAGCGCAGTCGACTGCGACGCCcacgcagcggaagtaataa
4   GGGAAACCAGAGAAATCCGGAGAGGCAACAGCGCAGTCGACTGCGACGGCCcacgcagcggaagtaataa
                                                         9999999111111111111

              220       230       240       250       260       270       280
               +         +         +         +         +         +         +
1   aaaagaaaagtgaaatcggaagtagggagagttcggtggaggaggagcagc---aggagttggaaacgca
2   aaaagaaaagtgaaatcggaagtagggagagttcggtggaggaggagcagcAGGaggagttggaaacgca
3   aaaagaaaagtgaaatcggaagtagggagagttcggtggaggaggagcagcAGGaggagttggaaacgca
4   aaaagaaaagtgaaatcggaagtagggagagttcggtggaggaggagcagcAGGaggagttggaaacgca
    1111111111111111111111111111111111111111111111111    111111111111111

              290       300       310       320       330       340       350
               +         +         +         +         +         +         +
1   gggggaactcgggctctagcattgtgcatcaggatctggaaacaaaggaaccgctaggagcagttgc---
2   gggggaactcgggctctagcattgtgcatcaggatctggaaacaaaggaaccgctaggagcagttgcCTC
3   gggggaactcgggctctagcattgtgcatcaggatctggaaacaaaggaaccgctaggagcagttgcCTC
4   gggggaactcgggctctagcattgtgcatcaggatctggaaacaaaggaaccgctaggagcagttgcCTC
    111111111111111111111111111111111111111111111111111111111111111111111

              360       370       380       390       400       410       420
               +         +         +         +         +         +         +
1   ------ctcacagccaccgaagtcatgccgctggccgattcgcaaaaggatctccgccagcagcctcagc
2   ACAACGctagcagccaccgaagtcatgccgctggccgattcgcaaaaggatctccgccagcagcctcagc
3   ACAACGctagcagccaccgaagtcatgccgctggccgattcgcaaaaggatctccgccagcagcctcagc
4   ACAACGctagcagccaccgaagtcatgccgctggccgattcgcaaaaggatctccgccagcagcctcagc
                                    M  P  L  A  D  S  Q  K  D  L  R  Q  Q  P  Q  Q
                  111111111111111111111111111111111111111111111111111111111111

              430       440       450       460       470       480       490
               +         +         +         +         +         +         +
1   agcagcag---cagcagcatgtgtcctccaccagcagcagcaacaatgcggagcagcaatgcagcagcag
2   agcagcagCAGcagcagcatgtgtcctccaccagcagcagcaacaatgcggagcagcaatgcagcagcag
3   agcagcaaCAGcagcagcatgtgtctcccaccagcagcagcaacaatgcggagcagcaatgcagcagcag
4   agcagcagCAGcagcagcatgtgtcctccaccagcagcagcaacaatgcggagcagcaatgcagcagcag
     Q  Q      Q  Q  H  V  S  S  T  S  S  S  N  N  A  E  Q  Q  C  S  S  S
    11119999    111111111111119111111111111111111111111111111111111111111

              500       510       520       530       540       550       560
               +         +         +         +         +         +         +
1   cggattgggtctgcagctgcgccagcgcatgcaaattacctcgtccggctgcagcagcctggcggtcacg
2   cggattgggtctgcagctgcgccagcgcatgcaaattacctcgtccggctgcagcagcctggcggtcacg
3   cggattgggtctgcagctgcgccagcgcatgcaaattacctcgtccggctgcagcagcctggcggtcacg
4   cggattgggtctgcagctgcgccagcgcatgcaaattacctcgtccggctgcagcagcctggcggtcacg
     G  L  G  L  Q  L  R  Q  R  M  Q  I  T  S  S  G  C  S  S  L  A  V  T
    111111111111111111111111111111111111111111111111111111111111111111111

              570       580       590       600       610       620       630
               +         +         +         +         +         +         +
1   cccatggagcacacgcccaccgaggacgaagagagtggcggaggcaacagcggtgtcacttcctcggtga
2   cccatggagcacacgcccaccgaggacgatgagagtggcggaggcaacagcggtgtcacttcctcggtga
3   cccatggagcacacgcccaccgaggacgatgagagtggcggaggcaacagcggtgtcacttcctcggtga
4   cccatggagcacacgcccaccgaggacgatgagagtggcggaggcaacagcggtgtcacttcctcggtga
     P  M  E  H  T  P  T  E  D  E  E  S  G  G  N  S  G  V  T  S  S  V  T
    111111111111111111111111111111111111111111111111111111111111111111111

              640       650       660       670       680       690       700
               +         +         +         +         +         +         +
1   ccactgtgacatcatcgcaacgccgccagcagctgcaacaggtgcaacagcaatctgctctgcaagcggc
2   ccactgtgacatcatcgcaacgccgccagcagctgcaacaggtgcaacagcaatctgctctgcaagcggc
3   ccactgtgacatcatcgcaacgccgccagcagctgcaacaggtgcaacagcaatctgctctgcaagcggc
```

```
4    ccactgtgacatcatcgcaacgccgccagcagctgcaacaggtgcaacagcaatctgctctgcaagcggc
       T  V  T  S  S  Q  R  R  Q  Q  L  Q  Q  V  Q  Q  Q  S  A  L  Q  A  A
     1111111111111111111111111111111111111111111111111111111111111111111111


            710       720       730       740       750       760       770
             +         +         +         +         +         +         +
1    cctcgagcagcatcacatctcaccaacagcagacttggattccgctaggaaaaggccgacgcatcgaacc
2    cctcgagcagcatcacatctcaccaacagcagacttggattccgctaggaaaaggccgacgcatcgaacc
3    cctcgagcagcatcacatctcaccaacagcagacttggattccgctaggaaaaggccgacgcatcgaacc
4    cctcgagcagcatcacatctcaccaacagcagacttggattccgctaggaaaaggccgacgcatcgaacc
       L  E  Q  H  H  I  S  P  T  A  D  L  D  S  A  R  K  R  P  T  H  R  T
     1111111111111111111111111111111111111111111111111111111111111111111111


            780       790       800       810       820       830       840
             +         +         +         +         +         +         +
1    ttgtgcccgccgccagaacttatggagctgagtgattccgagtcccaggggaggcgtggaaactggcggta
2    ttgtgcccgccgccagaacttatggagctgagtgattccgagtcccaggggaggcgtggaaactggcggta
3    ttgtgcccgccgccagaacttatggagctgagtgattccgagtcccaggggaggcgtggaaactggcggta
4    ttgtgcccgccgccagaacttatggagctgagtgattccgagtcccaggggaggcgtggaaactggcggta
       L  C  P  P  P  E  L  M  E  L  S  D  S  E  S  Q  G  G  V  E  T  G  G  R
     1111111111111111111111111111111111111111111111111111111111111111111111


            850       860       870       880       890       900       910
             +         +         +         +         +         +         +
1    ggagagaaggagctactgggcgaagtgcacctgatttagaagacacagagcccctatatgaaacagaaaa
2    ggagagaaggggctactgggcgaagtgcacctgatttagaagacacacgagcccctatatgaaacagaaaa
3    ggagagaaggggctactgggcgaagtgcacctgatttagaagacacacgagcccctatatgaaacagaaaa
4    ggagagaaggggctactgggcgaagtgcacctgatttagaagacacacgagcccctatatgaaacagaaaa
       R  E  G  A  T  G  R  S  A  P  D  L  E  D  T  E  P  L  Y  E  T  E  N
     1111111111111111111111111111111111111111111111111111111111111111111111


            920       930       940       950       960       970       980
             +         +         +         +         +         +         +
1    cgaattcgagcttaaggaagtcatcaaggagggtcacgacaaggccgatccttcccagttcgagctccta
2    cgaattcgagcttaaggaagtcatcaaggagggtcacgacaaggccgatccttcccagttcgagctccta
3    cgaattcgagcttaaggaagtcatcaaggagggtcacgacaaggccgatccttcccagttcgagctccta
4    cgaattcgagcttaaggaagtcatcaaggagggtcacgacaaggccgatccttcccagttcgagctccta
       E  F  E  L  K  E  V  I  K  E  G  H  D  K  A  D  P  S  Q  F  E  L  L
     1111111111111111111111111111111111111111111111111111111111111111111111


            990      1000      1010      1020      1030      1040      1050
             +         +         +         +         +         +         +
1    cgggttctgggcgaaggtagctttggaaaggtgtttctagtgcgaaagatcataggcaaagatgcaggaa
2    cgggttctgggcgaaggtagctttggaaaggtgtttctagtgcgaaagatcataggcaaagatgcaggaa
3    cgggttctgggcgaaggtagctttggaaaggtgtttctagtgcgaaagatcataggcaaagatgcaggaa
4    cgggttctgggcgaaggtagctttggaaaggtgtttctagtgcgaaagatcataggcaaagatgcaggaa
       R  V  L  G  E  G  S  F  G  K  V  F  L  V  R  K  I  I  G  K  D  A  G  T
     1111111111111111111111111111111111111111111111111111111111111111111111


           1060      1070      1080      1090      1100      1110      1120
             +         +         +         +         +         +         +
1    cactctatgccatgaaggtgctcaaaaaggccacccttaaagtaaaagatcgcgtaaggagcacaaatga
2    cactctatgccatgaaggtgctcaaaaaggccaccctaaaagtaaaagatcgcgtaaggagcacaaatga
3    cactctatgccatgaaggtgctcaaaaaggccacccttaaagtaaaagatcgcgtaaggagcacaaatga
4    cactctatgccatgaaggtgctcaaaaaggccaccctaaaagtaaaagatcgcgtaaggagcacaaatga
       L  Y  A  M  K  V  L  K  K  A  T  L  K  V  K  D  R  V  R  S  T  N  E
     1111111111111111111111111111111111111111111111111111111111111111111111


           1130      1140      1150      1160      1170      1180      1190
             +         +         +         +         +         +         +
1    acgaaaaatactagcggacgtgggtcatgctttcatcgtacgtcttcactatgccttccaaactcccgga
2    acgaaaaatactagcggacgtgggtcatgctttcatcgtacgtcttcactatgccttccaaactcccgga
3    acgaaaaatactagcggacgtgggtcatgctttcatcgtacgtcttcactatgccttccaaactcccgga
4    acgaaaaatactagcggacgtgggtcatgctttcatcgtacgtcttcactatgccttccaaactcccgga
       R  K  I  L  A  D  V  G  H  A  F  I  V  R  L  H  Y  A  F  Q  T  P  G
     1111111111111111111111111111111111111111111111111111111111111111111111


           1200      1210      1220      1230      1240      1250      1260
             +         +         +         +         +         +         +
1    aaactttacttgatactggattttcttcgtggcggtgatctgtttacccgtctatccaaagaagtaatgt
2    aaactctacttgatactggattttcttcgtggcggtgatctgtttacccgtctatccaaagaagtaatgt
3    aaactctacttgatactggattttcttcgtggcggtgatctgtttacccgtctatccaaagaagtaatgt
4    aaactctacttgatactggattttcttcgtggcggtgatctgtttacccgtctatccaaagaagtaatgt
       K  L  Y  L  I  L  D  F  L  R  G  G  D  L  F  T  R  L  S  K  E  V  M  F
     1111111111111111111111111111111111111111111111111111111111111111111111


           1270      1280      1290      1300      1310      1320      1330
             +         +         +         +         +         +         +
```

```
1   ttacggaagaagatgtcaagttctatttagcggaactggcgctagctatgaatcacctacacacattggg
2   ttacggaagaagatgtcaagttctatttagcggaactggcgctagctatgaatcacctacacacattggg
3   ttacggaagaagatgtcaagttctatttagcggaactggcgctagctatgaatcacctacacacattggg
4   ttacggaagaagatgtcaagttctatttagcggaactggcgctagctatgaatcacctacacacattggg
     T  E  E  D  V  K  F  Y  L  A  E  L  A  L  A  M  N  H  L  H  T  L  G
    1111111111111111111111111111111111111111111111111111111111111111111111
```

```
        1340      1350      1360      1370      1380      1390      1400
         +         +         +         +         +         +         +
1   cattatctacagggatctgaaaccggaaaatattctactggacgagcatggccatatagccttgacggac
2   cattatctacagggatctgaaaccggaaaatattctactggacgagcatggccatatagccttgacggac
3   cattatctacagggatctgaaaccggaaaatattctactggacgagcatggccatatagccttgacggac
4   cattatctacagggatctgaaaccggaaaatattctactggacgagcatggccatatagccttgacggac
     I  I  Y  R  D  L  K  P  E  N  I  L  L  D  E  H  G  H  I  A  L  T  D
    1111111111111111111111111111111111111111111111111111111111111111111111
```

```
        1410      1420      1430      1440      1450      1460      1470
         +         +         +         +         +         +         +
1   tttggtctatccaagcagcctttggatggctcaaaaacatatagcttttgtggaaccgtagaatacatgg
2   tttggtctatccaagcagcctttggatggctcaaaaacatatagcttttgtggaaccgtagaatacatgg
3   tttggtctatccaagcagcctttggatggctcaaaaacatatagcttttgtggaaccgtagaatacatgg
4   tttggtctatccaagcagcctttggatggctcaaaaacatatagcttttgtggaaccgtagaatacatgg
     F  G  L  S  K  Q  P  L  D  G  S  K  T  Y  S  F  C  G  T  V  E  Y  M  A
    1111111111111111111111111111111111111111111111111111111111111111111111
```

```
        1480      1490      1500      1510      1520      1530      1540
         +         +         +         +         +         +         +
1   cgccggagatcgtgaaccgaaagggacacgattttgccgctgattggtggagtttcggggtgctcatgta
2   cgccggagatcgtgaaccgaaagggacacgattttgccgctgattggtggagtttcggggtgctcatgta
3   cgccggagatcgtgaaccgaaagggacacgattttgccgctgattggtggagtttcggggtgctcatgta
4   cgccggagatcgtgaaccgaaagggacacgattttgccgctgattggtggagtttcggggtgctcatgta
     P  E  I  V  N  R  K  G  H  D  F  A  A  D  W  W  S  F  G  V  L  M  Y
    1111111111111111111111111111111111111111111111111111111111111111111111
```

```
        1550      1560      1570      1580      1590      1600      1610
         +         +         +         +         +         +         +
1   cgaaatgttaacggggaatttacccttttcatggccaaacccgccaagagactatgaatcagatccttaga
2   cgaaatgttaacggggaatttacccttttcatggccaaacccgccaagagactatgaatcagatccttaga
3   cgaaatgttaacggggaatttacccttttcatggccaaacccgccaagagactatgaatcagatccttaga
4   cgaaatgttaacggggaatttacccttttcatggccaaacccgccaagagactatgaatcagatccttaga
     E  M  L  T  G  N  L  P  F  H  G  Q  T  R  Q  E  T  M  N  Q  I  L  R
    1111111111111111111111111111111111111111111111111111111111111111111111
```

```
        1620      1630      1640      1650      1660      1670      1680
         +         +         +         +         +         +         +
1   agtaagctgggcatgccggagaatttgtcgccagaggcgcaatccctgctacgtgctctctcttcaaaagaa
2   agtaagctgggcatgccggagaatttgtcgccagaggcgcaatccctgctacgtgctctctcttcaaaagaa
3   agtaagctgggcatgccggagaatttgtcgccagaggcgcaatccctgctacgtgctctctcttcaaaagaa
4   agtaagctgggcatgccggagaatttgtcgccagaggcgcaatccctgctacgtgctctctcttcaaaagaa
     S  K  L  G  M  P  E  N  L  S  P  E  A  Q  S  L  L  R  A  L  F  K  R  N
    1111111111111111111111111111111111111111111111111111111111111111111111
```

```
        1690      1700      1710      1720      1730      1740      1750
         +         +         +         +         +         +         +
1   accccagaatcgtttgggtgcgggtgcccaaggaattctggacatcaaggcgcactgcttttt c gccac
2   accccagaatcgtttgggtgcgggtgcccaaggaattctggacatcaaggcgcactgctttttt t gccac
3   accccagaatcgtttgggtgcgggtgcccaaggaattctggacatcaaggcgcactgctttttt t gccac
4   accccagaatcgtttgggtgcgggtgcccaaggaattctggacatcaaggcgcactgctttttt t gccac
     P  Q  N  R  L  G  A  G  A  Q  G  I  L  D  I  K  A  H  C  F  F  A  T
    1111111111111111111111111111111111111111111111111111111111111111111111
```

```
        1760      1770      1780      1790      1800      1810      1820
         +         +         +         +         +         +         +
1   catcgactgggtgagattagaacgaaagcaggtgcgtccgcctttcataccggcggttagccgtgacgat
2   catcgactgggtgagattagaacgaaagcaggtgcgtccgcctttcataccggcggttagccgtgacgat
3   catcgactgggtgagattagaacgaaagcaggtgcgtccgcctttcataccggcggttagccgtgacgat
4   catcgactgggtgagattagaacgaaagcaggtgcgtccgcctttcataccggcggttagccgtgacgat
     I  D  W  V  R  L  E  R  K  Q  V  R  P  P  F  I  P  A  V  S  R  D  D
    1111111111111111111111111111111111111111111111111111111111111111111111
```

```
        1830      1840      1850      1860      1870      1880      1890
         +         +         +         +         +         +         +
1   gccttttactttgatgtggagtacacctcaaagtcccccagggattctcc c ggtggcccgatctccgcat
2   gccttttactttgatgtggagtacacctcaaagtcccccagggattctcc g ggtggcccgatctccgcat
3   gccttttactttgatgtggagtacacctcaaagtcccccagggattctcc g ggtggcccgatctccgcat
4   gccttttactttgatgtggagtacacctcaaagtcccccagggattctcc g ggtggcccgatctccgcat
     A  F  Y  F  D  V  E  Y  T  S  K  S  P  R  D  S  P  G  G  P  I  S  A  S
    1111111111111111111111111111111111111111111111111111111111111111111111
```

```
              1900       1910       1920       1930       1940       1950       1960
               +          +          +          +          +          +          +
1  ctgcccatgagatcttccgcgcgggttcagctttgtggctcctgtccttctggaaggtcagtgtgctggctc
2  ctgcccatgagatcttccgcgcgggttcagctttgtggctcctgtccttctggaaggtcagtgtgctggctc
3  ctgcccatgagatcttccgcgcgggttcagctttgtggctcctgtccttctggaaggtcagtgtgctggctc
4  ctgcccatgagatcttccgcgcgggttcagctttgtggctcctgtccttctggaaggtcagtgtgctggctc
    A  H  E  I  F  R  G  F  S  F  V  A  P  V  L  L  E  G  Q  C  A  G  S
   1111111111111111111111111111111111111111111111111111111111111111111111111

              1970       1980       1990       2000       2010       2020       2030
               +          +          +          +          +          +          +
1  gaatatgtgctccaccagcgccagtcctctgcatagtatagctcctattcctgctgctccagtgggagcc
2  gaatatgtgctccaccagcgccagtcctctgcatagtatagctcctattcctgctgctccagtgggagcc
3  gaatatgtgctccaccagcgccagtcctctgcatagtatagctcctattcctgctgctccagtgggagcc
4  gaatatgtgctccaccagcgccagtcctctgcatagtatagctcctattcctgctgctccagtgggagcc
    N  M  C  S  T  S  A  S  P  L  H  S  I  A  P  I  P  A  A  P  V  G  A
   1111111111111111111111111111111111111111111111111111111111111111111111111

              2040       2050       2060       2070       2080       2090       2100
               +          +          +          +          +          +          +
1  cctcgaacattacctggtgttcttcccggaaacttccatgcggaatataatctactgcaagaactgggac
2  cctcgaacattacctggtgttcttcccggaaacttccatgcggaatataatctactgcaagaactgggac
3  cctcgaacattacctggtgttcttcccggaaacttccatgcggaatataatctactgcaagaactgggac
4  cctcgaacattacctggtgttcttcccggaaacttccatgcggaatataatctactgcaagaactgggac
    P  R  T  L  P  G  V  L  P  G  N  F  H  A  E  Y  N  L  L  Q  E  L  G  R
   1111111111111111111111111111111111111111111111111111111111111111111111111

              2110       2120       2130       2140       2150       2160       2170
               +          +          +          +          +          +          +
1  gtggaacctttttcagtttgtcggttgtgcgagcatcgagcctccaagaaacattacgcagtaaaagtaat
2  gtggaacctttttcagtttgtcggttgtgcgagcatcgagcctccaagaaacattacgcagtaaaagtaat
3  gtggaacctttttcagtttgtcggttgtgcgagcatcgagcctccaagaaacattacgcagtaaaagtaat
4  gtggaacctttttcagtttgtcggttgtgcgagcatcgagcctccaagaaacattacgcagtaaaagtaat
     G  T  F  S  V  C  R  L  C  E  H  R  A  S  K  K  H  Y  A  V  K  V  I
   1111111111111111111111111111111111111111111111111111111111111111111111111

              2180       2190       2200       2210       2220       2230       2240
               +          +          +          +          +          +          +
1  cgaaaaggcagctgtggccgcagcatccacatccacttctgccgattgttgggaggaggtggagattatg
2  cgaaaaggcagctgtggccgcagcatccacatccacttctgccgattgttgggaggaggtggagattatg
3  cgaaaaggcagctgtggccgcagcatccacatccacttctgccgattgttgggaggaggtggagattatg
4  cgaaaaggcagctgtggccgcagcatccacatccacttctgccgattgttgggaggaggtggagattatg
    E  K  A  A  V  A  A  A  S  T  S  T  S  A  D  C  W  E  E  V  E  I  M
   1111111111111111111111111111111111111111111111111111111111111111111111111

              2250       2260       2270       2280       2290       2300       2310
               +          +          +          +          +          +          +
1  ctgaggtacggcaaccacccaaatatcgtcactctgtactctgtttacgaggatgcgggggtccgcatatc
2  ctgaggtacggcaaccacccaaatatcgtcactctgtactctgtttacgaggatgcgggggtccgcatatc
3  ctgaggtacggcaaccacccaaatatcgtcactctgtactctgtttacgaggatgcgggggtccgcatatc
4  ctgaggtacggcaaccacccaaatatcgtcactctgtactctgtttacgaggatgcgggggtccgcatatc
    L  R  Y  G  N  H  P  N  I  V  T  L  Y  S  V  Y  E  D  A  G  S  A  Y  L
   1111111111111111111111111111111111111111111111111111111111111111111111111

              2320       2330       2340       2350       2360       2370       2380
               +          +          +          +          +          +          +
1  ttgtgatggagctgcttaagggtggcgagcttctcgatcggatacttgccgtgggccagatgtgcgagag
2  ttgtgatggagctgcttaagggtggcgagcttctcgatcggatacttgccgtgggccagatgtgcgagag
3  ttgtgatggagctgcttaagggtggcgagcttctcgatcggatacttgccgtgggccagatgtgcgagag
4  ttgtgatggagctgcttaagggtggcgagcttctcgatcggatacttgccgtgggccagatgtgcgagag
     V  M  E  L  L  K  G  G  E  L  L  D  R  I  L  A  V  G  Q  M  C  E  S
   1111111111111111111111111111111111111111111111111111111111111111111111111

              2390       2400       2410       2420       2430       2440       2450
               +          +          +          +          +          +          +
1  tgaggccagcgcggtgttaaggacaattgcatctgcggtagcatatctccatgaacatggcgtggtccat
2  tgaggccagcgcggtgttaaggacaattgcatctgcggtagcatatctccatgaacatggcgtggtccat
3  tgaggccagcgcggtgttaaggacaattgcatctgcggtagcatatctccatgaacatggcgtggtccat
4  tgaggccagcgcggtgttaaggacaattgcatctgcggtagcatatctccatgaacatggcgtggtccat
    E  A  S  A  V  L  R  T  I  A  S  A  V  A  Y  L  H  E  H  G  V  V  H
   1111111111111111111111111111111111111111111111111111111111111111111111111

              2460       2470       2480       2490       2500       2510       2520
               +          +          +          +          +          +          +
1  cgagatcttaagcccttcaaatatgatatatgccagtatgcggcaaactcccgagaccctaaagctctgcg
2  cgagatcttaagcccttcaaatatgatatatgccagtatgcggcaaactcccgagaccctaaagctctgcg
3  cgagatcttaagcccttcaaatatgatatatgccagtatgcggcaaactcccgagaccctaaagctctgcg
```

```
4    cgagatcttaagccttcaaatatgatatatgccagtatgcggcaaactcccgagaccctaaagctctgcg
     R  D  L  K  P  S  N  M  I  Y  A  S  M  R  Q  T  P  E  T  L  K  L  C  D
     1111111111111111111111111111111111111111111111111111111111111111111111


           2530      2540      2550      2560      2570      2580      2590
            +         +         +         +         +         +         +
1    atttgggtttcgcgaagcagctgcgcgcggacaacggcctcctgatgacgccatgctacacagccaattt
2    atttgggtttcgcgaagcagctgcgcgcggacaacggcctcctgatgacgccatgctacacagccaattt
3    atttgggtttcgcgaagcagctgcgcgcggacaacggcctcctgatgacgccatgctacacagccaattt
4    atttgggtttcgcgaagcagctgcgcgcggacaacggcctcctgatgacgccatgctacacagccaattt
      L  G  F  A  K  Q  L  R  A  D  N  G  L  L  M  T  P  C  Y  T  A  N  F
     1111111111111111111111111111111111111111111111111111111111111111111111


           2600      2610      2620      2630      2640      2650      2660
            +         +         +         +         +         +         +
1    tgtggctcccgaggttctaaagagacagggctatgacctggcttgcgacatctggtcgctcggtgtgctg
2    tgtggctcccgaggttctaaagagacagggctatgacctggcttgcgacatctggtcgctcggtgtgctg
3    tgtggctcccgaggttctaaagagacagggctatgacctggcttgcgacatctggtcgctcggtgtgctg
4    tgtggctcccgaggttctaaagagacagggctatgacctggcttgcgacatctggtcgctcggtgtgctg
      V  A  P  E  V  L  K  R  Q  G  Y  D  L  A  C  D  I  W  S  L  G  V  L
     1111111111111111111111111111111111111111111111111111111111111111111111


           2670      2680      2690      2700      2710      2720      2730
            +         +         +         +         +         +         +
1    ctttacatcatgttatccggccggacgcctttcgccagcactccaaatgattcaccggacgtaatactga
2    ctttacatcatgttatccggccggacgcctttcgccagcactccaaatgattcaccggacgtaatactga
3    ctttacatcatgttatccggccggacgcctttcgccagcactccaaatgattcaccggacgtaatactga
4    ctttacatcatgttatccggccggacgcctttcgccagcactccaaatgattcaccggacgtaatactga
      L  Y  I  M  L  S  G  R  T  P  F  A  S  T  P  N  D  S  P  D  V  I  L  K
     1111111111111111111111111111111111111111111111111111111111111111111111


           2740      2750      2760      2770      2780      2790      2800
            +         +         +         +         +         +         +
1    agcgcatcggatcaggacaaattgacttcacaagcagtcgctgggcactgatcagtgtgccggccaaaga
2    agcgcatcggatcaggacaaattgacttcacaagcagtcgctgggcactgatcagtgtgccggccaaaga
3    agcgcatcggatcaggacaaattgacttcacaagcagtcgctgggcactgatcagtgtgccggccaaaga
4    agcgcatcggatcaggacaaattgacttcacaagcagtcgctgggcactgatcagtgtgccggccaaaga
       R  I  G  S  G  Q  I  D  F  T  S  S  R  W  A  L  I  S  V  P  A  K  E
     1111111111111111111111111111111111111111111111111111111111111111111111


           2810      2820      2830      2840      2850      2860      2870
            +         +         +         +         +         +         +
1    acttttgcgtcagatgctacacatagtaccggagaatcggccgacggcggcgcgaatacttgagcacgac
2    acttttgcgtcagatgctacacatagtaccggagaatcggccgacggcggcgcgaatacttgagcacgac
3    acttttgcgtcagatgctacacatagtaccggagaatcggccgacggcggcgcgaatacttgagcacgac
4    acttttgcgtcagatgctacacatagtaccggagaatcggccgacggcggcgcgaatacttgagcacgac
      L  L  R  Q  M  L  H  I  V  P  E  N  R  P  T  A  A  R  I  L  E  H  D
     1111111111111111111111111111111111111111111111111111111111111111111111


           2880      2890      2900      2910      2920      2930      2940
            +         +         +         +         +         +         +
1    tggctgcgggagcaattcgccggcggcgtacagcttacagagtatgcggtggcgcccggatcccaacttt
2    tggctgcgggagcaattcgccggcggcgtacagcttacagagtatgcggtggcgcccggatcccaacttt
3    tggctgcgggagcaattcgccggcggcgtacagcttacagagtatgcggtggcgcccggatcccaacttt
4    tggctgcgggagcaattcgccggcggcgtacagcttacagagtatgcggtggcgcccggatcccaacttt
      W  L  R  E  Q  F  A  G  G  V  Q  L  T  E  Y  A  V  A  P  G  S  Q  L  S
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           2950      2960      2970      2980      2990      3000      3010
            +         +         +         +         +         +         +
1    cgctgtgcgcccagcagcagcagcagaatcacatctccatggccttaagaggcgctgttgatgccacttt
2    cgctgggcgcccagcagcagcagcagaatcacatctccatggccttaagaggcgctgttgatgccacttt
3    cgctgggcgcccagcagcagcagcagaatcacatctccatggccttaagaggcgctgttgatgccacttt
4    cgctgggcgcccagcagcagcagcagaatcacatctccatggccttaagaggcgctgttgatgccacttt
       L  C  A  Q  Q  Q  Q  Q  N  H  I  S  M  A  L  R  G  A  V  D  A  T  F
     1111111111111111111111111111111111111111111111111111111111111111111111


           3020      3030      3040      3050      3060      3070      3080
            +         +         +         +         +         +         +
1    ccgggctattgccataccccaggcggcgaatgtgggacccgtagaactttccatgctcgccaagaggcgg
2    ccgggctattgccataccccaggcggcgaatgtgggacccgtagaactttccatgctcgccaagaggcgg
3    ccgggctattgccataccccaggcggcgaatgtgggacccgtagaactttccatgctcgccaagaggcgg
4    ccgggctattgccataccccaggcggcgaatgtgggacccgtagaactttccatgctcgccaagaggcgg
      R  A  I  A  I  P  Q  A  A  N  V  G  P  V  E  L  S  M  L  A  K  R  R
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           3090      3100      3110      3120      3130      3140      3150
            +         +         +         +         +         +         +
```

```
1   gccaaagatcgagccaacctgcactcctaatcctgggcggctgcatggtgtccgcggcgccaggccaagc
2   gccaaagatcgagccaacctgcactcctaatcctgggcggctgcatggtgtccgcggcgccaggccaagc
3   gccaaagatcgagccaacctgcactcctaatcctgggcggctgcatggtgtccgcggcgccaggccaagc
4   gccaaagatcgagccaacctgcactcctaatcctgggcggctgcatggtgtccgcggcgccaggccaagc
    A   K   D   R   A   N   L   H   S   *

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        3160      3170      3180      3190      3200      3210      3220
         +         +         +         +         +         +         +
1   ggcagatagtccgactacttttccgaatctatagtattctaatccaatgctgctgtgccaatccgcgtcg
2   ggcagatagtccgactacttttccgaatctatagtattctaatccaatgctgctgtgccaatccgcgtcg
3   ggcagatagtccgactacttttccgaatctatagtattctaatccaatgctgctgtgccaatccgcgtcg
4   ggcagatagtccgactacttttccgaatctatagtattctaatccaatgctgctgtgccaatccgcgtcg

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        3230      3240      3250      3260      3270      3280      3290
         +         +         +         +         +         +         +
1   tcttgtcaaggacaatgcgcagaagcttggttcacatccacaaacgctgggcaatcctcgccaacgctgc
2   tcttgtcaaggacaatgcgcagaagcttggttcacatccacaaacgctgggcaatcctcgccaacgctgc
3   tcttgtcaaggacaatgcgcagaagcttggttcacatccacaaacgctgggcaatcctcgccaacgctgc
4   tcttgtcaaggacaatgcgcagaagcttggttcacatccacaaacgctgggcaatcctcgccaacgctgc

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        3300      3310      3320      3330      3340      3350      3360
         +         +         +         +         +         +         +
1   cgcagcgccaagctgcgtgtggtttaggttttgggggaatcggcctaacctag-----------------
2   cgcagcgccaagctgcgtgtggtttaggttttgggggaatcggcctaacctaaAGTATTTGACAAGTGTT
3   cgcagcgccaagctgcgtgtggtttaggttttgggggaatcggcctaacctaaAGTATTTGACAAGTGTT
4   cgcagcgccaagctgcgtgtggtttaggttttgggggaatcggcctaacctaaAGTATTTGACAGGTGTT

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        3370      3380      3390      3400      3410      3420      3430
         +         +         +         +         +         +         +
1   ----------------------------------------------------------------------
2   AATTATTTATTTTATATGAAAGCATGCCAATATGCCAAGGCCATAACGCACTGTGCGGGGCATCTATATA
3   AATTATTTATTTTATATGAAAGCATGCCAATATGCCAAGGCCATAACGCACTGTGCGGGGCATCTATATA
4   AATTATTTATTTTATATGAAAGCATGCCAATATGCCAAGGCCATAACGCACTGTGCGGGGCATCTATATA

        3440      3450      3460      3470      3480      3490      3500
         +         +         +         +         +         +         +
1   ----------------------------------------------------------------------
2   CACATATATATAACTATACAAATACTGATATACATCTATATATAGCTATTTATATAACGAATCCTAAAGC
3   CACATATATATAACTATACAAATACTGATATACATCTATATATAGCTATTTATATAACGAATCCTAAAGC
4   CACATATATATAACTATACAAATACTGATATACATCTATATATAGCTATTTATATAACGAATCCTAAAGC

        3510      3520      3530      3540      3550      3560      3570
         +         +         +         +         +         +         +
1   ----------------------------------------------------------------------
2   AAGGGCCTAGAGAGCGCGTGTGAATGTGGCGCCAGGATCATCCCAGATGATTCCTGAAACCACATTATTT
3   AAGGGCCTAGAGAGCGCGTGTGAATGTGGCGCCAGGATCATCCCAGATGATTCCTGAAACCACATTATTT
4   AAGGGCCTAGAGAGCGCGTGTGAATGTGGCGCCAGGATCATCCCAGATGATTCCTGAAACCACATTATTT

        3580      3590      3600      3610      3620      3630      3640
         +         +         +         +         +         +         +
1   ----------------------------------------------------------------------
2   ATCCCAAACTGGAAATTAAAAAAAAGCCTCTCGCCCAAGTTTACATATTTTTAATTAAATTAATCGTAGC
3   ATCCCAAACTGGAAATTAAAAAAAAGCCTCTCGCCCAAGTTTACATATTTTTAATTAAATTAATCGTAGC
4   ATCCCAAACTGGAAATTAAAAAAAAGCCTCTCGCCCAAGTTTACATATTTTTAATTAAATTAATCGTAGC

        3650      3660      3670      3680      3690      3700      3710
         +         +         +         +         +         +         +
1   ----------------------------------------------------------------------
2   TAAATTTTTCAACATCACCGTGTTATTGGTGCTTAATTAAATTAATTAAAATGAAAAAAAAAATGGAAAA
3   TAATTTTTCAACATCACCGTGTTATTGTTGCTTAATTAAATTAATTAAAAA------------------
4   TAAATTTTTCAACATCACCGTGTTATTGTTGCTTAATTAAATTAATT---------------------
```

```
        3720        3730        3740        3750        3760        3770        3780
          +           +           +           +           +           +           +
1  ----------------------------------------------------------
2  AGTATTAGCAAAACAAACAAAAATTAAGAATAAAAGCCGCAATCCTTTTATAT
3  ----------------------------------------------------------
4  ----------------------------------------------------------
```

Figure 9: Sequencing result of precise jumpout line *ign*^{Δ1P1}. Primer 3 was used for sequencing reaction.

```
1              15 16           30 31           45 46           60 61           75 76           90
GCTGCTGCTGCTGAG GCTGCTGGCGGAGAT CCTTTTGCGAATCGG CCAGCGGCATGACTT CGGTGGCTGCTAGCG TTGTGAGGCAACTGC  90

91             105 106          120 121          135 136          150 151          165 166          180
TCCTAGCGGTTCCTT TGTTTCCAGATCCTG ATGCACAATGCTAGA GCCCGAGTTCCCCCT GCGTTTCCAACTCCT CCTGCTGCTCCTCCT  180

181            195 196          210 211          225 226          240 241          255 256          270
CCACCGAACTCTCCC TACTTCCGATTTCAC TTTTCTTTTTTATTA CTTCCGCTGCGTGGG CGTCGCAGTCGACTG CGCTGTTGCCTCTCC  270

271            285 286          300 301          315 316          330 331          345 346          360
GGATTTCTCTGGTTT CCCTGCTGCTCCTAC GGCCAACAAGCAGCG CTTTCCTAATGCGCC CACGTTTCGTACGCC GTCTGCTTTACTATC  360

361            375 376          390 391          405 406          420 421          435 436          450
CGCTTCGCAATCCGC ACAGCTGTGTTGCTT CTTATCACTATTATT TACACATTCACAATA AAGTTTTTTTAGAGC TGCAAAAAAATCGAT  450

451            465 466          480 481          495 496          510 511          525 526          540
GTTACCATCGATTAC ATCGATAGTTTCAGT TTTCCGAAAA       490
```

Figure 10: Sequencing result of precise jumpout line *ign*^{Δ2P1}. Primer 3 was used for sequencing reaction.

```
1              15 16           30 31           45 46           60 61           75 76           90
CTGCTGCTGCTGAGG CTGCTGGCGGAGATC CTTTTGCGAATCGGC CAGCGGCATGACTTC GGTGGCTGTGAGGCA ACTGCTCCTAGCGGT  90

91             105 106          120 121          135 136          150 151          165 166          180
TCCTTTGTTTCCAGA TCCTGATGCACAATG CTAGAGCCCGAGTTC CCCCTGCGTTTCCAA CTCCTGCTGCTCCTC CTCCACCGAACTCTC  180

181            195 196          210 211          225 226          240 241          255 256          270
CCTACTTCCGATTTC ACTTTTCTTTTTTAT TACTTCCGCTGCGTG GGCGTCGCAGTCGAC TGCGCTGTTGCCTCT CCGGATTTCTCTGGT  270

271            285 286          300 301          315 316          330 331          345 346          360
TTCCGTGTTGCTCCT ACGGCCAACAAGCAG CGCTTTCCTAATGCG CCCACGTTTCGTACG GCGTCTGCTTTACTA TCCGCTTCGCAATCC  360

361            375 376          390 391          405 406          420 421          435 436          450
ACACAGCTGTGTTGC TTCTTATCACTATTA TTTACACATTCACAA TAAAGTTTTTTTAGA GCTGCAAAAAAATCG ATGTTACCATCGATT  450

451            465 466          480 481          495 496          510 511          525 526          540
ACATCGATAGTTNAA GNTTTCCAAACATCA ATAACTTTTGCNCCG AGNTTTACTGGGCGN ATCANAAATTTNNT   524
```

8.2 Tables

Table 1: Performance of CantonS flies of different ages. Results of Mann-Whitney U-tests. Table includes age of tested groups (var 1; var 2; d = days), U value, Z value, p value, and sample sizes (N1; N2); only statistically significant results are shown.

var 1	var 2	U value	Z value	p value	N 1	N 2
2d	5d	20034.0	2.71	<0.01	222	212
2d	9d	24115.5	3.28	<0.01	222	262
5d	13d	22877.0	-3.47	<0.001	212	264
5d	17d	17303.0	-4.51	<0.00001	212	217
5d	21d	21417.5	-2.87	<0.01	212	239
5d	25d	19502.5	-4.42	<0.0001	212	241
5d	29d	18465.5	-3.59	<0.001	212	217
9d	13d	27660.5	-4.02	<0.0001	262	264
9d	17d	21043.0	-4.95	<0.00001	262	217
9d	21d	25842.5	-3.42	<0.001	262	239
9d	25d	23645.5	-4.94	<0.0001	262	241
9d	29d	22386.0	-4.07	<0.0001	262	217

Table 2: Walking activity of CantonS males versus females. Table shows results of Mann-Whitney U-tests. Table indicates age of tested groups (age_{male}; age_{female}; d = days), U value, Z value, p value, and sample sizes (N_{male}; N_{female}); only statistically significant results are shown.

age_{male}	age_{female}	U value	Z value	p value	N_{male}	N_{female}
2 d	2 d	4446.5	-3.54	<0.001	118	104
5 d	5 d	4099.0	-3.39	<0.001	109	103
9 d	9 d	6555.5	-3.24	<0.01	140	122
17 d	17 d	4472.5	-3.02	<0.01	102	115
25 d	25 d	5544.0	-3.15	<0.01	115	126
29 d	29 d	4298.5	-3.42	<0.001	112	105

Table 3: Walking activity of CantonS males of different ages. Results of Mann-Whitney U-tests. Table includes age of tested groups (var 1; var 2; d = days), U value, Z value, p value, and sample sizes (N1; N2); only statistically significant results are shown.

var 1	var 2	U value	Z value	p value	N 1	N 2
2 d	5 d	5333.0	-2.22	<0.05	118	109
5 d	17 d	3721.5	4.15	<0.0001	109	102
5 d	21 d	5686.0	2.54	<0.05	109	129
5 d	25 d	4517.0	3.61	<0.001	109	115
5 d	29 d	4426.0	3.53	<0.001	109	112
9 d	17 d	5549.0	2.96	<0.01	140	102
9 d	25 d	6744.5	2.23	<0.05	140	115
9 d	29 d	6744.5	2.23	<0.05	140	115
13 d	17 d	5750.5	2.97	<0.01	145	102
13 d	29 d	6949.0	1.98	<0.05	145	112
17 d	21 d	5412.5	-2.31	<0.05	102	129

Table 4: Walking activity of CantonS females of different age. Results of Mann-Whitney U-tests. Table includes age of tested groups (var 1; var 2; d = days), U value, Z value, p value, and sample sizes (N1; N2); only statistically significant results are shown.

var 1 females	var 2 females	U value	Z value	p value	N 1	N 2
2 d	13 d	5138.0	2.18	<0.05	104	119
2 d	17 d	4681.0	2.77	<0.01	104	115
2 d	21 d	4740.5	2.16	<0.05	104	110
2 d	25 d	5383.0	2.33	<0.05	104	110
2 d	29 d	4472.5	2.26	<0.05	104	105
5 d	13 d	4264.5	3.91	<0.0001	103	119
5 d	17 d	3858.0	4.44	<0.00001	103	115
5 d	21 d	3805.0	4.14	<0.0001	103	110
5 d	25 d	4174.0	4.64	<0.0001	103	126
5 d	29 d	3595.5	4.18	<0.0001	103	105
9 d	13 d	5905.5	2.50	<0.05	122	119
9 d	17 d	5382.0	3.10	<0.01	122	115
9 d	21 d	5411.5	2.54	<0.05	122	110
9 d	25 d	6021.5	2.95	<0.01	122	126
9 d	29 d	5411.5	2.52	<0.05	122	105

Table 5: Performance of mutant flies *dunce* (dnc^{ML}), *rutabaga* (rut^{2080}) and *amnesiac* (amn^1) compared to wild-type CantonS flies. Table shows results of Mann-Whitney U-tests, including U value, Z value, p value, and sample sizes (N1; N2); only statistically significant results are shown. tr = last training minute, te 1 = first test minute, te 2 = second test minute, te 3 = third test minute.

line	PI	U value	Z value	p value	N1	N2
dnc^{ML}	tr	3347.0	2.05	p<0.05	76	107
	te 2	3302.0	2.18	p<0.05	76	107
	te 3	3164.0	2.57	p<0.05	76	107
rut^{2080}	tr	5853.0	3.70	p<0.001	150	107
	te 1	5861.5	3.70	p<0.001	150	107
	te 2	5770.5	3.85	p<0.001	150	107
	te 3	6472.5	2.65	p<0.01	150	107
amn^1	tr	3892.5	5.85	p=0.0	130	107
	te 1	5197.0	3.36	p<0.001	130	107
	te 2	5571.5	2.64	p<0.01	130	107
	te 3	5906.5	2.00	p<0.05	130	107

Table 6: Behavioral results of 49 candidate lines. Table shows P-element line, Performance Index of last training minute (tr) and first test minute (te 1; te 2; each 30 sec), classification of the P-element line (class; H = heat avoidance candidate, M = memory candidate, out = disqualified as candidate, (H) = border line heat avoidance candidate, (M) = border line memory candidate), sample sitze (n), training duration (train dur) and indicates the heat-box version (orig = original, mod = modified) which was used for the experiment. value indicates the consistency of the behavioral phenotype (first, second and last choice).

value	line	tr	te 1	te 2	class	n	tain dur	Heat-box version
10 candidates first choice	**5054/3**	0.748	0.262	-0.018	M	24	2 min	orig
		0.376	0.167	-0.029	H	24	2 min	orig
		0.416	0.447	-0.040	(M)	24	2 min	orig
		0.549	0.291	0.349	M	23	4 min	orig
		0.488	0.197	0.234	M	21	3 min	orig
		0.465	0.187	0.225	M	68	4 min	**mod**
		0.576	0.321	0.165	out	70	4 min	**mod**
	6139/2	0.499	0.254	0.107	M	26	2 min	orig
		0.594	0.119	0.011	M	23	2 min	orig
		0.517	0.574	0.309	out	18	4 min	orig
		0.460	0.311	0.054	(M)	19	3 min	orig
		0.560	0.237	0.141	M	21	3 min	orig
		0.525	0.183	0.093	M	102	4 min	**mod**
	8466/2	0.307	0.173	0.216	H	~20	4 min	orig
		0.401	0.313	0.379	(H)	16	4 min	orig
		0.200	0.346	0.141	H	22	4 min	orig
		0.450	0.341	0.124	out	18	4 min	orig
		0.402	0.240	0.154	M	~20	3 min	orig
		0.724	0.287	0.030	M	20	3 min	orig
		0.577	0.260	0.075	M	104	4 min	**mod**
		0.578	0.271	0.222	M	56	4 min	**mod**
	8522/1	0.468	0.199	0.114	M	21	2 min	orig
		0.416	0.219	0.000	M	19	2 min	orig
		0.606	0.289	0.104	M	17	4 min	orig
		0.472	0.261	0.015	M	23	4 min	orig
		0.452	0.120	0.173	M	95	4 min	**mod**
		0.519	0.272	0.259	M	82	4 min	**mod**
		0.466	0.251	0.151	M	67	4 min	**mod**
		0.546	0.398	0.313	out	69	4 min	**mod**
	8570/1	0.425	0.283	0.005	M	23	2 min	orig
		0.583	0.275	0.139	M	21	4 min	orig
		0.674	0.501	0.437	out	21	4 min	orig
		0.548	0.266	0.288	M	26	4 min	orig
		0.453	0.246	0.182	M	80	4 min	**mod**
	8631/4	0.472	0.261	-0.097	M	23	2 min	orig
		0.426	0.185	0.180	M	~20	2 min	orig
		0.455	0.310	-0.008	(M)	18	3 min	orig
		0.498	0.150	0.080	M	95	4 min	**mod**
		0.573	0.225	0.110	M	74	4 min	**mod**
		0.641	0.394	0.258	out	69	4 min	**mod**
		0.668	0.350	0.346	out	66	4 min	**mod**
	48-8657/1	0.586	0.013	-0.014	M	17	2 min	orig

		0.478	0.248	0.136	M	25	2 min	orig
		0.366	0.121	0.276	H	23	4 min	orig
		0.541	0.343	0.248	out	22	3 min	orig
		0.419	0.255	0.118	M	21	3 min	orig
		0.502	0.226	0.219	M	96	4 min	**mod**
		0.330	0.085	0.067	H	71	4 min	**mod**
	9530/1	0.463	0.274	0.298	M	22	3 min	orig
		0.301	0.212	-0.007	H	18	3 min	orig
		0.466	0.226	0.005	M	22	3 min	orig
		0.419	0.179	0.168	M	101	4 min	**mod**
		0.357	0.143	0.152	H	82	4 min	**mod**
		0.302	0.100	0.094	H	66	4 min	**mod**
		0.401	0.239	0.171	M	72	4 min	**mod**
	9690/3	0.374	0.094	0.061	H	18	4 min	orig
		0.380	0.301	0.126	H	~20	4 min	orig
		0.431	0.064	0.099	M	23	3 min	orig
		0.322	0.312	0.158	H	23	3 min	orig
		0.363	0.102	0.032	H	88	4 min	**mod**
		0.604	0.345	0.357	out	77	4 min	**mod**
	9885/1	0.405	0.259	0.309	M	24	3 min	orig
		0.425	0.342	0.080	(M)	23	3 min	orig
		0.303	0.169	0.154	H	18	3 min	orig
		0.381	0.167	0.145	H	105	4 min	**mod**
		0.362	0.137	0.131	H	83	4 min	**mod**
		0.326	0.182	0.052	H	72	4 min	**mod**
		0.454	0.191	0.129	M	74	4 min	**mod**
19 candidates second choice	**185/1**	0.463	0.275	0.076	M	24	2 min	orig
		0.453	0.215	0.095	M	22	2 min	orig
		0.497	0.266	0.441	M	22	3 min	orig
		0.560	0.298	0.146	M	104	4 min	**mod**
	36-1726/2	0.303	0.300	0.054	H	26	2 min	orig
		0.426	0.274	-0.043	M	21	2 min	orig
		0.289	0.325	-0.011	H	24	2 min	orig
		0.450	0.430	0.234	out	19	4 min	orig
		0.431	0.452	0.251	(H)	32	4 min	orig
		0.370	0.154	0.045	H	86	4 min	**mod**
		0.372	0.126	0.157	H	79	4 min	**mod**
	54-1946/1	0.526	0.255	0.038	M	27	2 min	orig
		0.494	0.187	0.092	M	23	2 min	orig
		0.454	0.095	-0.131	M	~20	4 min	orig
		0.526	0.267	0.174	M	102	4 min	**mod**
	29-1998/2	0.262	0.407	0.161	H	23	2 min	orig
		0.578	0.206	-0.031	M	21	2 min	orig
		0.468	0.145	0.361	M	~20	3 min	orig
		0.392	0.205	0.184	H	97	4 min	**mod**
	28-2253/2	0.355	0.284	-0.040	H	26	2 min	orig
		0.442	0.224	-0.012	M	22	2 min	orig
		0.529	0.354	0.073	(M)	21	3 min	orig

	0.383	0.097	0.173	H	22	3 min	orig
	0.561	0.303	0.215	out	21	3 min	orig
	0.455	0.131	0.155	M	107	4 min	**mod**
	0.573	0.379	0.287	out	60	4 min	**mod**
50-3121/1	0.453	0.249	-0.062	M	22	2 min	orig
	0.383	0.240	0.141	H	23	2 min	orig
	0.653	0.179	0.113	M	17	4 min	orig
	0.490	0.251	0.197	M	88	4 min	**mod**
3223/3	0.425	0.317	0.002	(M)	~20	2 min	orig
	0.484	0.259	0.125	M	32	2 min	orig
	0.314	0.200	0.167	H	20	4 min	orig
	0.466	0.208	0.082	M	86	4 min	**mod**
	0.623	0.222	0.024-	M	64	4 min	**mod**
3449/1	0.440	0.269	0.057	M	27	2 min	orig
	0.647	0.292	0.003	M	14	2 min	orig
	0.512	0.261	0.375	M	21	3 min	orig
	0.469	0.066	0.068	M	75	4 min	**mod**
50-3587	0.510	0.276	0.022	M	24	2 min	orig
	0.679	0.189	0.158	M	21	2 min	orig
	0.675	0.294	0.454	M	22	4 min	orig
	0.454	0.269	0.265	M	19	3 min	orig
	0.524	0.222	0.146	M	95	4 min	**mod**
4114/2	0.325	0.357	-0.024	H	19	2 min	orig
	0.325	0.410	0.063	H	17	2 min	orig
	0.553	0.357	0.469	out	22	3 min	orig
	0.487	0.271	0.164	M	101	4 min	**mod**
5128	0.541	0.202	0.089	M	24	2 min	orig
	0.688	0.217	0.243	M	~20	4 min	orig
	0.347	0.183	0.074	H	98	4 min	**mod**
	0.447	0.296	0.213	M	82	4 min	**mod**
5446/2	0.262	0.131	0.040	H	16	4 min	orig
	0.384	0.180	0.187	H	~20	4 min	orig
	0.373	0.164	0.076	H	24	3 min	orig
	0.424	0.122	0.082	M	112	4 min	**mod**
	0.390	0.121	0.073	H	71	4 min	**mod**
5459/1	0.499	0.288	0.069	M	23	2 min	orig
	0.552	0.229	-0.007	M	23	2 min	orig
	0.406	0.223	0.127	M	25	4 min	orig
	0.634	0.420	0.344	out	17	3 min	orig
	0.503	0.273	0.169	M	96	4 min	**mod**
5865/2	0.352	0.244	0.323	H	20	4 min	orig
	0.724	0.079	0.161	M	23	3 min	orig
	0.490	0.219	0.129	M	96	4 min	**mod**
816/1	0.533	0.283	0.094	M	24	2 min	orig
	0.456	0.222	0.099	M	24	2 min	orig
	0.711	0.414	0.397	out	~20	4 min	orig
	0.680	0.219	0.104	M	18	4 min	orig

		0.437	0.167	0.004	M	19	3 min	orig
		0.486	0.229	0.051	M	110	4 min	**mod**
		0.548	0.241	0.076	M	66	4 min	**mod**
	850/1	0.376	0.165	0.136	H	23	3 min	orig
		0.476	0.196	0.257	M	20	3 min	orig
		0.540	0.196	0.075	M	91	4 min	**mod**
	9725/3	0.637	0.360	0.008	(M)	19	4 min	orig
		0.380	0.057	-0.003	H	16	4 min	orig
		0.609	0.580	0.129	out	18	4 min	orig
		0.694	0.291	0.132	M	23	3 min	orig
		0.462	0.349	0.146	(M)!!!	99	4 min	**mod**
	9910/4	0.396	0.396	0.330	H	18	4 min	orig
		0.346	0.177	0.143	H	22	4 min	orig
		0.361	0.177	0.087	H	22	3 min	orig
		0.376	0.221	0.180	H	99	4 min	**mod**
		0.337	0.176	0.101	H	82	4 min	**mod**
	noname4	0.405	0.254	-0.057	M	24	2 min	orig
		0.521	0.408	0.156	out	21	4 min	orig
		0.577	0.381	0.120	out	22	3 min	orig
		0.570	0.257	0.264	M	110	4 min	**mod**
20 candidates last choice	**1545/2**	0.517	0.245	0.163	M	25	3 min	orig
		0.601	0.187	0.148	M	22	3 min	orig
		0.591	0.191	0.142	M	18	3 min	orig
		0.654	0.414	0.312	out	98	4 min	**mod**
	1872/1	0.339	0.160	0.034	H	22	3 min	orig
		0.495	0.385	0.058	(M)	19	3 min	orig
		0.585	0.365	0.181	out	21	3 min	orig
		0.532	0.338	0.265	out	91	4 min	**mod**
	2163/1	0.382	0.321	0.132	H	23	2 min	orig
		0.499	0.171	0.134	M	23	2 min	orig
		0.312	0.160	0.178	H	32	4 min	orig
		0.630	0.351	0.211	out	110	4 min	**mod**
	2705/2	0.397	0.242	-0.213	H	11	2 min	orig
		0.497	0.292	0.143	M	21	2 min	orig
		0.572	0.296	0.210	M	~20	2 min	orig
		0.700	0.384	0.417	out	84	4 min	**mod**
	2739/1	0.360	0.219	0.038	H	23	2 min	orig
		0.439	0.210	0.070	M	18	2 min	orig
		0.659	0.318	0.288	out	~20	4 min	orig
		0.281	0.083	0.120	H	20	4 min	orig
		0.641	0.338	0.316	out	101	4 min	**mod**
		0.420	0.235	0.149	M	76	4 min	**mod**
	4175/2	0.539	0.207	0.018	M	20	2 min	orig
		0.438	0.284	0.115	M	24	2 min	orig
		0.504	0.343	0.394	out	16	4 min	orig
		0.596	0.473	0.063	(M)	~20	3 min	orig
		0.473	0.306	0.111	out	94	4 min	**mod**

	0.498	0.237	0.202	M	77	4 min	**mod**
4742/2	0.599	0.293	0.130	M	26	2 min	orig
	0.599	0.240	-0.022	M	22	2 min	orig
	0.441	0.271	0.108	M	31	4 min	orig
	0.619	0.371	0.275	out	92	4 min	**mod**
5170/2	0.598	0.165	0.242	M	~20	4 min	orig
	0.645	0.247	0.392	M	~20	4 min	orig
	0.454	0.243	0.237	M	20	4 min	orig
	0.414	0.238	0.190	M	~20	4 min	orig
	0.653	0.463	0.389	out	97	4 min	**mod**
5835/2	0.480	0.214	0.117	M	21	2 min	orig
	0.405	0.332	0.437	(H)	17	4 min	orig
	0.620	0.452	0.292	out	108	4 min	**mod**
6099/3	0.515	0.058	-0.112	M	17	4 min	orig
	0.362	-0.031	0.033	H	17	4 min	orig
	0.443	0.339	0.189	out	20	4 min	orig
	0.483	0.252	0.141	M	23	3 min	orig
	0.545	0.174	0.116	M	19	3 min	orig
	0.557	0.318	0.161	out	92	4 min	**mod**
708/3	0.316	0.449	0.058	H	18	4 min	orig
	0.387	0.448	0.313	H	17	4 min	orig
	0.404	0.298	0.359	M	18	3 min	orig
	0.620	0.461	0.202	out	98	4 min	**mod**
	0.514	0.391	0.321	out	77	4 min	**mod**
7837/2	0.449	0.307	0.048	M	16	4 min	orig
	0.481	0.170	0.200	M	~20	4 min	orig
	0.388	0.138	0.171	H	21	3 min	orig
	0.701	0.476	0.537	out	22	3 min	orig
	0.738	0.552	0.407	out	93	4 min	**mod**
7920/4	0.490	0.277	0.244	M	19	3 min	orig
	0.377	0.269	0.363	H	20	3 min	orig
	0.380	0.353	0.256	H	20	3 min	orig
	0.605	0.384	0.281	out	107	4 min	**mod**
8036/1	0.361	0.269	0.153	H	20	2 min	orig
	0.672	0.289	0.210	M	36	4 min	orig
	0.595	0.418	0.310	out	~20	4 min	orig
	0.560	0.168	0.006	M	25	4 min	orig
	0.655	0.403	0.179	out	100	4 min	**mod**
8405/2	0.559	0.268	0.325	M	~20	3 min	orig
	0.543	0.297	0.076	M	24	3 min	orig
	0.521	0.152	0.287	M	20	3 min	orig
	0.663	0.329	0.283	out	102	4 min	**mod**
8743/2	0.343	0.161	0.260	H	20	4 min	orig
	0.439	0.398	0.288	out	22	3 min	orig
	0.528	0.265	0.194	M	19	3 min	orig
	0.580	0.386	0.221	out	106	4 min	**mod**

8756/2	0.448	0.281	0.164	M	~20	4 min	orig
	0.399	0.471	0.398	H	22	3 min	orig
	0.443	0.379	0.121	out	19	3 min	orig
	0.625	0.331	0.235	out	105	4 min	**mod**
9353/1	0.594	0.225	0.078	M	23	3 min	orig
	0.521	0.512	0.133	out	21	3 min	orig
	0.515	0.277	0.021	M	20	3 min	orig
	0.745	0.502	0.386	out	97	4 min	**mod**
9519/1	0.481	0.199	0.041	M	24	2 min	orig
	0.466	0.212	-0.090	M	21	2 min	orig
	0.422	0.284	0.210	M	20	3 min	orig
	0.635	0.394	0.209	out	108	4 min	**mod**
9879/3	0.554	0.322	0.324	out	21	4 min	orig
	0.506	0.164	0.055	M	23	3 min	orig
	0.558	0.530	0.099	(M)	17	3 min	orig
	0.556	0.359	0.275	out	100	4 min	**mod**

LEBENSLAUF

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WISSENSCHAFTLICHER BEITRAG

- Beitrag zu akademischen Konferenzen

Putz, G., S. Kramer, T. Zars, and M. Heisenberg, (2000). Characterization of new learning and memory mutants in an operant conditioning paradigm. Neurofly 2000. 8. Europäisches Symposium in *Drosophila* Neurobiologie, Alicante, Spanien.

Putz G., T. Zars, and M. Heisenberg (2001). Mutants of the *Drosophila ignorant/S6KII* gene: learning/memory defect in P-insertion and viability of null allele. 28. Göttinger Neurobiologentagung, Göttingen, Deutschland.

Putz G., T. Zars, and M. Heisenberg (2001). Mutants of the *Drosophila ignorant*/*S6KII* gene: learning/memory defect in P-insertion and viability of null allele. Gordon Research Conference, Neural Plasticity, Newport, RI (USA).

- Einladungen zur mündlichen Präsentation

20 Oct. 1999 Universität Hohenheim, Institut für Physiologie, Deutschland. Seminar in Insect Neurobiology. Visual and spatial learning in *Drosophila*.

19 Jan. 2002 Julius Maximilians-Universität Würzburg, Neurologie, Deutschland. Symposium on Molecular and Cellular Basis of Higher Brain Function. *Drosophila* as model organism for learning and memory studies: Defects of mutant line *ignorant* /S6KII in operant conditioning.

- Publikationsliste

Putz, G. and M. Heisenberg (accepted in Learning & Memory). Memories in *Drosophila* Heat-box learning.

Putz, G., T. Zars, and M. Heisenberg (in prep). The *Drosophila ignorant/S6KII* gene.

ERKLÄRUNG

Erklärungen gemäß § 4 Absatz 3 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität 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 benutzt zu haben. Alle aus der Literatur entnommenen Stellen sind als solche kenntlich gemacht. Desweiteren erkläre ich, daß die vorliegende Arbeit weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat. Zuvor habe ich keine akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 15.August. 2002   Gabriele Putz