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## Apoptosis and Immune System Adaptation in *Drosophila melanogaster* following Space Flight and Irradiation

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# Apoptosis and Immune System Adaptation in *Drosophila melanogaster* following Space Flight and Irradiation

Diplomarbeit zur Erlangung des akademischen Grades Magister rer. nat. am Zentrum für Molekulare Biologie an der Universität Wien

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The author of this thesis was not involved in any of the work conducted prior the space flight or directly after the landing of the Space Shuttle at Kennedy Space Center.

# I. ABSTRACT

Living and working in space produces new challenges to astronauts, engineers and scientists due to several unique properties of this environment. For instance, weightlessness or microgravity is responsible for reduced exercise of skeletal muscles resulting in muscle atrophy as well as osteoporosis-like bone loss. Away from the Earth's protective magnetic field, space radiation can damage nucleic acids, cells and tissues resulting in radiation sickness or cancer. The NASA FIT project – **F**ungal Pathogenesis, **I**mmunity and **T**umorigenesis Studies – involved a 12-day Space Shuttle experiment with *Drosophila melanogaster* flies. Additionally, ground based studies involved the exposure of flies to proton irradiation to investigate the genetic, cellular and behavioural effects.

The aims of this project, as part of FIT, were to characterize adaptations of apoptosis and immune system functions following space flight as well as ground based proton radiation exposure. Furthermore, it was of interest if changes in immune system function can be linked to an altered level of apoptosis. RT-PCR gene expression studies of Minibrain, Morgue and Wengen, proteins involved in cell death and immune system functions, showed a trend towards increased apoptotic activity of space flown flies after a bacterial infection in comparison to infected ground control flies. Those trends were also observed by measurement of caspase enzyme activities in space flown animals. Proton irradiation increased fragmentation of DNA in *Drosophila* hemocytes, which was investigated with the TUNEL assay. Tumor suppressor p53 activation in response to proton treatment was shown with a *Drosophila* strain containing a p53 radiation response element in front of a GFP protein. Phagocytosis activity was investigated with the Alexa Fluor E.coli Phagocytosis Assay and the Clearance Assay, both showing that high proton irradiation exposure levels can be responsible for an elevated activity level in hemocytes.

# II. INTRODUCTION

## *2.1. Space Biology and Space Medicine*

### 2.1.1. Why send humans to space?

Over the past centuries the infinity of space has inspired countless science fiction authors to write about humans travelling to other planets, searching for alien life and experiencing exciting adventures. However, scientists were also inspired and began to develop necessary technologies and skills to enable humans to leave Earth to explore the unknown. The achievements over the past 50 years have shown that we are capable of travelling to space, sending astronauts to the Moon, and exploring other planets.

Today's space technology is incorporated into our everyday life, e.g. television broadcasting, money transfers, the Global Positioning System, the internet, the telephone, the weather forecast and many other applications. Several space agencies around the world have focused their space exploration programmes towards new goals of sending humans back to the Moon, to Mars and beyond. Although it will take some time until the first human being will set foot on Mars, it is already possible for someone with sufficient capital to make a trip to space.

### 2.1.2. Definition – Space Biology

Space Biology research is a fundamental scientific research field studying the effects of the space environment on living organisms. These effects are caused by the lack of gravity, presence of space radiation and possible other undiscovered factors. Studying these effects expands our fundamental knowledge about life on Earth and in space, and enables the development of countermeasures to prepare astronauts for long duration space flights to the Moon and Mars. Current manned long duration missions last about six months onboard the International Space Station (ISS), a joint project of five space agencies; the National Aeronautics and Space Administration (NASA, USA), the Russian Federal Space Agency (Roskosmos, Russian Federation), the Japanese Aerospace Exploration Agency (JAXA, Japan), the Canadian Space Agency (CSA, Canada) and the European Space Agency (ESA, Europe). Future Moon and Mars missions will expose humans to the space environment for up to two years without the possibility to return them quickly to Earth

in case of technical or medical incidents. Therefore, fields like Space Biology and Space Medicine will have to pave the way for these next steps in space exploration to ensure the health of astronauts by conducting the necessary research and developing the respective countermeasures.

### 2.1.3 History of Space Biology and Space Medicine

Space Biology research began to expand as a field of practical interest shortly after the end of World War II. An early example of a space biological experiment was the exposure of *Drosophila melanogaster* fruit flies to the extreme upper limits, about 60 km, of the atmosphere using a balloon. After parachuting back to Earth the flies were recovered alive and in apparent good health. In 1948, before the first manned space flight in 1961, the monkey, “Albert”, was sent to space. Meanwhile, soviet scientists launched nine dogs before the historic flight of the dog “Laika” in 1957. But even prior these experiments, which are considered to be the first in Space Biology, there has been an interest in the gravitational impact on organisms. Knight in 1806 and Pfluger in 1883 reported about growing plants and oocytes under hypergravity conditions using a centrifuge. In the 1960s the first automated missions with biological payloads were launched carrying bacteria, plants, and animal cells to space. The breakthrough in systematic and extensive investigations happened in 1983 with the first Space Shuttle – Spacelab mission. The integration of the Biorack into the Space Shuttle provided a wide range of laboratory facilities, for instance 2 incubators, a -20℃ freezer and a glove box (Moore et al., 1996).

With the increased capability of conducting experiments in space, the question of good control samples arose. An Earth-bound control was not sufficient enough due to additional effects like high acceleration at the spacecraft launch and re-entry, strong vibration and acoustic variables. To further distinguish the impacts of all these effects, a centrifuge spinning at Earth gravity, 1g, was necessary to be included aboard a spacecraft. Due to the high costs and risk involved in space flight, other options to study or simulate the effects of low gravity were investigated. Successful methods for small experiments included the clinostat, the random positioning machine, the sounding rocket, and drop towers (Cogoli, 1993; Moore et al., 1996). To study small animals like rodents the hindlimb suspension is used to simulate space flight induced muscle atropy and bone loss (Morey-Holton and Globus, 2005). Bed-

rest-study and parabolic flights with an aircraft are also conducted with humans to simulate the effects of weightlessness (Klaus, 2001; Schmitt and Angerer, 2001).

### 2.1.4. Medical Issues on Long Duration Missions

Bioastronautics, as a discipline, is the study of biological and medical effects of space flight on humans and development of countermeasures to overcome them (NASA Bioastronautic Roadmap). The NASA Bioastronautic Roadmap (http://bioastroroadmap.nasa.gov) provides information for making informed decisions about determining research priorities, setting exposure standards, and allocating resources. Since the beginning of the manned space flight era hundreds of experiments on astronauts, model organisms and cell cultures have been conducted to address the medical risks for astronauts on long-duration missions (Bikle et al., 2003; Ohnishi and Ohnishi, 2004; Boonyaratanakornkit et al., 2005; Sonnenfeld, 2003; Grindeland et al., 2005).

| Medical Issue | Possible Cause |
|---|---|
| Reduced muscle mass, strength, and endurance | Weightlessness<br>Exercise not sufficient as countermeasure |
| Accelerated bone loss and fracture fisk | Weightlessness<br>Reduced muscle use |
| Carcinogenesis | Space radiation |
| CNS damage | Space radiation |
| Immune dysfunction | Combined space effects<br>Negative effect on immune system cells |
| Alterations in microbes and host interactions | Infector properties change in space |
| Renal stone formation | Changes in urine chemistry |

***Table 2.1.*** Examples for medical challenges arising during space flight (http://bioastroroadmap.nasa.gov)

Studies on gene and protein expression unfolded some of the mechanisms behind microgravity-induced problems (Nichols et al., 2006). Space radiation, consisting of ionizing radiation in the form of charged high-energy particles, seems to pose the biggest threat for humans travelling in space. It is directly associated with the production of reactive oxygen species such as peroxides and hydroxyl radicals, which can attack cellular lipids, proteins and DNA (von Deutsch et al., 2005). Gene

expression studies in mice and human blood cells showed that exposure to radiation can be predicted by gene expression profiles (Dressman et al., 2007).

Biological countermeasures, like dietary additives, have been used to reduce the levels of some biological consequences resulting from exposure to charged high-energy particles (Kennedy and Todd, 2003). Ground-based simulation of space radiation components and space based measurement of the radiation environment are required to understand the associated hazard on organisms and are necessary to develop effective countermeasures (Schimmerling, 2003). Lessons learned from the International Space Station will contribute to the development of countermeasures including shielding and medication consisting of radical scavengers, antioxidant consumption, cytokines, and cell transplants (Todd, 2003).

### 2.1.5. *Drosophila melanogaster* – a Model for Space Biology Research

The small fruit fly *Drosophila melanogaster* is a well-characterized organism that is ideal for the study of molecular, cellular, developmental, and physiological biology (Beckingham et al., 2005). Its size, low maintenance requirements and short life-cycle are important characteristics representing the advantages of using *Drosophila* as a tool for space biological research. In 1983, two experiments with *Drosophila melanogaster* males' onboard the Russian Salyut 6 orbital station showed an increased frequency of chromosome nondisjunction and recombination (Flatova et al., 1983). In 1995, the IML-2 spaceflight of female fruit flies resulted in a stimulation of oogenesis and a slightly delayed development compared to ground controls (Marco et al., 1995). Also, young male *Drosophila* flies showed acceleration in aging and an increase in locomotor activity. A decreased amount of mitochondrial 16S ribosomal RNA was found in the microgravity exposed flies, as well as an increase in food uptake suggesting an increase in metabolism (Benguria et al., 1996). Following another Space Shuttle flight with *Drosophila*, post mission analysis of sex-linked recessive lethal mutations was two to three times higher in flight than in ground control samples (Ikenaga et al., 1997). In a ground based experiment with *Drosophila,* Schneider S-1 cells grown in a clinostat, mitochondria abnormalities and clustering similar to space flown Jurkat cells were observed, indicating a microtubular dysfunction (Schatten et al., 2001).

In 2006, The NASA FIT experiment – Fungal Pathogenesis, Immunity and Tumorigenesis studies – was conducted to study the effects of the space

environment on higher organisms, of particular interest was immune system function. During a 12-day space flight *Drosophila melanogaster* fruit flies were allowed to complete all or part of their development in space (Fahlen et al., 2006). Currently, post mission analysis is still under way, but some of the first results are presented as part of this thesis. Another part of the FIT experiment involved ground based studies of the effects of proton irradiation on fruit flies. Ionizing radiation poses a threat to cells, tissues and organs potentially resulting in DNA damage or cancer. Human space travel away from Earth's protective magnetic field consequently results in a higher exposure of astronauts to ionizing space radiation. Results of these ground based studies with *Drosophila* are also part of this work. This approach will help to distinguish the multiple effects of the space environment on cells by exposure of model organisms to space radiation without the additional microgravity condition.

## *2.2. Apoptosis*

Heterophagic programmed cell death or apoptosis is an important mechanism in development and homeostasis of tissues for the removal of either superfluous, infected, transformed, or damaged cells by activation of an intrinsic suicide program. Maintaining an intact cell membrane during apoptosis allows adjacent cells to engulf dying cells, while avoiding the release of its contents, which would trigger a local inflammatory reaction. Characteristic morphologies of apoptotic cells include fragmentation of the cell into membrane-bound apoptotic bodies, nuclear and cytoplasmic condensation, and endolytic cleavage of the DNA. During embryonic development and metamorphosis in *Drosophila melanogaster*, apoptosis occurs in many cells to remove or remodel larval tissues and organs. Molecular characterization of mutants with defects in cell death has led to the identification of a region in the *Drosophila* genome (75C), which contains the three main initiators for apoptosis in *Drosophila*: Reaper, Head involution defective (Hid), and Grim. Independent regulation of these genes allows fine control of developmental apoptosis and activation of the subsequent caspases (Bangs et al., 2000). The Reaper gene encodes a protein that contains a putative sequence, the death domain, which appears to be essential for conferring the cell death-promoting activity of the Reaper protein. Similar death domains have been identified in vertebrate

proteins including the Tumour-necrosis-factor-receptor (TNFR) family. The Hid protein does not contain a death domain, but co-expression of Hid and Reaper results in increased cell death compared to Reaper alone, suggesting that Hid acts in synergy with Reaper to regulate *Drosophila* cell death. Grim appears to have a similar role like Hid, since it also lacks a death domain (Vernooy et al., 2000).

Apoptosis leads to the activation of a family of cysteine proteases known as caspases. The only family of proteins able to inhibit caspase activation or activity is IAP, inhibitors-of-apoptosis. IAPs contain a special motif known as a baculovirus-IAP-repeat (BIR) as well as a RING finger domain. The *Drosophila* genome encodes four BIRPs, including Diap-1, Diap-2, Deterin, and Bruce (Jones et al., 2000). For example, Diap-1 is bound and regulated by the main apoptosis initiator proteins forming a complex, which inactivates its functions (Goyal et al., 2000). IAP function is essential for cell survival, and its deregulation is observed in many forms of human cancer (Hay, 2000). A fine balance in regulating apoptosis activators and inhibitors is required for proper development, metamorphosis, aging and homeostasis in *Drosophila* (Yin and Thummel, 2004). Comparison of the apoptotic mechanisms in the fruit fly *Drosophila melanogaster*, the worm *Caenorhabditis elegans,* and vertebrates shows structural similarities in caspases, IAPs, IAP antagonists and caspase activators. However, differences in their functions have also been shown, for instance, the regulation of the apoptosome by cytochrome c and the role of the Bcl-2 family (Kornbluth and White, 2005).

### 2.2.1. The Caspase Family

Caspase proteases play important roles in the regulation of apoptotic cell death. They are synthesized as inactive zymogens, known as pro-caspases, and activated in response to death stimuli. They transform and amplify these signals by cleaving and thereby activating effector caspases. Caspases that act as signal transducers (apical or upstream caspases) have long pro-domains containing specific sequence motifs, death-effector-domains (DEDs), or caspase-recruitment-domains (CARDs). Some caspases also become activated as a consequence of pro-domain-dependent homodimerization. Once activated, long pro-domain caspases cleave and activate short pro-domain caspases, known as downstream or executioner caspases, that rely on cleavage by other caspases for activation (Vernooy et al., 2000).

*Drosophila* encodes three long pro-domain caspases, Dredd, Dronc (Dorstyn et al., 1999a), and Dream, as well as four caspases with short pro-domains, Dcp-1, Drice (Fraser et al., 1997), Decay (Dorstyn et al., 1999b), and Daydream. The initiator caspase Dronc cleaves and activates two short pro-domain caspases, Dcp-1 and Drice. Drice plays an important, non-redundant role as a cell death effector, whereas Dcp-1-null mutants show normally occurring cell death (Muro et al., 2006). In specific tissues the transcription of Dronc during programmed cell death is regulated by the hormone ecdysone (Kumar and Doumanis, 2000).

The apoptosome refers to the adaptor protein complex that mediates the activation of an initiator caspase. In mammalian cells, Caspase-9, Caspase-8, and Caspase-2 rely on the Apaf-1-apoptosome, DISC, and PIDDosome, respectively, for activation. In *Drosophila*, activation of the Caspase-9 homolog Dronc requires assembly of an apoptosome comprised of the proteins Dark, Hac-1 and dApaf-1. The induced conformation model suggests that the activated conformation of a given initiator caspase is attained through direct interaction with the apoptosome or through homo-oligomerization facilitated by the apoptosome (Bao and Shi, 2007).

Diap-1, *Drosophila*-inhibitor-of-apoptosis-protein-1 also referred to as Thread, a member of the inhibitor of apoptosis (IAP) family, is required to prevent excessive accumulation of the first continuously produced form of processed Dronc by ubiquitinating and thus, targeting it for proteasomal degradation. Pro-apoptotic signalling stimulates autoubiquitination of Diap-11 and frees Dronc to activate downstream caspases (Stafford, 2005; Olson et al., 2002).

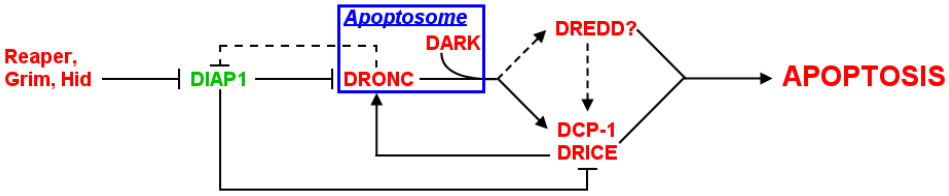


***Figure 2.1.*** The *Drosophila* apoptosis pathway. Red proteins are pro-apoptotic and green are anti-apoptotic. Dashed lines represent less well-established interactions. (Stafford et al., 2005)

Mitochondrial release of Cytochrome-c constitutes a pro-apoptotic output as part of the apoptosome complex. In mammals, a second protein, Apoptosis-inducing-factor (AIF), is released from mitochondria, which translocates from the mitochondria into the nucleus upon receipt of a death signal and causes large-scale fragmentation

of the DNA. The *Drosophila* homologue of AIF is CG7263 (Vernooy et al., 2000). The Bcl-2 family of proteins represents a group of major cell death regulators, directly engaging caspases through the apoptosome complex. *Drosophila* encodes two clear Bcl-2 family members, which are known as Debcl, a pro-apoptotic factor, and Buffy (Colussi et al., 2000; Brachmann et al., 2000), which exhibits a weak anti-apoptotic function. After cell death initiation, degradation of DNA occurs by the Caspase-activated-DNase, CAD (Inohara et al., 1998; Inohara et al., 1999; Yokoyama et al., 2000).

Caspases can also play important non-apoptotic roles, such as the differentiation of sensory organ precursors (Kuranaga et al., 2006), sperm individualization (Arama et al., 2003), border cell migration (Geisbrecht and Montell, 2004), and the morphology of sensory and tracheal cells (Oshima et al., 2006). Recent investigations in *Caenorhabditis elegans*, *Drosophila* and mice suggest that caspases also function as regulatory molecules for immunity and cell-fate determination. In the developing imaginal discs, a large number of cells undergo apoptosis and proliferation to regulate the organ size. Caspase activation in dying cells might induce the secretion of Wg or Dpp towards neighboring cells to increase their proliferation (Kuranaga and Miura, 2007).

| **Drosphila:** | | |
|---|---|---|
| **DCP-1** | Knockout | Larval lethality |
| **Dredd** | Loss of Function | Compromised immune response |
| **Dronc** | RNAi | Absence of PCD during development |
| **dBorg 1** | Knockout | Surplus glial cells |
| **Reaper** | Knockout | Absence of embryonic cell death |
| **d-IAP1, d-IAP2** | Over expression | Inhibit death induced by RHG proteins |

***Table 2.2.*** Apoptois involved proteins and the consequences of changing their gene expression (Twomey and McCarthy, 2005)

### 2.2.2. Minibrain, Morgue, Wengen

The three genes Minibrain, Morgue and Wengen are part of the apoptotic machinery in *Drosophila*. Microarray analysis by a colleague has indicated changes

in gene expression levels of space flown animals. Therefore, these three genes were further investigated in this study.

*Minibrain* is the founding member of the Dual-specificity tyrosine-phosphorylation-regulated kinases (DYRKs) family, which are involved in regulating key developmental and cellular processes such as neurogenesis (Fischbach and Heisenberg, 1984), cell proliferation, cytokinesis and cellular differentiation. Its human orthologue maps to the Down Syndrome critical region, belongs to the nuclear subclass and affects post-embryonic neurogenesis (Lochhead et al., 2003) According to Flybase, Minibrain is also involved in olfactory learning, circadian rhythm, visual behaviour (Tejedor et al., 1995), protein amino acid phosphorylation, and induction of apoptosis.

*Morgue* serves as an enhancer of grim-reaper-induced apoptosis by regulating ubiquitiniation processes. It encodes an F box and an ubiquitin E2 conjugase domain that lacks the active site required for ubiquitin linkage. Morgue can target Inhibitor-of-Apoptosis-Proteins (IAPs) for ubiquitination and proteasome-dependent turnover by acting either in an SCF ubiquitin E3 ligase complex, or as an ubiquitin E2 conjugase enzyme variant in conjunction with a catalytically active E2 conjugase (Wing et al., 2002).

*Wengen*, a member of the tumor necrosis factor (TNF) receptor family, is a type I membrane protein that can physically interact with dTraf2, an upstream activator of Dif and Relish. In mammals, the TNF family proteins play an important role in the regulation of cellular proliferation, differentiation and programmed cell death. Eiger is a type II membrane protein, which can be cleaved and released from the cell surface and bind to the receptor Wengen. Eiger and Wengen are expressed in distinctive patterns during embryogenesis and Eiger is also responsive to genotoxic stress. Overexpression of Eiger or Wengen causes apoptotic cell death (Kauppila et al., 2003).

### 2.2.3. p53

Although the p53 tumor suppressor protein was identified nearly three decades ago and plays a pivotal role in human cancer, its complexity continues to surprise the research community. p53 derives from a multigene family that also includes p63 and p73 (Kaghad et al. 1997; Schmale and Bamberger 1997). All three proteins express an array of isoforms as a result of multiple promoter usage and alternative splicing

adding further complexity to their role in development, cancer, and aging (Zaika et al. 2002; Mills, 2005; Bourdon et al. 2005).

The mammalian p53 protein functions as a tumor suppressor by controlling cell cycle progression and cell survival in response to genotoxic stresses like DNA damage and hypoxia. Cell cycle arrest occurs through induction of p21, which prevents entry into S phase by inhibiting G1 cyclin-dependent kinase activity. However, p21 is not required for p53-dependent apoptosis, but rather protects against it in some cell types. Induction of apoptosis is critical for the tumor suppressor function of p53 and initiated by transcriptional activation of pro-apoptotic genes Fas, IGF-BP3, and Bax as well as a set of genes that may promote apoptosis through the formation of reactive oxygen species (Ollmann et al., 2000).

*Drosophila's* Dmp53 is required for radiation-induced apoptosis, but not for normal levels of cell death that occur in the absence of DNA-damaging agents. It activates transcription of pro-apoptotic targets including Hid, Reaper, Sickle, and the Tumor-necrosis-factor-family member Eiger (Sogame et al., 2003; Brodsky et al., 2000; Brodsky et al., 2004). p53 and Chk2 also regulate DNA repair genes, including two components of the non-homologous end-joining repair pathway, Ku70 and Ku80. Additionally, Chk2- and p53-independent pathways can activate caspases and induce apoptosis in response to ionizing radiation (Wichmann et al., 2006).

p63 and p73 encode proteins with transactivation, DNA-binding, and tetramerization domains, and some isoforms are capable of transactivating p53 target genes and inducing apoptosis. The high level of sequence similarity between p63, p73 and p53 proteins, particularly in the DNA binding domain, allows p63 and p73 to transactivate p53-responsive genes causing cell cycle arrest and apoptosis. However, they are not functionally entirely redundant and the primary role of each p53 family member illustrates that each protein has its own unique functions (Murray-Zmijewski et al., 2006).

As cells undergo apoptosis, they are recognized and removed from the body by phagocytosis. This final step in the cell-death program protects tissues from exposure to the toxic contents of dying cells and also serves to prevent further tissue damage by stimulating production of anti-inflammatory cytokines and chemokines. The clearance of apoptotic-cell corpses is important for normal development during embryogenesis, the maintenance of normal tissue integrity and function, and the resolution of inflammation (deCathelineau and Henson, 2003).

## *2.3. Immune system*

Because it lacks an adaptive immune response, *Drosophila melanogaster* serves as a great model for studying aspects of the innate immune system, which is divided into cellular and humoral defense mechanisms. The cellular defense responses are carried out by three classes of circulating immune surveillance cells, called hemocytes: plasmatocytes, crystal cells, and lamellocytes.

Like humans, *Drosophila* protects itself against microbes and parasites via epithelial barriers, but once within the body cavity, microbes may be phagocytosed by plasmatocytes. Larger pathogens, such as eggs of parasitic wasps, are inactivated by encapsulation, carried out by lamellocytes (Govind and Nehm, 2004). Crystal cells participate in immune responses and wound healing through melanization. Plasmatocytes and crystal cells are found in all developmental stages, while lamellocytes have only been observed in larvae and increase during immune challenge (Milchanowski et al., 2004). Hemocyte-mediated defense responses are regulated by signaling factors and effector molecules that control cell adhesion and cytotoxicity (Lavine and Strand, 2002). The transcription factors GATA, Friend-of-GATA, and Runx family proteins, as well as the signal transduction pathways Toll/NF-κB, Serrate/Notch, and JAK/STAT, are required for specification and proliferation of blood cells during normal hematopoiesis, as well as during the hematopoietic proliferation (Evans et al. 2003; Meister et al., 2004).

Humoral defense mechanisms include the production of antimicrobial peptides, the cascades that regulate coagulation and melanization of hemolymph, and the production of reactive oxygen and nitrogen intermediates. Potentially damaging endogenous and/or exogenous challenges sensed by specific receptors initiate signals via the Toll and/or the Imd signaling pathway. Toll does not act as a pattern recognition receptor in *Drosophila*, but instead its activation depends on the presence of the processed, active form of the growth-factor-like polypeptide Spätzle. The effector NF-κB transcription factor of the Imd pathway is Relish, which upon immune activation is cleaved by the Dredd Caspase (Elrod-Erickson et al., 2000). Sickle is also required for Relish activation, while Defense-repressor-1 acts as an inhibitor of the Dredd caspase. Transcription factors Dorsal, Dorsal-related immune factor Dif, and Relish activate genes that are involved in the production of

antimicrobial peptides, melanization, phagocytosis, and the cytoskeletal rearrangement required for appropriate responses (Kim and Kim, 2005).

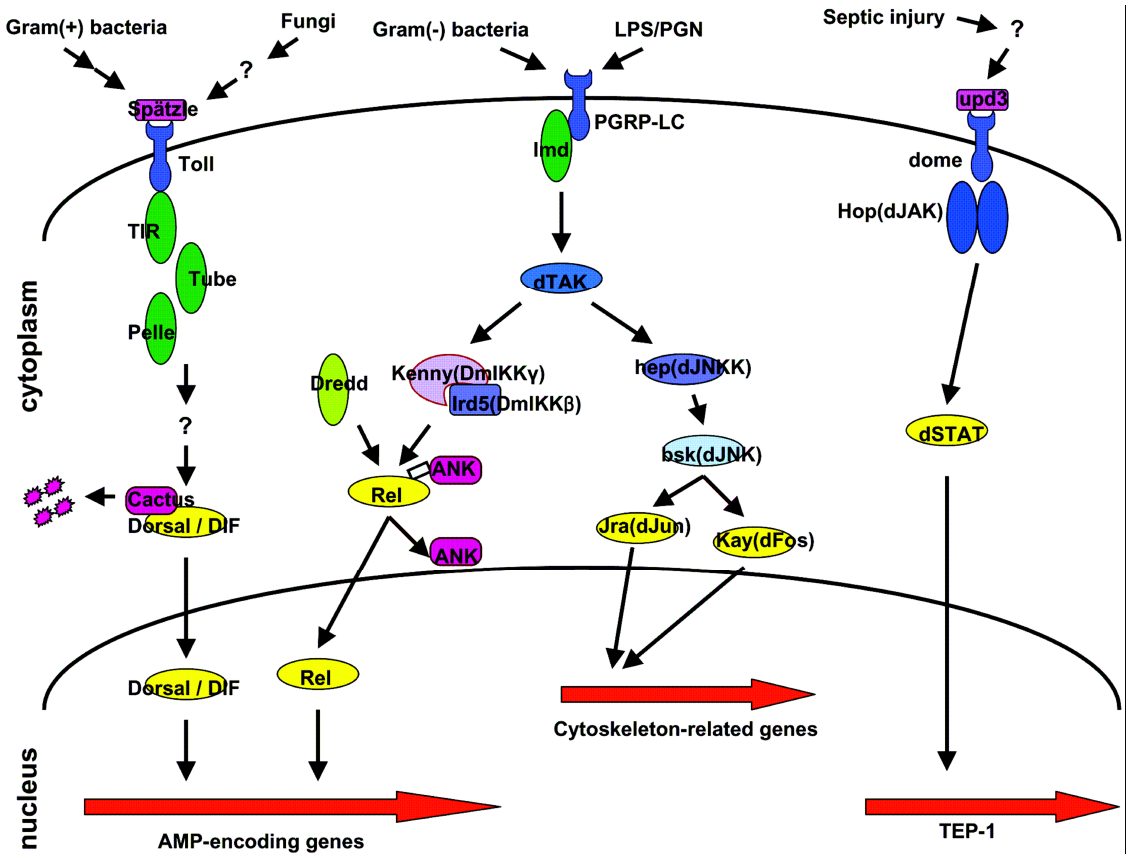


***Figure 2.2.*** The Toll, Imd and JAK-STAT pathways controlling the expression of genes related to physiological responses to infection. Spätzle is processed by a serine protease cascade upon infection and binds to Toll. This activates an intracellular signalling cascade culminating in degradation of Cactus and nuclear translocation of the NF-κB-like transcription factors, Dorsal and Dif. The Imd pathway branches into the JNK and IKK signalling modules at dTAK1. Relish, phosphorylated by the IKK complex, is cleaved to a smaller, active form with transactivating activity in the nucleus. The JNK pathway leads to activation of dAP1 (Kim and Kim 2005).

# Objectives

The aim of this work was to extend fundamental space life sciences research in apoptosis and immune system adaptations in response to space flight conditions and ground based proton irradiation. Studying gene expression of apoptosis relevant proteins, activity measurement of caspases and measurement of phagocytotic activity were used to characterize the differences between space flown, proton irradiated and ground control *Drosophila melanogaster* flies. It was also of interest, if an altered level of apoptosis could be linked to a change in phagocytotic activity of hemocytes.

# III. MATERIALS AND METHODS

## *3.1. Common materials and fly lab methods*

### 3.1.1. Chemicals

All chemicals were of analytical or molecular biology grade.

### 3.1.2. Common Solutions

PBS (10x)

| | |
|---|---|
| NaCl | 81.8 g |
| KCl | 2.01 g |
| $KH_2PO_4$ | 2.04 g |
| $K_2HPO_4$ | 11.32 g |
| pH 7.5 | |
| $dH_2O$ to 1000 ml | |

Tegosept

10% Nipagin (Methyl-4-hydroxy benzoate)
in 95% Ethanol.

## *3.2. Common Drosophila laboratory methods*

### 3.2.1. Fly food

**Dextrose medium**

| | |
|---|---|
| Torula yeast | 64 g |
| Cornmeal | 122 g |
| Agar | 18.6 g |
| Dextrose | 258 g |
| Tegosept | 40 ml |

All solid components were mixed in 2000 ml distilled water and heated on a hotplate while being stirred with a magnetic stirring bar. After boiling for about 5

minutes, the mixture was allowed to cool to 60℃. 4 0 ml of the antifungal agent Tegosept were added to the food, mixed, and poured either into bottles or vials, which were closed with cotton or foam plugs. The dextrose food was used as standard food for most assays.

**Semi-defined or blue food**

| | |
|---|---|
| Agar | 2 g |
| Brewer's yeast | 16 g |
| Yeast extract | 4 g |
| Peptone | 4 g |
| Sucrose | 6 g |
| Glucose | 12 g |
| $MgSO_4$ | 0.1 g |
| $CaCl_2$ | 0.1 g |
| Propionic acid | 1.2 ml |
| 10% p-hydroxybenzoate in 95% ethanol | 2.0 ml |
| Blue food dye | 1.0 ml |

All solid components were mixed in 2000 ml distilled water and heated on a hotplate while being stirred with a magnetic stirring bar. After boiling for about five minutes, the mixture was allowed to cool to 60℃. 1 .2 ml of propionic acid, 2 ml of 10% p-hydroxybenzoate and 1 ml of food dye were mixed with the food and poured either into bottles or vials, which were closed with cotton or foam plugs. Late 3$^{rd}$ instar larvae will be distinguished from earlier 3$^{rd}$ instar larvae by the absence of blue food dye in the gut. Late 3$^{rd}$ instar larvae stop feeding and wander for about 12 hours before pupating and the food containing the visible blue dye is cleared from the gut after about 6 hours, thus making it possible to distinguish 3$^{rd}$ instar larvae that are close to pupation from earlier 3$^{rd}$ instar larvae.

### 3.2.2. Keeping stocks

Mucus-producing bacteria, mites and molds belong to the worst enemies of *Drosophila* stocks. To avoid contamination and overcrowded cultures only about 20 or so flies per vial were transferred, or flipped, on a regular schedule into a new vial containing fresh food. Stocks, that were not in everyday use, were stored

at 18ºC on a 4-5-week generation cycle. Inspection of the flies on transfer was necessary to ensure that both sexes were present and that their phenotype was as expected.

### 3.2.3 Egg collection

Two to four day old flies were allowed to lay eggs on apple juice agar plates containing some yeast paste. Eggs were washed off the plates with distilled water into a filter, transferred onto a microscope glass slide and counted under the dissection microscope. The desired number of eggs was collected with a piece of filter paper and put onto food in a vial.

### 3.2.4 Virgin collection

Virgin collection was performed to collect females that had not mated with a male, which was important for experiments, where the animals need to be developed to specific stages at a certain time point like in the irradiation procedure. Although variations between stocks exist, the general rule is that females will not accept a male mate until they are 10-12 hours old (i.e., after eclosion from the pupa). Thus, flies were collected between 8 and 10 hr after eclosion, anesthetized, separated into males and females, and stored until needed.

### 3.2.5 Sexing flies

Sorting flies by their gender was needed for most assays. As well, the set up for crosses required the sexing of flies. Males and females were distinguished from each other by three main rules.
Only males have a sex comb, a fringe of black bristles on the forelegs. The tip of the abdomen is elongate and somewhat pointed in females and more rounded in males. The abdomen of the female has seven segments, whereas that of the male has only five segments.

### 3.2.6 Line expansion

To increase the number of flies of a certain strain to conduct experiments, these animals were transferred into bottles. The egg-laying can be accelerated by adding some yeast paste to the food. Storing the flies at 25℃ and flipping them

into new bottles every one or two days resulted in the fast expansion in the number of flies.

### *3.3. Irradiation setup*

On three separate occasions fruit flies were irradiated at a synchrotron housed at the Loma Linda University Medical Center in California. This proton irradiation facility is primarily used to treat cancer patients, however, is also available for animal research, and was used in this study. Vials of flies were transported by car to and from Loma Linda. The facility was used for proton irradiation with an energy of 250 MeV, a proton rate of about 1 Gy/minute and proton dosages between 0 to 60 Gy, respectively. Embryos were collected and irradiated on apple juice plates, while larvae up to 3rd instar, grown in polystyrene vials, were exposed in a vertical position, parallel to the proton path. Wandering late 3rd instar larvae and pupae were irradiated in vials set in horizontal position.

***Figure 3.1***. Proton irradiation setup at Loma Linda University Medical Center

The following table presents the necessary timing for preparation prior to irradiation. This setup was used to expose animals at different developmental stages depending on the desired assay.

| | hrs | STAGE | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Wed 08 | 0 | Eggs | | | | | | |
| Thu 09 | 24 | 1sts | Eggs | | | | | |
| Fri 10 | 48 | 2nds | 1sts | Eggs | | | | |
| Sat 11 | 72 | 3rds | 2nds | 1sts | Eggs | | | |
| Sun 12 | 96 | 3rds | 3rds | 2nds | 1sts | Eggs | | |
| Mon 13 | 120 | Pupae | 3rds | 3rds | 2nds | 1sts | Eggs-11pm | |
| Tue 14 *2-6am | | Pupae | Pupae | 3rds | 3rds | 2nds | 1sts | Eggs-11pm |
| Wed 15 | | Pupae | Pupae | Pupae | 3rds | 3rds | 2nds | 1sts |
| Thu 16 | | Pupae | Pupae | Pupae | Pupae | 3rds | 3rds | 2nds |
| Fri 17 | | Pupae | Pupae | Pupae | Pupae | Pupae | 3rds | 3rds |
| Sat 18 | | Adults | Pupae | Pupae | Pupae | Pupae | Pupae | 3rds |
| Sun 20 | | | Adults | Pupae | Pupae | Pupae | Pupae | Pupae |
| Mon 21 | | | | Adults | Pupae | Pupae | Pupae | Pupae |
| Tue 22 | | | | | Adults | Pupae | Pupae | Pupae |
| Wed 23 | | | | | | Adults | Pupae | Pupae |
| Thu 24 | | | | | | | Adults | Pupae |
| Fri 25 | | | | | | | | Adults |
| AGE AT IRRADIATION 11/14-4am 10,20,30Gy | | 0-24 hour-old pupae | wandering 3rds, with food in the gut | early 3rds, still crawling in food | 2nds | 1sts | Eggs | |
| AGE AT IRRADIATION 11/15-4am 40, 60Gy | | 24-48 hour-old pupae | 0-24 hour-old pupae | wandering 3rds, with food in the gut | early 3rds, still crawling in food | 2nds | 1sts | Eggs |

***Table. 3.1.*** Setup for preparation, timing and developmental stage of animals used in irradiation experiments. 1st, 2nd, 3rd corresponds to the respective larval instar stage.

## *3.4. Nucleic Acid Methods*

### 3.4.1. RNA isolation from adult flies

RNA extraction from adults was performed following the Qiagen RNeasy Mini Handbook 06/2001. Three to four flies were put into an Eppendorf tube containing 350 µl RLT-buffer (see protocol, contains guanidine thiocyanate) and 3.5 µl betamercaptoethanol. Using sterile, RNase-free pestles and a battery powered homogenizer the prepared flies were homogenized by grinding them up against the tube inside and the following solution spun done in microfuge at 11000g for 3 minutes. The supernatant was transferred to a new tube, about 300 µl 70 % ethanol was added, mixed and the solution transferred to a column tube, which was spun for 30 seconds at 8000g. The resulting elute was discarded and 700 µl of RW1-buffer (see protocol, contains ethanol) were added to the column. Spinning was repeated and the column was transferred onto a new tube. To wash the column 500 µl RPE-buffer (see protocol) was added twice and removed by spinning at 8000g for 30 seconds. The last spinning step was performed to dry

the filter and the RNA is eluted out of the column into a fresh tube by adding 50ul of RNase-free water and spinning at 8000g for 1 minute.

### 3.4.2. cDNA from RNA

Master Mix cDNA Archive Kit: 1x

| | |
|---|---|
| 10X Reverse transcription Buffer | 10 µl |
| 25X dNTPs | 4 µl |
| 10X random primers | 10µl |
| MultiScribe™ Reverse Transcriptase, 50 U/µL | 5 µl |
| Nuclease-free $H_2O$ | 21µl |
| Total Volume per Reaction | 50 µl |

The High-Capacity cDNA Archive Kit from Applied Biosystems was used for reverse transcription of total extracted RNA to single-stranded cDNA, which was subsequently needed for Real-Time-Polymerase Chain Reaction (RT-PCR). First, a master mix depending on the number of samples was prepared. Next, 50 µl of extracted RNA were added to 50 µl master mix in a PCR tube and the following program was run in a thermal cycler: 10 minutes at 25℃, 2 hours at 37℃. The resulting cDNA product was either stored at 4℃ for immediate use or at -20℃ for long duration storage.

### 3.4.3. Quantification of cDNA concentration

cDNA concentration was measured via optical density (OD) at a wavelength of 260 nm. 2 µl of the respective sample were added to 98 µl DNase-free water, transferred into a quartz cuvette and the absorbance at 260nm was determined using a spectrophotometer. cDNA concentration was calculated using the conversion factor, OD260 = 1 => 50 µg/ml, and the respective sample was diluted to the desired concentration.

### 3.4.3. Real-Time Polymerase Chain Reaction – RT-PCR

DNA samples for RT-PCR were obtained according to 3.4.1 and 3.4.2. The reaction was performed following Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Manual (www.appliedbiosystems.com).

Master Mix for genes of interest: *wengen*, *minibrain* and *CG1887* 1x

| | |
|---|---|
| Taqman Mix | 12.5 µl |
| Forward-Primer Ribo | 0.225 µl |
| Reverse-Primer Ribo | 0.225 µl |
| Probe Ribo | 0.0625 µl |
| Primer/Probe | 1 µl |
| RNase free $H_20$ | 6 µl |
| cDNA 1µg/µl | 5 µl |

Master Mix for gene of interest: *morgue* 1x

| | |
|---|---|
| Taqman Mix | 12.5 µl |
| Forward-Primer Ribo | 0.225 µl |
| Reverse-Primer Ribo | 0.225 µl |
| Probe Ribo | 0.0625 µl |
| Forward-Primer morgue | 0.225 µl |
| Reverse-Primer morrgue | 0.225 µl |
| Probe morgue | 0.0625 µl |
| RNase free $H_20$ | 6 µl |
| cDNA 1µg/µl | 5 µl |

Samples were diluted to the desired cDNA concentration of 1 µg/µl and used for RT- PCR analysis. A master mix was prepared depending on the number of samples as well as on the gene of interest and loaded onto a 96-well RT-PCR plate. Taqman mix refers to the TAqMan Universal PCR Master Mix by Applied Biosystems. This master mix also includes the primer/probe set for a ribosomal protein, RpS15Ab-RA, which serves as an internal control and is need for normalization of the sample results.

ATAAGTAATTTGACATTTTCATTAATTAGTTTTGAACTACATCTAAGAGAAGTACTTACGGAAGTTTTCAAGTAAAGGTTT
TTTCCCTTTTTCACGTTTGCGTGACGGTCGTGTAAAACAGTTTTGGCAAACAAATCCAGCTATGGTGCGTATGAACGTA
TTGGCCGATGCCCTGAAGTGCATAAACAACGCCGAGAAGCGTGGCAAGCGGCAGGTGCTGCTGCGTCCCTGCTCCA
AGGTGATCATCAAGTTCCTGACCGTGATGATGAAGCATGGCTATATCGGCGAATTCGAGATCGTCGAGGATCACCGT
GCCGGCAAGATCGTTGTCAACCTGACCGGTCGGCTAAACAAGTGCGGCGTCATCTCGCCCCGCTTCGATGCGCCCA
TCAACGACATCGAGAAGTGGACCAACAATCTGTTGCCCTCGCGTCAGTTTGGTTACGTTGTGCTCACCACCTCTGGCG
GCATCATGGACCACGAGGAGGCTAGGAGAAAACATTTGGGAGGCAAAATTCTCGGCTTCTTCTTCTAGAGACACCAA
GTTCACATCGTAGAGGGATGGAGTTTGATATGTAGATTGACGTTTTATTTCACCGACCATGCATCCAACTATGTACTTT
GTGCACAGGAAACAAAGGGCGAAAACGCTACTACGTTCATTCAGAAAACAAACGACGATGAATGTTCACAAAGTTACG
CGAAATAAAATGAAAAATTTGGACATCTAATATCGAAA

DNA Sequence of the ribosomal Protein RpS15Ab-RA

cDNA was transferred into the respective wells either with a single or a multi-channel pipette. The plate was loaded onto the Real-Time 7500 PCR System machine, Applied Biosystems, and the following amplification program was run:

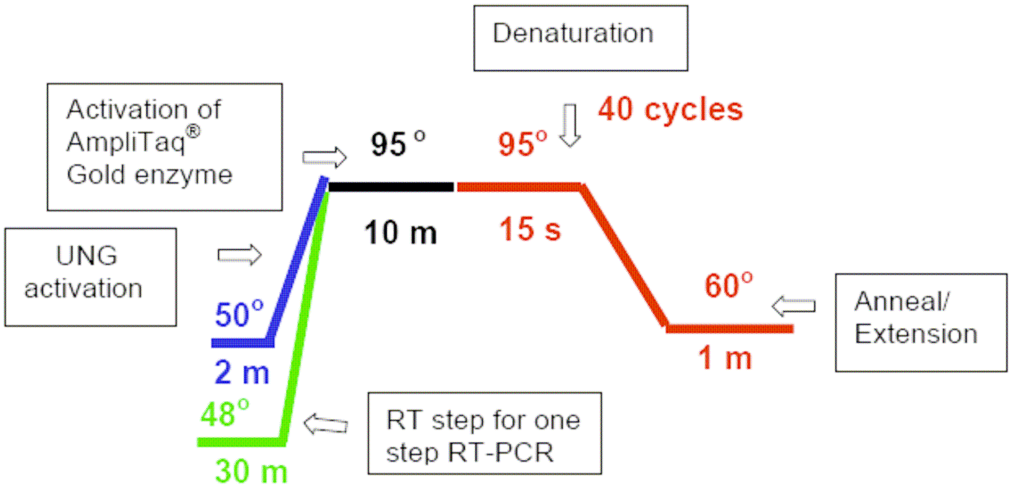


***Figure 3.2.:*** by Applied Biosystems Applied Biosystems Real-Time PCR Rapid Assay Development Guidelines. Publication 117GU16-01.

After the run was completed, the Ct values for the gene of interest and the internal control were exported into an Excel spread sheet for analysis. The Comparative Ct Method or $2^{-\Delta\Delta}$Ct Method has been used to calculate and visualize the changes in gene expression in between different samples or treatments (Livak et al 2001). For the $2^{-\Delta\Delta}$Ct Method, first, the Ct- value of the internal control is subtracted from the Ct-value of the gene of interest. Next, this ΔCt of the untreated sample is subtracted from the ΔCt of the treated sample. This ΔΔCt value is used to calculate the $2^{-\Delta\Delta}$Ct, which represents the fold change of the gene of interest between the treated and untreated sample. The values between 0 and 1 need to be converted for a simplified visulaisation, since they represent negative fold changes. For instance, a fold change of 0.5 can also be expressed as a fold change of –2.

For the herein presented experiments expression changes of specific genes in response to a bacterial infection in a flight and a ground samples were analysed. Therefore, the $2^{-\Delta\Delta}$Ct Method was used to show the difference of an infected flight sample to a PBS infected flight sample and similar calculation was

conducted for the ground sample. The two data points were plotted as fold changes next to each other for direct comparison.

## 3.5. Protein Methods

### 3.5.1. Fatbody Dissection and Protein Extraction

CAB= Caspase Assay Buffer

| | |
|---|---|
| HEPES-KOH pH 7.5 | 20 mM |
| KCl | 10 mM |
| $MgCl_2$ | 1.5 mM |
| Sodium EDTA | 1 mM |
| Triton X-100 | 0.5 % |
| PMSF | 2 mM |

(PMSF = **p**henyl**m**ethyl**s**ulphonyl **f**luoride)

Six wandering 3rd instar larvae grown in blue food were rinsed in water and placed on a microscope slide, which was sitting on the lid of a bench-top-cooler to maintain constant low temperature during dissection. A few drops of CAB were added to the slide, the bench-top-cooler-lid placed under a Leica MZ 125 dissection microscope, and the posterior tip was cut open to extract all organs of the larva. Carefully the fatbodies of all six larvae were separated and collected in an Eppendorf tube containing 100 µl cold CAB. Using a cold sterile pestle the fatbodies were homogenized and spun down for 5 minutes at 12000 g and 4℃. The supernatant was removed to a fresh tube and stored on ice for immediate use or in the -80℃ freezer.

### 3.5.2. Caspase Enzymatic Activity Measurement

Caspase reaction mix

| | |
|---|---|
| CAB | 85 µl |
| Protein lysate | 10 µl |
| Substrate | 5 µl |

Negative control

| | |
|---|---|
| CAB | 95 µl |
| Substrate | 5 µl |

Caspase activity was assayed using the chromophore para-Nitroaniline (pNA) attached to the substrate sequence. Cleavage of the substrate by the caspase results in the release of the chromogen, which can be monitored as a change in absorbance at 405 nm.

The frozen substrate obtained from the “Calbiochem Caspase Substrate Set III, colorimetric Kit” was warmed to RT to be liquid and ready for the assay. 10 µl of protein lysate obtained from the fatbody dissections contains about 30-40 µg protein, which was sufficient for a good signal. CAB and lysate were added to a well on a 96-well plate, followed by the substrate, which activates the enzymatic reaction. After brief mixing the plate-reader was started to execute the following program:

Platereader measurements at OD405:

| Intervall | Repeats | Elapsed time |
|---|---|---|
| 10 sec | 12 | 2 min |
| 30 sec | 56 | 30 min |
| 1 min | 30 | 1 h |
| 10 min | 3 | 1 h 30 min |

In all negative control runs no change in absorbance was observed, therefore no subtraction of negative control values from measured sample data was necessary. Each supernatant obtained from one fatbody dissection contained about 80 µl protein lysate. 70 µl were needed to test each of the seven Caspase substrates in a separate reaction. The remaining 10 µl were use to measure the protein concentration.

### 3.5.3. Protein concentration

Protein concentration was measured following the QuantiPro BCA Assay protocol by Sigma. First, a Bovine Serum Albumin (BSA) working solution with a concentration of 50µg/ml BSA was prepared. QuantiPro Buffer QA and QuantiPro Buffer QB were mixed with Copper(II) sulfate solution according to the protocol to produce the QuantiPro Working Reagent, which needs to be freshly prepared before every use. The protein samples obtained from larval fatbody dissections were diluted 1:250 in 250µl PBS. The BSA working solution is used to prepare six standards with protein concentrations 0, 1, 2, 5, 10 and 20 µg/ml, respectively, in PBS. All samples and standards were mixed 1:1 with the QuantiPro Working Reagent and incubated at 60℃ for 1 hour. The tubes were allowed to acclimate to room temperature and absorbance of the reactions was measured at 562nm. The values for the standards were plotted in Excel and a linear regression served as a standard curve. The equation of this regression was used to determine the absolute protein concentration in the samples.

## *3.6. Immunohistochemistry*

### 3.6.1. Fixing of embryos

Fixative solution

| | |
|---|---|
| EGTA (pH 7.4) | 50 mM |
| Formaldehyde | 4 % |
| In PBS | |

*Drosophila* embryos were washed in distilled water, dechorionated for 2.5 minutes in 50% bleach (v/v in water) and rinsed thoroughly with water again. The fixative solution was mixed 1:1 with heptane and about 1.5-2 ml was added to a screw-top glass tubes. The embryos were fixed in these glass tubes for 20-30 minutes while being gentle shaken. Then, the top aqueous layer was removed leaving the embryos in the interphase. One volume of methanol was added and devitellinization of embryos performed by strong shaking. Successfully devitellinized embryos were sunken to the bottom of the tube. The heptane and methanol were aspirated and and the embryos washed with four to five times with

methanol. In this condition the embryos can be stored at -20℃ in methanol or ethanol for at least one year.

### 3.6.2. Antibodies

All antibodies purchased at Santa Cruz Biotechnologies.

PBT

| | |
|---|---|
| Triton X-100 | 0.1% |
| In PBS | |

PBT + BSA

| | |
|---|---|
| BSA | 2% |
| In PBT | |

Antibody staining against GFP is necessary, since the fixative solution used in 5.6.1 quenches the individual fluorescence of the GF protein. The first antibody binds against GFP and the secondary antibody against the primary to re-establish fluorescence. The embryos were rehydrated in a 1:1 PBT and methanol solution for 15 minutes and another 15 minutes in pure PBT. Blocking was performed in PBT + BSA for 1-2 hours on the rocker. The incubation with the first antibody was conducted over night at 4℃ and was followed by an intense washing step with PBT for 1 hour the next day. Incubation with the second antibody was performed for 2 hours at 4℃, as well as covered from light t o protect the fluorescence of the secondary antibody. The same washing step was conducted as after the first antibody incubation and mounting the embryos in propidium iodide on a microscope glass slide resulted in an additional DNA staining. The embryos were sealed with a coverslip and kept at 4℃ in the dark until analysis with the fluorescence microscope.

### 3.6.3. TUNEL

PPP solution

| | |
|---|---|
| Phenylthiourea | 1 crystal (~1mg) |
| PMSF | 1mM |
| PBS | 600 µl |

Fixation solution

| | |
|---|---|
| PPP | 600 µl |
| Formaldehyde | 16% |

Permeabilisation solution

| | |
|---|---|
| Triton X-100 | 10% |
| Na citrate | 10% |
| $dH_2O$ | |

TUNEL mix (by Roche Applied Sciences)

| | |
|---|---|
| Enzyme solution | 5 µl |
| Label solution | 45 µl |

DNA staining solution

| | |
|---|---|
| DAPI | 1.5 µg/ml |
| In Vectashield | |

The TUNEL kit protocol (Manual Version June 2005) and solutions used in this experiment were purchased from Roche Applied Sciences; In Situ Cell Death Detection Kit, TMR red. First, 3rd instar larvae were washed with PBS in a 9-well plate. Three to four larvae were bled per well on a 10-well slide, which was prepared in a wet-chamber (pertidish with wet kimwipes). Bleeding was conducted by peeling back the cuticle of a larva with a pair of forceps into 30 µl of PPP prepared in the well. Within 15-30 minutes the cells adhere to the surface of the slide. 30-50 µl of the freshly made fixation solution is added to the well after removal of the PPP. After 1 hour of incubation the wells were rinsed two times with PBS and the slide was put directly on ice. 50 µl of permebilisation solution were added to the well for exactly 2 minutes. To stop the reaction the well was rinsed three times with PBS and the slide put back into the wetchamber. The area around the well was dried and tilting slide further removed PBS in the well. 5 µl Tunel mix were added to the well, which was covered with parafilm and aluminium foil and incubated at 37℃ for 15-20 minu tes. Finally, the wells were washed with PBS, each mounted with a drop of Vectashield solution containing DAPI for DNA staining and stored at 4℃ in the dark until analysis with the fluorescence microscope.

## *3.7. Infection methods*

### 3.7.1. Preparation of bacterial culture

Luria broth liquid medium (LB medium)

| | |
|---|---|
| LB Broth BP 1427-500 by Fisher | 20g |
| $dH_2O$ | 1L |

Luria broth plates (LB plates)

| | |
|---|---|
| LB Agar BP1425-500 by Fisher | 40g |
| $dH_2O$ | 1L |

For preparation of Luria Broth plates and liquid medium the solid components were mix, stirred and then autoclaved in distilled water for 30 minutes. Medium for the plates was allowed to cool to 55℃ before streptomycin was added. For the liquid medium streptomycin (Sigma) was added at room temperature.

Inoculation of a single colony of *E.coli* strain HB101 from an agar plate or glycerol stock into 5 ml of LB containing 5 µl of 50 mg/ml streptomycin solution was conducted under sterile conditions in fume hood.

Bacteria were grown overnight at 37℃ with vigorous shaking. On the next day the bacterial culture is transferred to a 15 ml tube and pelleted by centrifugation. The supernatant was discarded and the cell-pellet resuspended in 5 ml PBS. This washing step was repeated and 50 µl of the remaining cell suspension were diluted with 950 µl of PBS to determine the optical density at 600 nm wavelength. Using the conversion factor, OD600 = 1 => 1 x $10^9$ cells/ml, the concentration of the original bacterial culture in PBS was calculated and diluted to the desired concentration.

### 3.7.2. Storage of bacterial culture

Glycerol stocks were prepared for long term storage of E.coli bacteria at –80℃. Therefore, 5 ml LB media (see 3.7.1.) were in oculated from a single colony and incubated over night at 37℃ with vigorous shak ing. 800 µl of these growing E.coli culture were transferred to a sterile screw-cap tube and 200 µl of 80% autoclaved glycerol added. After brief vortexing the stock was stored at -80℃. To

grow cells out of this stock, do not thaw it but rather touch the surface with a sterile tip and place it into 5 ml of LB medium.

### 3.7.3. Clearance Assay

LB + 1% Trition 100

The infection protocol for adult *Drosophila* flies was adapted from Dionne et al., 2002. 12 female flies per individual time point and a bacterial solution of E.coli HB101 (5.7.1.) with a concentration of 2 x $10^8$ cells/ml were required. First, the injection volume of a pulled glass needle attached to the picospritzer injection system was calibrated. Using a vaccum set at 60 psi approximately 5 µl of the bacterial solution were drawn up into the needle. In the field of view under a dissecting the tip was placed into a small Petri dish containing hydrocarbon oil and a bubble is injected into the oil. By adjusting the injection time a sphere of 457 µl in diameter was created containing a volume of 50nl. The calibrated needle was used to inject CO2 anaesthetized flies with the bacterial solution as well as control flies with PBS. The flies were moved to fresh vials and allowed to recover. After 3-6 hours three septic and three antiseptic flies were homogenized in separate Eppendorf tubes that contained 200 µl of LB + 1% Trition 100. Proper dilutions were plated onto streptomycin LB- agar plates and incubated over night at 37℃. The grown colonies were counted and calcul ated back to remaining bacteria per fly. 24 and 72 hours after infection the homogenization and plating steps were representing two additional timepoints. As further controls, uninfected flies were used at least once to insure the absence of contamination. The actual amount of injected bacteria was tested by direct inoculation of the LB medium with the calibrated needle and picospritzer.

### 3.7.4. Alexa Fluor E.coli Phagocytosis Assay

Schneider's Culture Medium with FBS

Fetal Bovine Serum (FBS) 12%

The following protocol was developed and tested in this lab. Three siliconized 1.5 ml Eppendorf tubes were prepared with 350 µl chilled Schneider's

Culture Medium with FBS. As well, 50 µl chilled Schneider's Culture Medium was added to one well of a 9-well Pyrex dish, where five larvae were bled into respectively. 50 µl of this mix was transferred into one of the prepared Eppendorf tubes. These steps were continued two more times and all tubes placed on a rocker in a 25℃ incubator for 20 minutes. 0.5 µl of an Alexa Fluor 594-prelabelled bacterial suspension purchased from Invitrogen containing about 109cells/ml was added to the first Eppendorf tube, which was labelled as the 45-minute time point, and returned to the rocker. 20 minutes later the second tube was infected, labelled as 25 minute time point and returned to the rocker. Another 20 minutes later the last tube was treated the same and labelled as 5 minute time point. Five minutes after treatment of the last tube 100 µl Trypan Blue were added to all Eppendorf tubes, which were returned to the rocker for another 45 minutes. Next, two times 100 µl aliquots of each tube were transferred to a single well of a Teflon coated glass slide, where the hemocytes adhered to for 30 minutes, while being protected from light. 200 µl were aspirated from each well and discarded. Gently, the cells were washed with 10 µl PBS and 5 µl 0.2% Trypan Blue in PBS were added to each well quenching any extracellular Alexa Fluor labelled E.coli bacteria. Finally, a coverslip was placed over the slide and images for analysis were taken under the fluorescence microscope.

### 3.8. Behavioural study – Climbing assay

A 10 cm high, clear, plastic-vial containing 10-15 female flies was set up in front of a digital camera and next to an upright ruler. The vial was hit with the bottom onto the desk to return all moving flies back onto the food. 10 seconds later a picture of the vial is taken next to the ruler focusing on the flies. Subsequent image analysis was performed to determine how many flies travelled how far up the vial within the 10 seconds after the bump.

### *3.9. Fluorescence Microscopy and Data analysis*

Specimens were viewed in a Zeiss Axiophot 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Digital colour or black and white photographs were taken using an Optronics microscope camera, and the Magnafire Application Software 2.0 (Optronics®). All digital images were processed using the Image Pro Version 6 and Adobe Photoshop Version 8.0 (Adobe Systems Inc.). The data obtained from all experiments was processed and analysed with Microsoft Excel and SAS JMP 6 (www.jmp.com).

# IV. RESULTS

## *4.1. Gene expression analysis following space flight*

*Drosophila melanogaster* strain *Hml∆-GAL4, UAS-eGFP, UAS-eGFP/ Hml∆-GAL4, UAS-eGFP, UAS-eGFP* (Sinenko and Mathey-Prevot, 2004), herein referred to as HML, was used for a 12-day Space Shuttle flight experiment. One aspect of the post-flight-analysis involved the characterization of gene expression differences in response to a bacterial infection performed with space flown and ground control animals. RNA for microarrays and quantitative Real-Time-PCR was extracted on three different days after landing of the Shuttle. Flies of day one after landing have completed development and eclosed in space. They contain animals of mixed ages including virgins and non-virgins, but only females were used for the infection and RNA isolation. Day three flies returned as pupae and eclosed three days after landing. Only virgin females were selected for further processing. The last group of flies spent most of their pupae stage on Earth, eclosed five days after landing and was handled like the day three flies. Except for negative controls, all flies being used for RNA extraction were infected with *E.coli* HB 101 bacteria according to the Clearance Assay protocol (3.7.2.). The different immune responses following space flight were shown by comparison of bacterial with PBS (3.1.2) infected flies, which served as negative controls to compensate for the wound healing response triggered by the infection needle.

| | | E.coli | | PBS | |
|---|---|---|---|---|---|
| | | Flight | Ground | Flight | Ground |
| DAY1 | time 0 | FE 1- 0 | GE 1- 0 | FP 1- 0 | GP 1- 0 |
| | time 1.5 | FE 1- 1.5 | GE 1- 1.5 | FP 1- 1.5 | GP 1- 1.5 |
| | time 4 | FE 1- 4 | GE 1- 4 | FP 1- 4 | GP 1- 4 |
| | | | | | |
| DAY3 | time 0 | FE 3- 0 | GE 3- 0 | FP 3- 0 | GP 3- 0 |
| | time 1.5 | FE 3- 1.5 | GE 3- 1.5 | FP 3- 1.5 | GP 3- 1.5 |
| | time 4 | FE 3- 4 | GE 3- 4 | FP 3- 4 | GP 3- 4 |
| | | | | | |
| DAY5 | time 0 | FE 5- 0 | GE 2- 0 | FP 5- 0 | GP 2- 0 |
| | time 1.5 | FE 5- 1.5 | GE 2- 1.5 | FP 5- 1.5 | GP 2- 1.5 |
| | time 4 | FE 5 - 4 | GE 2- 4 | FP 5- 4 | GP 2- 4 |

***Table 4.1.*** Samples from flight and ground control and the respective day after landing. *E.coli* column shows flies that were infected after landing. Control flies were infected with PBS. The time next to the day indicates the hours after infection that RNA was extracted. FE = Flight E.coli; GE = Ground E.coli; FP = Flight PBS; GP = Ground PBS. Numbers = day – timepoint.

The microarray experiments were conducted at Stanford University and analysed by a member of this group. In support of this analysis, gene lists were prepared to highlight the genes and pathways of interest. These gene lists were produced using Flybase (http://flybase.bio.indiana.edu/), an internet platform for *Drosophila* research, and additional publications about the respective pathways of interest (see Introduction).

| Symbol | FlyBase_ID | Full_name | Synonyms |
|---|---|---|---|
| skl | FBgn0036786 | sickle | CG13701; CG13701; CG13701; CG13701; CG13701; Skl; Sickle; veto; BcDNA:RE14076; meph: mephisto; mephisto |
| rpr | FBgn0011706 | reaper | CG4319; CG4319; CG4319; Rpr; rp; Reaper L; RPR; anon-WO0162936.19; reaper |
| grim | FBgn0015946 | grim | CG4345; Grim; BcDNA:RE28551; grim |
| corp | FBgn0030028 | companion of reaper | CG10965; CG10965 |
| dar | FBgn0082821 | defender against reaper | Defender against reaper |

***Table 4.2.*** Examples of some genes involved in apoptosis. As part of the gene lists all synonym names for each gene were collected to avoid complications with the analysis.

Following the results of the microarray analysis four genes involved in apoptosis and immune system functions showed significant gene expression changes and were selected for further analysis: Morgue, Wengen, Minibrain and CG1887. Quantitative Real-Time-PCR (RT-PCR) was conducted with the same RNA samples obtained for microarrays. The RT-PCR results were normalized against the ribosomal protein RpS15Ab-RA, chosen to serve as an internal control. It was amplified simultaneously together with every sample and every run (Fig. 4.0.).
First, the ordered primer/probe sets for Morgue, Wengen, Minibrain and CG1887 were tested with positive controls respectively, before the flight samples were amplified. Therefore, three different ways to induce apoptosis were conducted in laboratory grown animals that served as positive controls:

- Heatshock of a heatshock sensitive strain, hs-Rpr, expressing the apoptosis initiating protein Reaper.
- Heatshock of a heatshock sensitive strain, hs-Hid, expressing the apoptosis initiating protein Hid.
- Irradiation of HML flies with UV-light.

Flies were heatshocked in their bottles in a 37℃ water bath for one hour. For the irradiation control clear vials with flies were exposed to the UV-light in the laminar flow for one hour. The flies were examined following the protocols explained in 3.4. These positive control experiments confirmed that the primer/probe sets for Minibrain, Wengen and Morgue worked as expected and caused a respective fold change compared to the untreated samples (Fig. 4.1; 4.5.; 4.9.). In the positive control experiment for CG1887 the fluorescence for this primer/probe set did not cross the threshold or Ct-value. Since the primer/probe set for the ribosomal protein, which ran in parallel in the same tube, crossed the Ct- value, a problem with the primer/probe set for CG1887 was assumed. As well, a repeat with an increased amount of initial cDNA was not successful leading to the decision to skip CG1887 for further sample analysis.

The normalized changes in gene expression due to infection compared to the PBS control are shown for each gene of interest respectively, and the altered response in space flown animals compared to ground controls is being discussed.

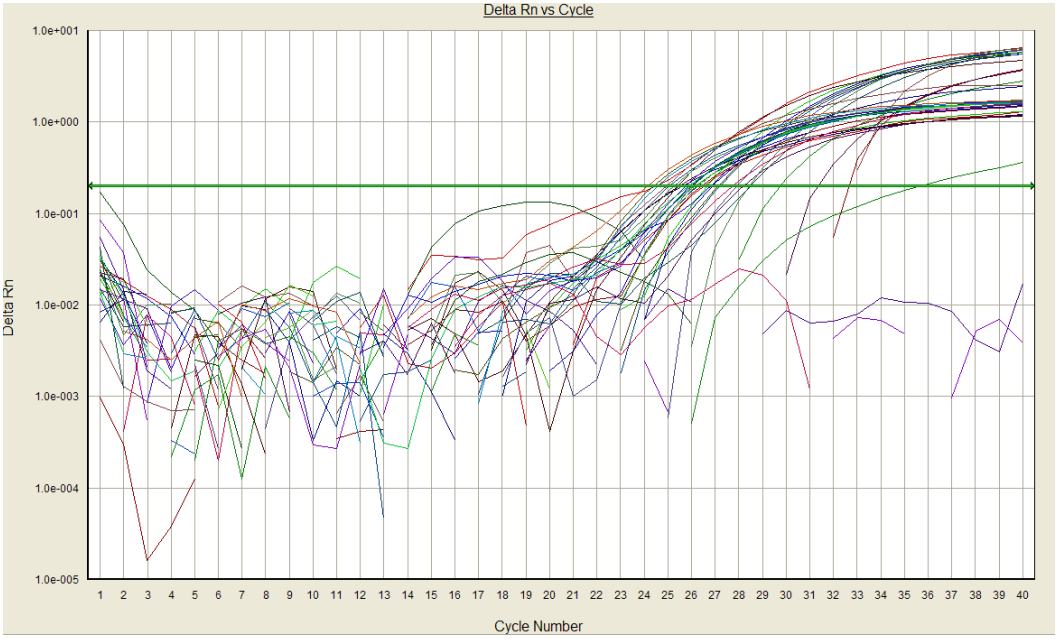


***Fig. 4.0.*** Positive control sample results from a RT-PCR run. The amplification curves of each sample are shown, with cycle number on the X-axis. Several positive control samples were loaded and measured in a special RT-PCR 96-well plate. Increase in fluorescence, representing the respective amplification, (Y-axis) measured for each well, for both the gene of interest and the internal control gene RpS15Ab-RA. Green line shows the threshold level. Ct- value of a sample is the cycle number when the fluorescence crosses the threshold level.

### 4.1.1. Minibrain

The positive control results for Minibrain are presented in Fig.4.1. and show a 4-6 fold reduction in expression compared to an untreated negative control

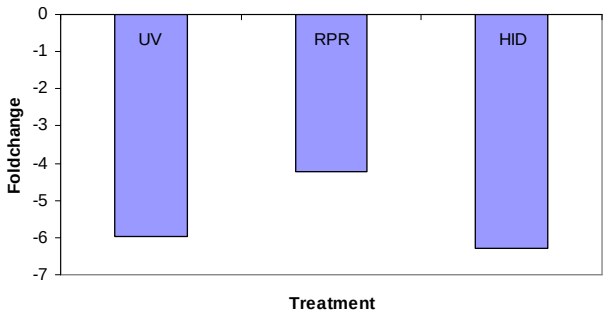


***Figure 4.1***. All three positive controls for Minibrain show decreases in gene expression. A fold change of 1 or -1 would indicate the same expression level as in the untreated sample (baseline). UV = UV-treated flies; Rpr = heatshocked hs-Rpr flies; Hid = heatshocked hs-Hid flies. Foldchange: -2 = -200%

The day one RNA samples were obtained from mixed age as well as mixed virgin and non-virgin female flies that had completed their full development in space. After landing flies were infected with *E.coli* bacteria following the Clearance Assay protocol (3.7.2). Immediately, 1.5 and 4 hours after infection the samples were homogenized and the RNA was isolated (3.4.1). Each data point represents an *E.coli* infected sample versus its respective PBS infected control. At time point 0 the flight sample shows no change, since a fold change of 1 (or -1) represents the same amount of gene expression in the infected and the PBS animal. The ground control animals show an increase in expression of about 1.5 fold (Fig. 4.2.). In the following sampling the ground group maintains positive change in response to the infection, while the change in the flight sample decreases.

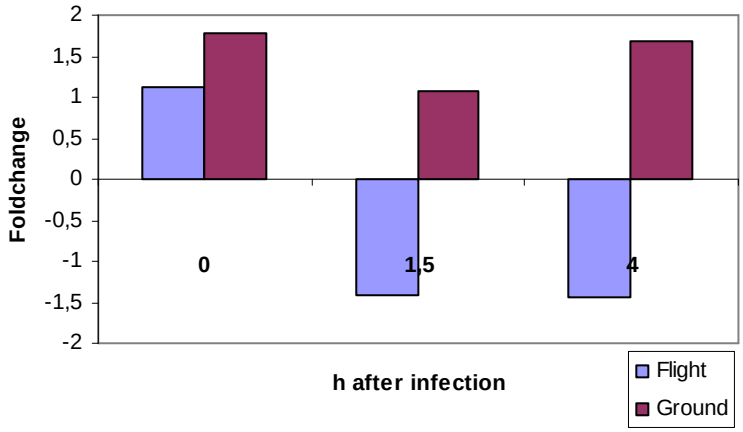


***Figure 4.2***. Day 1 infection, Minibrain. Blue columns = flight sample. Purple column = ground sample. Both data sets show gene expression changes of infected animals in comparison to the respective PBS infected animals. As shown in Table 4.1.

Day three flies used for RNA isolation consisted only of virgin females. Following the same protocol as for day 1 flies, RNA was extracted and RT-PCR performed. Minibrain shows a slight decrease in the flight group and no change in the ground control at time 0. At 1.5 hours as well as at 4 hours the flight group expression increases slightly, while the ground sample results indicate no change in gene expression of Minibrain (Fig. 4.3.).

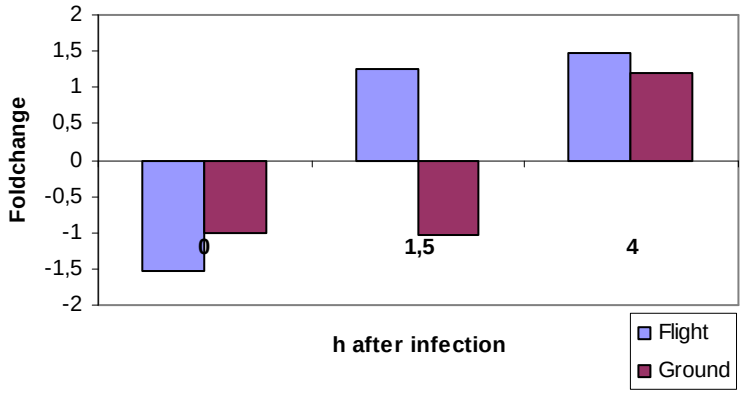


***Figure 4.3***. Day 3 infection, Minibrain. Blue columns = flight sample. Purple column = ground sample. Both data sets show gene expression changes of infected animals in comparison to the respective PBS infected animals. As shown in Table 4.1.

Day five flies spent most of their pupae stage already on Earth. Five days after landing the eclosed virgin female adults were infected and show just a no change at time 0 (Fig. 4.4.). At time 1.5 and 4 hours after infection, the ground control group shows a 3 and 4 time positive fold change in Minibrain gene expression compared to its PBS treated controls, while the flight group decreases at 1.5 hours and increases slightly at 4 hours.

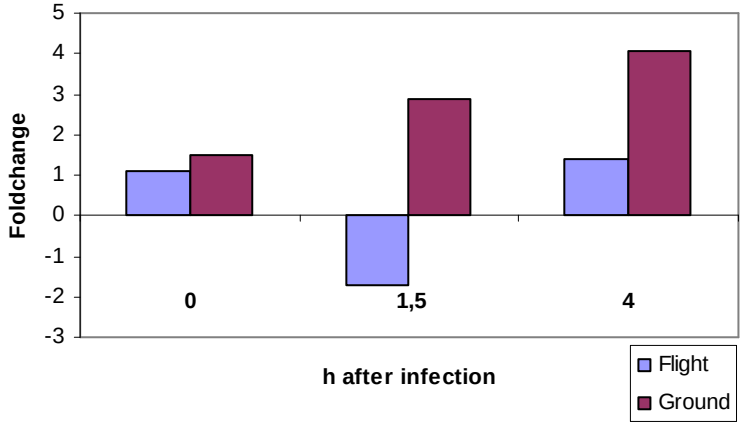


***Figure 4.4***. Day 5 infection, Minibrain. Blue columns = flight sample. Purple column = ground sample. Both data sets show gene expression changes of infected animals in comparison to the respective PBS infected animals. As shown in Table 4.1.

To date only the first set of flight and ground samples was tested for Minibrain gene expression changes resulting in the lack of error bars for the calculation. This first set of data still gives an idea about potential difference in gene expression Minibrain between flight and ground samples triggered by the bacterial infection. Only day 1

flies (Fig. 4.2.) of the flight sample show a decrease in Minibrain expression, which has also been observed in the apoptosis positive controls of Minibrain (Fig. 4.1.). The ground control maintains a positive expression showing a clear difference between flight and ground

At day 3, (Fig. 4.3.) the overall trend in Minibrain gene expression seems to increase faster in flight than in the ground sample. Day 5 (Fig. 4.4.) shows a faster increase in Minibrain expression in the ground sample than in the flight sample, which seems to be relatively stable. Except for day 1 in the flight sample there is no obvious gene expression change pointing towards an apoptosis initiation, as seen in the positive controls. Since Minibrain is involved in a variety of cellular processes (2.2.2.), a change in expression might not primarily be due to an altered apoptosis rate or reaction to the infection but also be triggered by other stress factors. This mix of inputs might be responsible that no clear pattern can be seen in Minibrain gene expression. Further repeats of the experiment are necessary for better interpretation of the data as well as to include standard errors for the data points.

### 4.1.2. Morgue

The positive control tests for Morgue (Fig. 4.5.) show a negative change of 2 - 3 fold compared to an untreated negative control, which confirms the functionality of the primer/probe set.

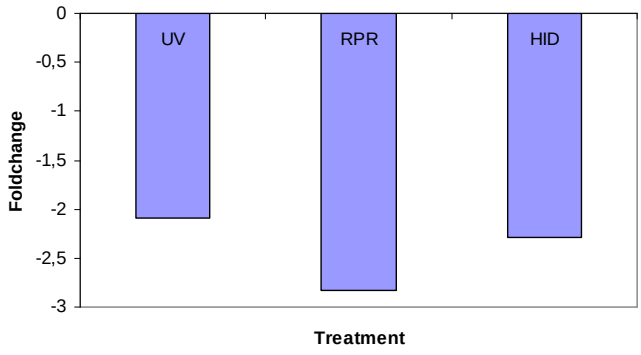


***Figure 4.5***. Positive control test of primer/probe set for Morgue. All three positive controls for Morgue show decreases in gene expression. A fold change of 1 or -1 would indicate the same expression level as in the untreated sample (baseline). UV = UV-treated flies; Rpr = heatshocked hs-Rpr flies; Hid = heatshocked hs-Hid flies. Foldchange: -2 = -200%

The same RNA samples used for Minibrain were tested for gene expression of Morgue. Initially, the flight sample shows a huge decrease in gene expression, while

the ground sample does not change greatly (Fig. 4.6.). 1.5 hours after infection the flight sample shows a slight positive increase in expression, while the ground sample's gene expression is still decreased compared to its respective PBS control. 4 hours after infection, both samples show a positive change, with the ground sample being slightly higher than the flight group.

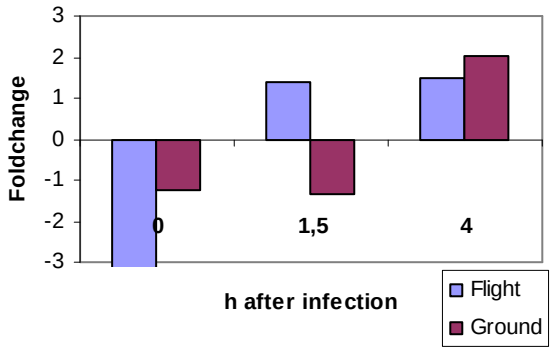


***Figure 4.6.*** Day 1 infection, Morgue. Blue columns = flight sample. Purple column = ground sample. Both data sets show gene expression changes of infected animals in comparison to the respective PBS infected animals. As shown in Table 4.1.

Day three shows an initial difference at time 0 with almost two times decreased Morgue expression in flight. While the ground sample remains at a constant positive expression level over time, the flight sample shows an increase after 1.5 hours followed by another decrease at 4 hours after infection (Fig. 4.7.).

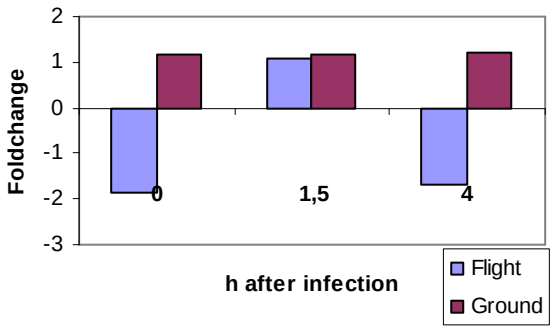


***Figure 4.7.*** Day 3 infection, Morgue Blue columns = flight sample. Purple column = ground sample. Both data sets show gene expression changes of infected animals in comparison to the respective PBS infected animals. As shown in Table 4.1.

At day five, with flies that spent most of their development stages back on Earth, the initial difference in expression between flight and ground groups is relatively small (Fig. 4.8.). Over time, the ground groups shows a stronger decrease than the flight

sample, indicating a possible effect in the flight samples taken place during the embryonic and larval stages. Time 1.5 of flight is missing due to an undetermined Ct value.

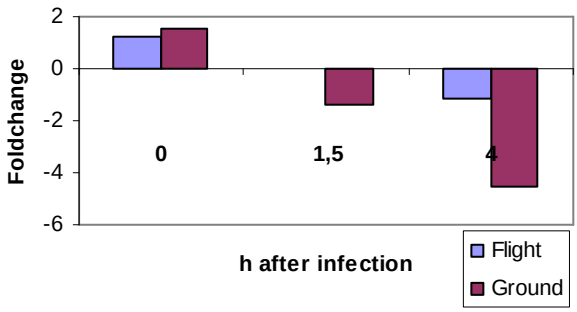


***Figure 4.8***. Day 5 infection, Morgue. Blue columns = flight sample. Purple column = ground sample. Both data sets show gene expression changes of infected animals in comparison to the respective PBS infected animals. As shown in Table 4.1.

As for Minibrain, to date only the first set of flight and ground samples was tested for Morgue gene expression resulting in the lack of error bars for the calculation. This first set of data still shows potential differences in gene expression of Morgue between flight and ground samples triggered by the bacterial infection. Day 1 (Fig. 4.6.) and day 3 (Fig. 4.7.) flies from flight both show a significantly decreased expression of Morgue at time point 0 hours after infection. This negative fold change is similar to the trend seen in the apoptosis positive controls for Morgue (Fig. 4.5.). In the course of the reaction both flight and ground samples show a positive fold change in day 1 and day 3. Only the flight sample in day 3 at the 4 hour time point returns to a negative fold change of Morgue expression. In day 5 (Fig. 4.8.) only the ground sample shows a steady expression decrease of Morgue in course of the reaction indicating possible increase in apoptosis, which is not recognizable in the flight sample. Overall, day 1 and day 2 time point 0 data points show the only strong evidence for Morgue reacting in combination with apoptosis, similar to the positive control experiments. The rest of the data does not indicates any clear patterns, making it difficult to draw any conclusions. As well, further repeats of the experiment are necessary to verify the data, for better interpretation and to include standard errors for the data points.

### 4.1.3. Wengen

The positive control for the Wengen primer/probe set shows a positive change of thirty to forty fold compared to an untreated negative control (Fig. 4.9) indicating that the primer/probe set is functioning.

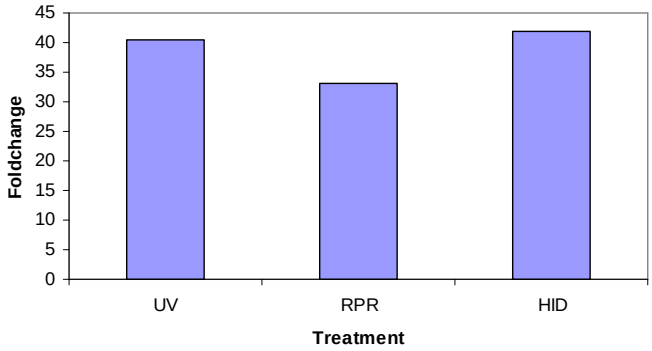


***Figure 4.9***. Primer/Probe set test for Wengen. All three positive controls for Wengen show decreases in gene expression. A fold change of 1 or -1 would indicate the same expression level as in the untreated sample (baseline). UV = UV-treated flies; Rpr = heatshocked hs-Rpr flies; Hid = heatshocked hs-Hid flies. Foldchange: -2 = -200%

An increased expression of Wengen in the flight sample at time 0 is followed by a constant decrease over time. In contrast, the ground sample shows no significant fold changes in the beginning, but increases by two times, 4 hours after infection (Fig. 4.10).

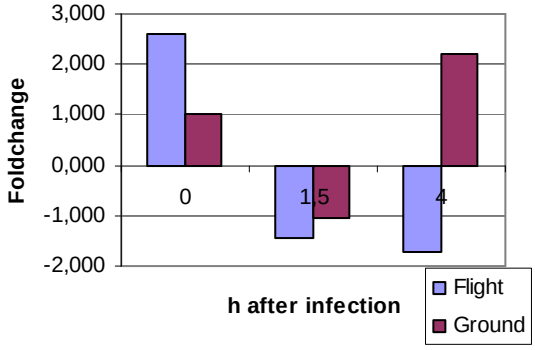


***Figure 4.10.*** Day 1 infection, Wengen. Blue columns = flight sample. Purple column = ground sample. Both data sets show gene expression changes of infected animals in comparison to the respective PBS infected animals. As shown in Table 4.1.

Day three flies show a decreased expression of Wengen in flight and ground during the first two time points, which is converted to a two times positive change in the

flight group. The ground group returns from two times negative to a slightly positive expression (Fig. 4.11.).


***Figure 4.11.*** Day 3 infection, Wengen. Blue columns = flight sample. Purple column = ground sample. Both data sets show gene expression changes of infected animals in comparison to the respective PBS infected animals. As shown in Table 4.1.

Day 5 post infections shows an initial negative change in the flight group, which is followed by a positive and a slightly negative expression at subsequent sampling times (Fig. 4.12.). On the other hand, the ground group increases its Wengen gene expression up to nine times during the last sampling time.

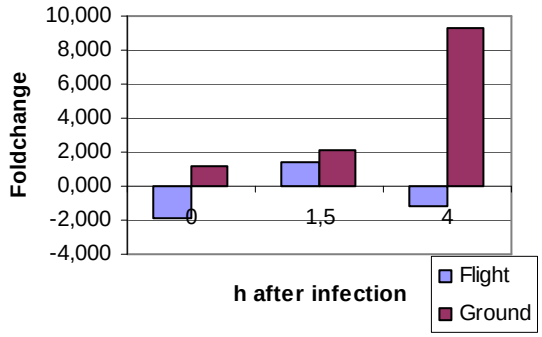


***Figure 4.12.*** Day 5 infection, Wengen. Blue columns = flight sample. Purple column = ground sample. Both data sets show gene expression changes of infected animals in comparison to the respective PBS infected animals. As shown in Table 4.1.

As for Minibrain and Morgue, to date only the first set of flight and ground samples was tested for Wengen gene expression resulting in the lack of error bars for the calculation. This first set of data still shows potential trends in gene expression differences in Wengen between flight and ground samples triggered by the bacterial infection. Day 1 flight sample at time point 0 (Fig. 4.10.) shows a positive fold change

showing a possible involvement of apoptosis elements. The increase is not as strong as seen before in the positive control but could still be part of an apoptosis initiation reaction. Over the course of the reaction to the infection this change returns to normal and only the ground sample shows a positive increase after 4 hours. Day 2 flight sample (Fig. 4.11.) shows an increase in Wengen expression after 4 hours, whereas the ground sample expression is slightly negative after 1.5 hours. At day 5 (Fig. 4.12.), only the ground sample shows a strong positive fold change after 4 hours. Since no clear pattern of the data can be observed, it is difficult to draw detailed conclusions. As well, further repeats of the experiment are necessary to verify the data, for better interpretation and to include standard errors for the data points.

## *4.2. Caspase activity in space*

The activity of cysteine-containing-aspartate-specific proteases, the caspases, was measured by the cleavage-speed of enzyme-specific substrates (3.5.2). Increased caspase activity typically indicates an elevated level of apoptosis, which can be triggered by various stress factors, including space flight related factors. The described assay can be performed on adult flies or 3$^{rd}$ instar larvae, but to date just one larval flight sample has been run due to the long assay development phase necessary to adapt the provided protocol, which was originally designed for mammalian samples.

The activity of caspases was measured by mixing protein extracts from 3$^{rd}$ instar larval fatbody dissections with enzyme-specific substrates (3.5.2.) (Thornberry et al., 1997). These substrates are part of the “Calbiochem Caspase Substrate Set III, colorimetric” and the specific caspase activity can be assayed by using the chromophore para-Nitroaniline (pNA) attached to the substrate sequence. Cleavage of the substrate results in the release of the chromogen, which is measured at 405 nm on a 96 well spectrophotometer plate-reader. The space flown larvae were dissected at NASA Kennedy Space Center within 24 hours after landing of the shuttle and the protein lysate extracted from the fatbodies stored at –80℃ until measurement at NASA Ames Research Center.

| Sequence | M.W. | Known Target Caspases |
|---|---|---|
| Ac-YVAD-*p*NA | 628.6 | 1, 4 |
| Ac-VDVAD-*p*NA | 679.7 | 2 |
| Ac-WEHD-*p*NA | 747.7 | 1, 4, and 5 |
| Ac-DEVD-*p*NA | 638.6 | 3, 6, 7, 8, and 10 |
| Ac-VEID-*p*NA | 636.7 | 6 |
| Ac-IETD-*p*NA | 638.6 | 8 and Granzyme B |
| Ac-LEHD-*p*NA | 674.7 | 4, 5, and 9 |

***Table 4.3***. This table shows the used substrates together with their mammalian target caspases. Protocol adaptations for use with Drosophila were conducted. (Calbiochem user manual, Caspase Substrate Set III, Colorimetric, Cat. No. 218808)

The first step towards measurement of caspase activity is the establishment of a reliable positive control proving that the caspases are able to cleave their respective substrates and that the assay is functioning. Therefore, a heatshock sensitive strain of *Drosophila*, hs-Rpr, expressing the apoptosis initiating protein Reaper was used. One hour heatshock of 3$^{rd}$ instar larvae in a bottle using a 37℃ water bath triggered the production of the Reaper protein and initiated the apoptotic machinery. Subsequent protein extraction (3.5.1) was conducted using the Caspase Assay Buffer (CAB) and the isolated caspases were tested for their activity measuring the changing optical density at 405nm on a plate reader (3.5.2). Therefore, each sample was incubated separately in a 96 well plate with each of the seven Calbiochem substrates (Tab. 4.3.). and measured. For easier visualization of the reaction progression polynomial regressions of fourth order were applied. The results obtained from the heatshocked hs-Rpr strain, the positive control, were compared to freshly dissected, laboratory grown HML 3$^{rd}$ instar larvae, the same strain that was used for the space flight experiment. The plate reader results are shown in Fig. 4.13. and 4.14., and the normalized results are presented and compared in Fig. 4.17.

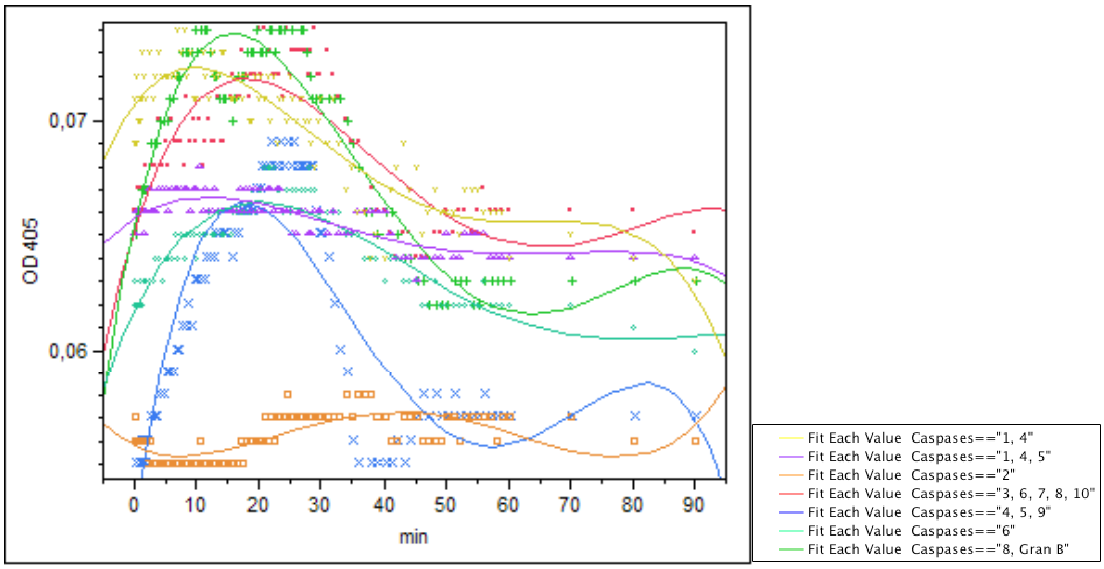


***Figure 4.13***. Plate reader results for one hs-Rpr sample, the positive control. OD at 405nm. Caspase activity measured using seven different substrates, each color represents one substrate. Corresponding caspases for each substrate shown in left box. High activity in most caspases due to steep increase at beginning of the reaction, indicating that the positive control works. Results still need to be normalized to a protein concentration of 1mg/ml.

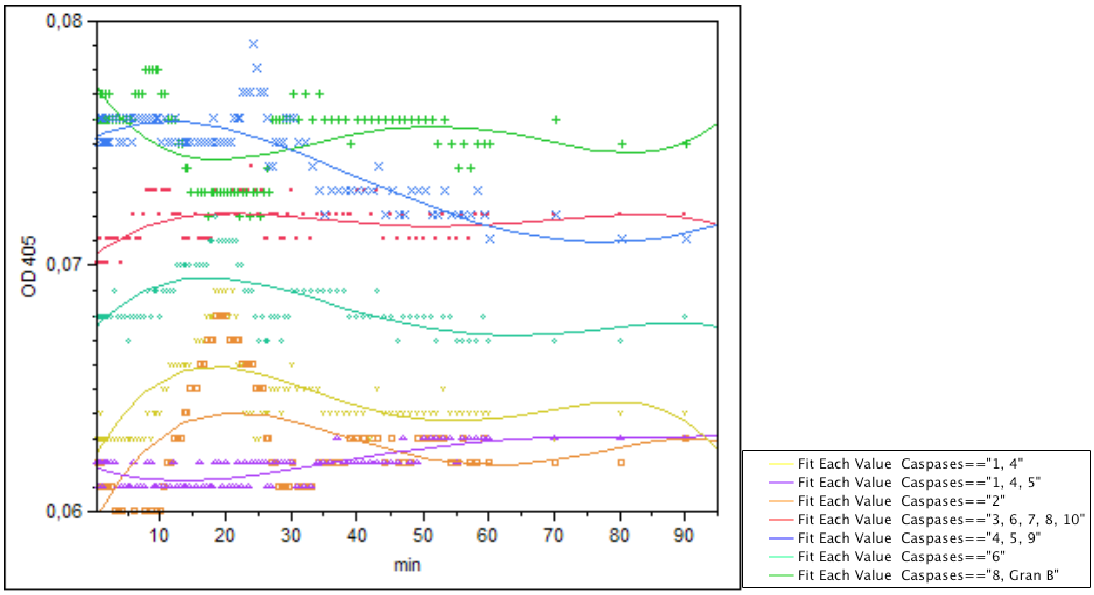


***Figure 4.14.*** Plate reader results for one HML sample, the baseline for the caspase activity assy, OD at 405nm. Caspase activity measured using seven different substrates, each color represents one substrate. Corresponding caspases for each substrate shown in left box. HML caspases show flatter curves than positive control, indicating less activity. Results still need to be normalized to a protein concentration of 1mg/ml.

Absolute caspase enzyme activity is normally expressed as moles of a substrate converted per unit time. Since the goal for this experiment was the measurement of any relative changes in caspase activates triggered by space flight conditions, the comparison of the steepness of the slopes after normalization

representing the activity of a caspase was sufficient. Therefore, the initial slopes for each sample were selected and calculated by applying a linear regression through the data points. Since protein concentration of the extracted lysates influences caspase activities, the obtained slopes needed to be normalized. Therefore, protein concentration measurements for each sample were performed using the Bicinchonic Acid (BCA) Protein Assay Kit from Sigma. The slopes were divided through the protein concentration of each sample, normalizing the data to 1 mg/ml. Slopes of the same caspase substrate in multiple samples of the same strain and condition, for instance the positive control hs-Rpr, were taken together and mean and standard error were plotted (Fig. 4.15.).

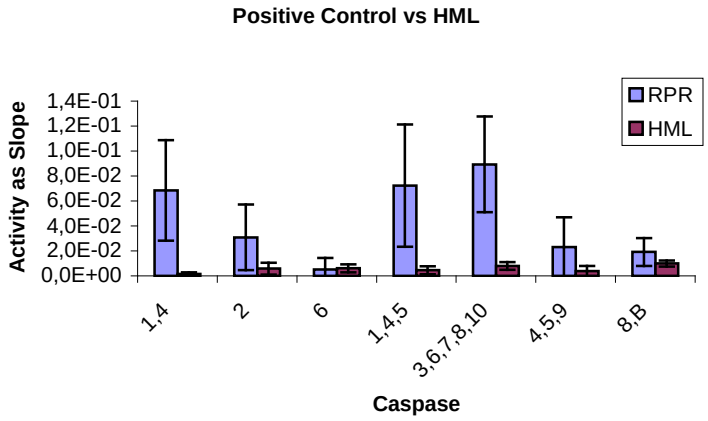


***Figure 4.15***. hs-Rpr and HML sample. Normalized (1 mg/ml) caspase activities, as steepness of slopes, for each substrate. Corresponding caspases on x-scale. Blue bars = hs-Rpr, the positive control. Purple bars = HML, the baseline control. Error bars for positive control big due to fluctuation. Sample number, N, for each

As explained above, caspase activity is compared by the cleavage speed of each respective caspase substrate. In Fig. 4.15., the positive control hs-Rpr shows significantly higher cleavage activities of three caspase substrates, YVAD, WEHD and DEVD (Tab. 4.3.), in comparison to the untreated HML sample. The high error bars result from fluctuations in-between repeats of the positive control. Showing a significant difference between the positive control and the baseline indicated that the assay is working.

Next, laboratory (standard conditions) grown HML flies, not the ground controls, from NASA Kennedy Space Center, containing the same generation of animals as the flight ones, had been dissected at Kennedy Space Center and the protein lysate been stored at –80℃. Therefore, these samples experienced the same time and

conditions in storage as the flight and ground samples. These laboratory-grown HML samples were the first to be tested for caspase activity, as well as to be checked for potential effects due to the long storage time. The results showed slightly less activity after normalization than the freshly dissected HML samples indicating a possible decrease in activity due to long duration freezing, but further quantification of potential effects can only be investigated with long duration storage of activated positive controls, like hs-Rpr, which was not performed.

Finally, the first flight sample (Fig. 4.16) was tested for caspase activity together with its respective ground control sample (Fig. 4.17). The normalized results are compared in Fig. 4.18. Limitation of time is responsible that to date only one sample of flight and its respective ground control were tested resulting in the lack of error bars (Fig. 4.18.). Repeats with further samples will be necessary to verify these initial results.

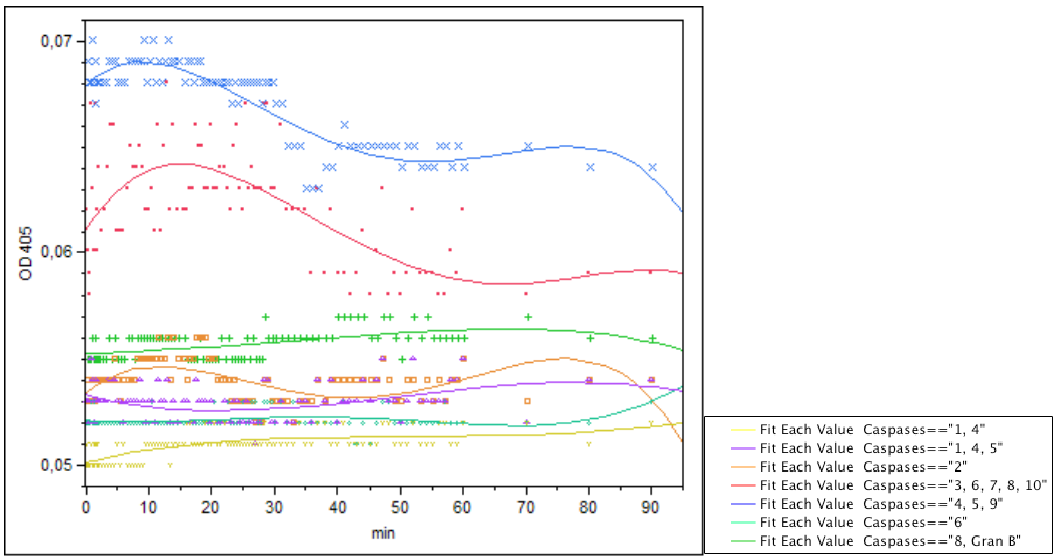


***Figure 4.16***. Plate reader results for first ground sample, OD at 405nm. Caspase activity measured using seven different substrates, each color represents one substrate. Corresponding caspases for each substrate shown in left box. Results still need to be normalized to a protein concentration of 1mg/ml.

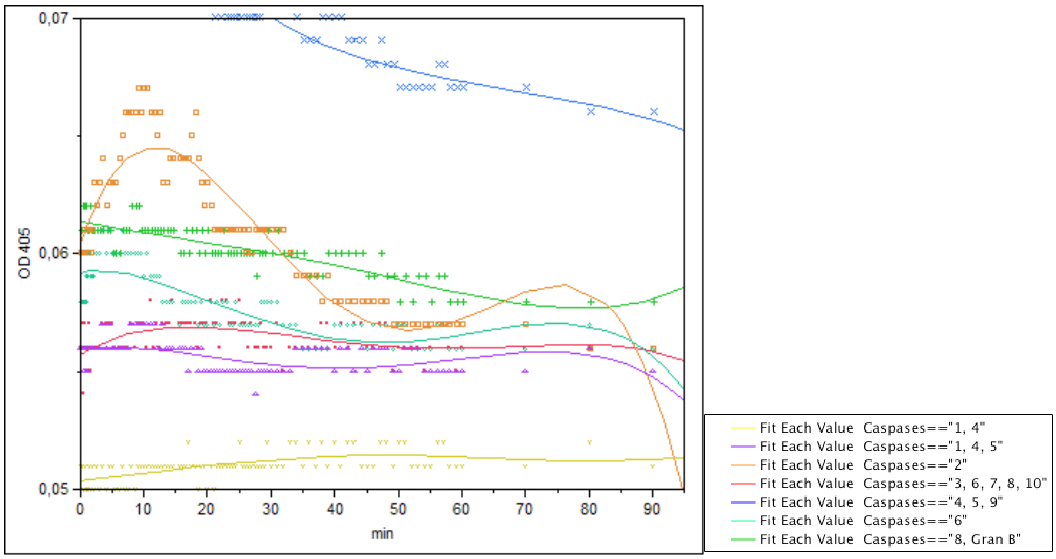


***Figure 4.17***. Plate reader results for first flight sample. OD at 405nm. Caspase activity measured using seven different substrates, each color represents one substrate. Corresponding caspases for each substrate shown in left box. Results still need to be normalized to a protein concentration of 1mg/ml.

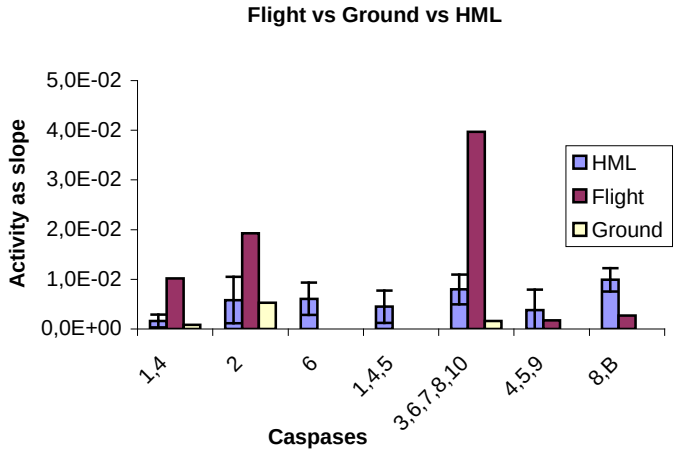


***Figure 4.18.*** Flight, Ground, HML sample. Normalized (1 mg/ml) caspase activities, as steepness of slopes, for each substrate. Corresponding caspases on x-scale. Blue bars = HML, the baseline. Purple bars = Flight sample. White bars = Ground sample. No error bars in flight and ground due to lack of repeats.

In the flight sample substrates YVAD (1,4), VDVAD (2) and DEVD (3, 6, 7, 8, 10) appeared to be cleaved faster than in the ground and HML sample (Fig. 4.18.). Caspase 2 substrate VDVAD is specific only for this caspase and therefore, the observed increase in activity must be due to this enzyme. DEVD can be digested by caspases 3, 6, 7, 8, and 10 and also showed increased activity as well, but all of these enzymes could be responsible for this effect. Only caspases 6 and 8 can be ruled out since their activity was tested with two other substrates, VEID and IETD.

Substrate YVAD was also processed faster than its ground and HML sample, although caspases 1 and 4 showed no cleavage activity of substrate WEHD.
Calbiochem caspase substrates were to date mainly used for mammalian samples. Therefore, the manual only explains which provided substrate would be cleaved by which mammalian caspase. Homologies between mammalian and *Drosophila* caspases are shown in Tab. 4.4. and used to interpret the results of this assay. The flight sample shows activated caspases 1, 2, 3, 4, 7 and 10. According to Tab 4.4., *Drosophila* caspases Dronc, Dredd, Drice, and Dcp-1 could have the necessary functional homologies to cleave substrates YVAD, VDVAD and DEVD. As explained in 2.2.1. caspase activation in *Drosophila* involves initiator caspases, which activate the executioner caspases. The latter group of proteases are the actual effector enzymes responsible for the break down process. Since substrates for initiator (Dronc, Dredd) and effector caspases (Drice, Dcp-1) were digested at an elevated level in the flight compared to the ground sample, it is reasonable to assume that the apoptotic machinery seems to be in an activated state due to the space flight conditions.

| Subfamily | *Drosophila* | Mammals |
|---|---|---|
| CARD-containing caspases (initiator caspases) | Dronc | Caspase-1, Caspase-2, Caspase-4, Caspase-5, Caspase-9, Caspase-11, Caspase-12 |
| DED containing caspase (initiator caspase) | Dredd | Caspase-8, Caspase-10 |
| Short prodomain caspase (executioner caspase) | Drice, Dcp-1 | Caspase-3, Caspase-6, Caspase-7 |
| Others | Strica (also called Dream), Decay, Damm | Caspase-14 |

***Table 4.4.*** Homologue caspases in *Drosophila* and mammals (Kuranaga et al., 2007)

Lastly, a difference in protein concentration in the flight sample compared to the rest of the samples was observed. While all samples normally showed protein concentrations of 1-3 mg/ml after fatboday dissection and caspase extraction, the flight sample only contained 0.57 mg/ml protein. This difference was balanced out by normalization of the data for the caspase activity measurement. Further samples need to be measured to verify this trend, but if confirm, it would be consistent with an observed reduction in size of larvae that developed in space.

## *4.3. Apoptosis following Irradiation*

In ground based studies of the FIT experiment effects of proton irradiation, a major component of space radiation, on cells and organs in *Drosophila* were investigated. The applied proton irradiation covers a wide range of exposure levels that would be experienced by astronauts in low-Earth-orbit, potential flights to the Moon and Mars and during increased solar activity.

Apoptosis or programmed cell death is essential for development and maintenance of tissues and organs. Apoptosis is also initiated in damaged cells to remove them and replace them by new ones. At the cellular level ionizing radiation produces reactive oxygen species or other radicals that can attack nucleic acids and proteins resulting in cell cycle arrest or apoptosis. The cellular response to proton irradiation exposure was studied with focus on apoptosis initiation.

### 4.3.1. The Climbing assay

The Climbing Assay, as we discovered in our laboratory, is an easy phenotypic indicator to quantify irradiation effects. Following proton irradiation of 3$^{rd}$ instar larvae the eclosed female flies showed a decreased activity in jumping and overall motility with respect to increasing dosage (Fig. 4.20.). The 0 Gy control demonstrated that the activity of flies decreases with days after eclosion. Irradiation exposure decreases the initial activity of flies after eclosure. For instance, at day 2 40% untreated flies remain within the first centimeter in the vial, while 30 Gy exposure results in 80% flies being at the lowest level in the vial. The decrease in motility of the proton irradiated flies is most obvious at day 1 after hatching, but also monitored at the subsequent days, indicating progressive neurodegeneration.

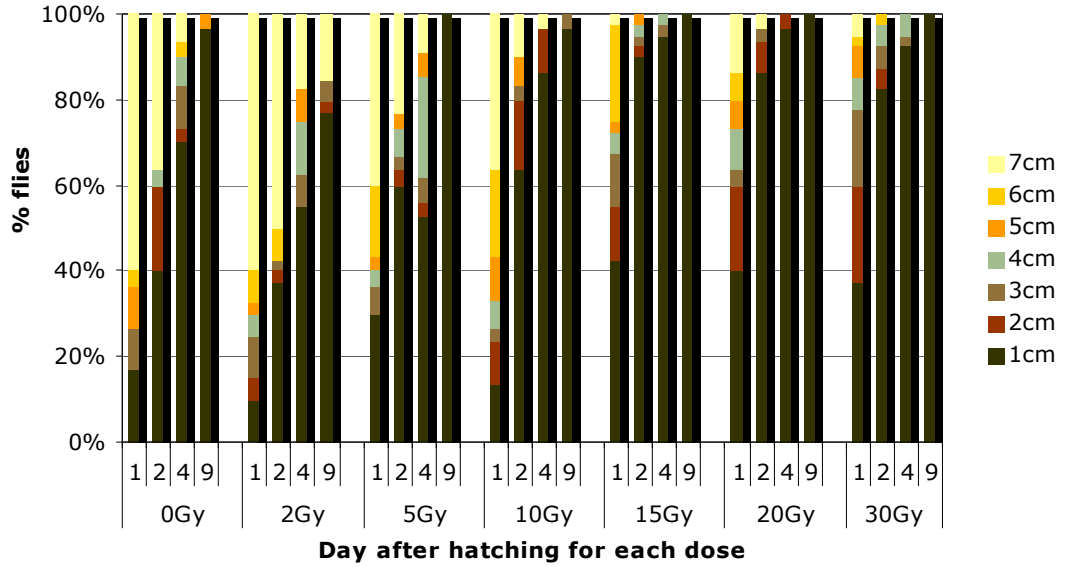


***Figure 4.20***. Climbing Assay data. Y-axis: %flies observed at specific height in a vial 10sec after being forced to the vial bottom by knocking it on a table. Color code on the left: 1cm = flies within the first centimeter, 2 cm = flies within the second centimeter; and so on. X-axis: numbers 1,2,4,9 are days after hatching when the assay was performed. 0-30Gy shows proton irradiation exposure level.

The exposure to proton irradiation can result in multiple effects on various cells, tissues and systems of the fly affecting its ability for locomotion. Changes in signal transduction, synaptic transmission as well as neurodegeneration and neuronal death are possibly affected by irradiation (Ohnishi and Ohnishi. 2004; Kennedy and Todd, 2003). To further pinpoint the place of defect, electroretinograms or ERG's, measuring the neuronal activity, were conducted by a colleague, showing no difference between irradiated and untreated control samples, suggesting that signal transduction and synaptic transmission is not a problem at these exposed levels.

### 4.3.2. The TUNEL assay

Terminal transferase dUTP nick end labeling (TUNEL) is a common method for detecting DNA fragmentation that results from apoptotic signalling cascades. Nicks are breaks of the DNA backbone, potentially caused by irradiation or other space flight effects. They can be identified by the terminal transferase, an enzyme that catalyzes the addition of dUTPs, which are secondarily labelled with a fluorescent marker. The Tunel assay was performed on hemocytes collected from 3$^{rd}$ instar larvae at different time points after proton irradiation. Fig.4.22. shows the number of apoptotic cells at different time points after irradiation treatment with various

intensities. Immediately as well as 12 hours after irradiation no significant change in apoptotic cells was observed. At 24 hours the number of cells undergoing programmed cell death increased in all three irradiation levels. At 72 hours this change disappeared and the cells showed relatively low apoptosis activity.

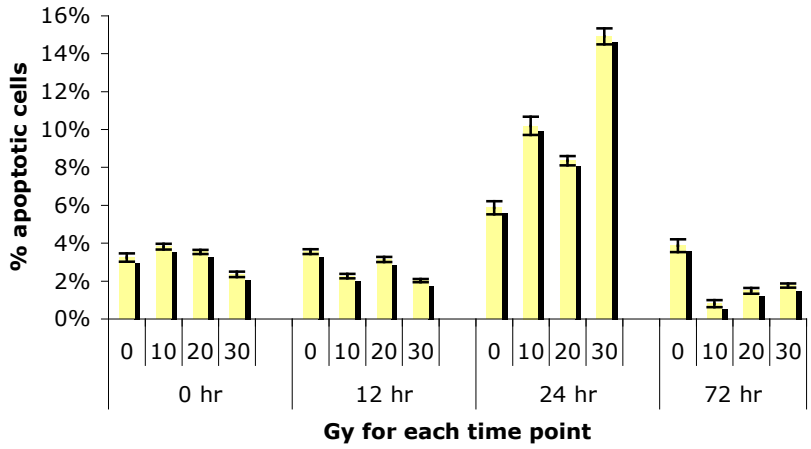


***Figure 4.22***. TUNEL assay data. Percent of apoptotic hemocytes extracted from 3rd instar larvae at different time points and treatments. Healthy and apoptotic cells counted on a microscopic slide. Repeats averaged and mean/standard error plotted. X-axis: 0, 10, 20, 30 = intensity of irradiation in Gy; 0, 12, 24, 72 = hours after irradiation when TUNEL assay was performed.

The observed delay of 24 hours could indicate the lag time needed for cells to respond to the treatment and to activate the apoptotic machinery. Another important factor to be considered is the developmental stage of the animals during irradiation treatment. Since the Tunel assay can only be performed with 3rd instar larvae, the animals used for the later time points must be younger during irradiation (Tab.3.1.). Therefore, the larvae used for the 24 hour time point were irradiated as 2nd instar larvae, the larvae for the 72 hour time point were 1st instar larvae. It is possible that receptivity to irradiation is different depending on the developmental stage of the animals.

### 4.3.3. p53 assay

When animal cells are exposed to stressful conditions, the tumor suppressor protein p53 restrains growth by promoting an arrested cell cycle or initiating a cell death program. Dmp53 function is required for γ-irradiation-induced transcription of the apoptosis initiating Reaper region (Sogame et al., 2003). To determine the effects of proton irradiation on p53 induction, *Drosophila* embryos provided by Dr. John Abrams, University of Texas, containing a p53 radiation response element in front of a Green-Fluorescence-Protein (GFP) were exposed. This response element, also referred to as p53RE, is constructed of a 150 bp radiation inducible enhancer containing a 20 bp potential p53 binding site. It was characterised and used by Brodsky et al., 2000 to understand p53 function. The 2, 5, 10, 15, 20 and 30 Gy proton irradiated embryos were examined following protocol 3.6.2. and are shown in Fig. 4.23.

Propidium iodide DNA staining was performed to roughly sort the embryos according to their developmental stage (http://flymove.uni-muenster.de/). Only embryos containing most of their DNA at the edge (until about stage 9 in embryonic development), were used for the analysis. The DNA and GFP images were overlaid using Photoshop. The 0 Gy image shows a faint green background of GFP expression. The increased expression of GFP with respect to increased irradiation intensity is observed in the majority of the embryos. This effect indicates that the p53RE element drives the expression of GFP in response to ionising proton irradiation. Therefore, it can be assumed that this element, in its natural position, would drive the expression p53 in response to proton irradiation. The faint embryo in the 20 Gy image serves as an example that not all embryos show the same increased response to the irradiation exposure.

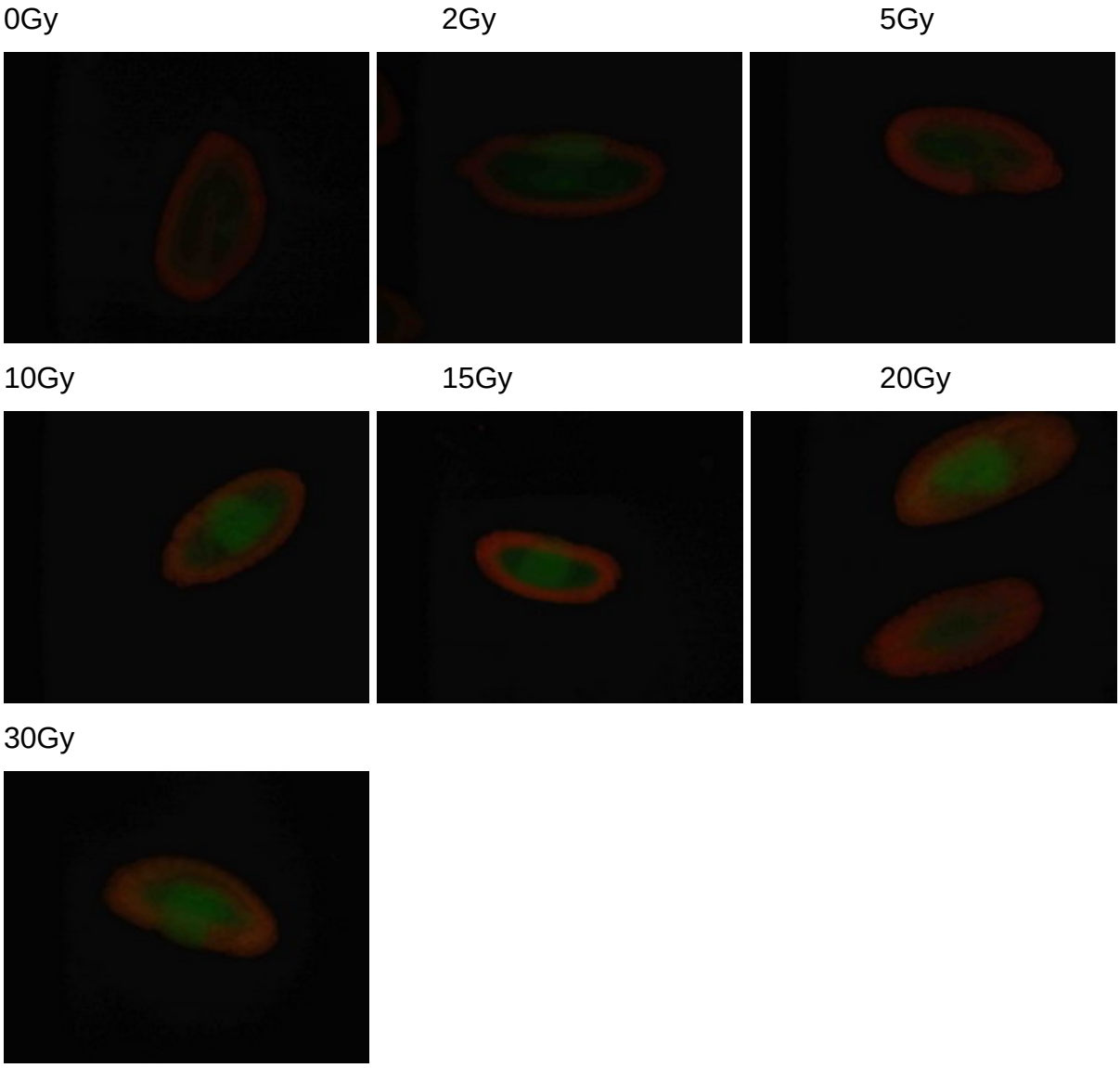


***Figure 4.23***. Merged pictures of propidium iodide stained DNA (red) and p53RE induced GFP (green) in embryos of various irradiation levels.

## *4.4. Immune System following Irradiation*

Apoptosis initiation in response to proton irradiation was shown in 4.3.3. by activation of the tumor suppressor protein p53 and DNA fragmentation (4.3.2.). To remove these damaged and dying cells, phagocytosing blood cells or hemocytes need to be activated. Hemocyte activity in *Drosophila* flies exposed to proton irradiation was examined and a potential link between the response to infection and prior irradiation was investigated.

### 4.4.1. Alexa Fluor E.coli Phagocytosis Assay

To study the activity of larval hemocytes, the Alexa Fluor E.coli Phagocytosis Assay was performed after the Space Shuttle flight and after proton irradiation treatment (3.7.3). *Drosophila melanogaster* strain HML was used in flight as well as in the ground studies. A Hemolectin promoter drives the expression of the Green-Fluoresence-Protein (GFP) specifically in flies' plasmatocytes, and is particularly bright due to the 4 copies of GFP in the homozygous line (Goto et al., 2001). Herein, the results of the ground based proton irradiation experiments are presented. The animals were irradiated at different developmental stages and the Alexa Fluor E.coli Phagocytosis Assay was performed at four time points after exposure. Hemocytes extracted from 3$^{rd}$ instar larvae were infected with dead Alexa Fluor 594-labelled *Escherichia coli* bacteria from Invitrogen and the respective activity and amount of phagocytosing cells was analysed (3.7.4). Remaining external bacteria were quenched with the presents of Trypan Blue (Fig 4.24.).

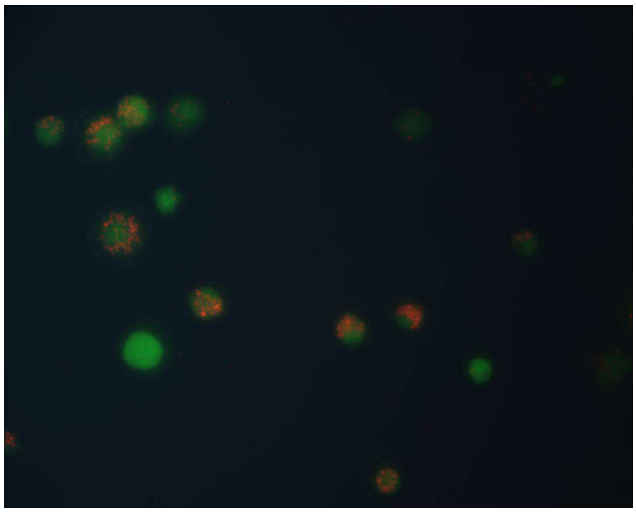

***Figure 4.24***. This image shows extracted hemocytes from HML-larvae expressing the green fluorescence protein GFP. Only engulfed red Alexa Fluor 594 labelled bacteria are shown within the blood cells, since the external cells were quenched with Trypan Blue.

The first question addressed with this assay was: *How many hemocytes contain engulfed E.coli bacteria with respect to irradiation level as well as hours after exposure?* The absolute number of hemocytes with *E.coli* bacteria was compared to the number of "empty" hemocytes and the ratio calculated. Each time the assay was performed, hemocytes were inoculated with *E.coli* bacteria for 5, 25, and 45 minutes and the level of uptake was analysed for each inoculation period. The assay was

carried out at 4 time points after irradiation, at 2 hours, 14 hours, 35 hours and 57 hours. Due to time limitations the assay could not be repeated at another irradiation run resulting in the lack of error bars. Still, the presented data show two interesting trends helping to understand a potential connection between apoptosis and immune system function after irradiation.

At 2 hours after irradiation the number of phagocytosing hemocytes is relatively constant, except for a slightly decreased activity at the 10 Gy level and 5 minutes of *E.coli* inoculation (Fig. 4.25.). Due to a technical failure of the irradiation facility the 40 and 60 Gy exposure levels for the 2 hour time point could not be completed.

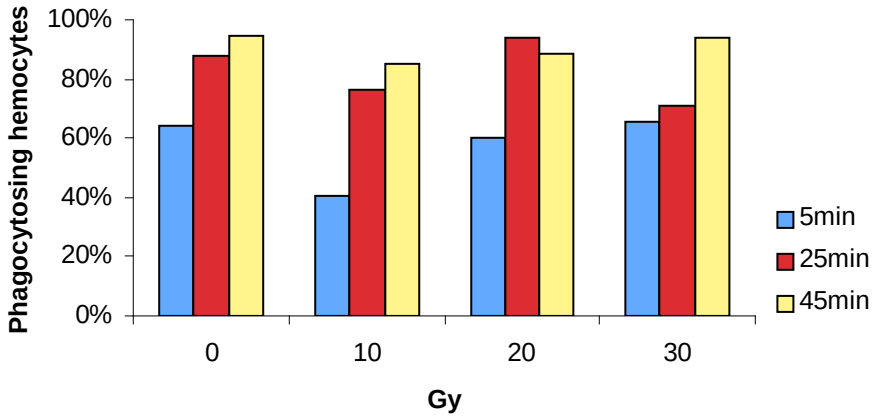


***Figure 4.25.*** 2 hour time point. Number of hemocytes containing engulfed bacteria two hours after irradiation exposure. For each irradiation level 0, 10, 20, and 30 Gy, the hemocytes were inoculated with E.coli bacteria for 5, 25 and 45 min resulting in an increased level of "engulfing" hemocytes, showing the time course of the reaction to a bacterial infection.

At 14 hours after irradiation, the assay was repeated and the data presented in Fig. 4.26. Phagocytosis activity seems to be constant at 0, 10, 20 and 30 Gy exposures, similar to the results of the 2 hour time point. Only at 40 Gy larvae show a decreased number of "active" hemocytes at the 5-minute incubation indicating a possible delayed activation response, since the overall number of hemocytes did not decrease (data not shown). This decrease is recovered at the 25 and 45 min inoculation time points at 40 Gy. At 60 Gy and 45 min of inoculation with E.coli, the number of hemocytes containing bacterial cells after 45 minutes decreases below the 25 and 45 min inoculation time point. Further investigation is needed to interpret this last effect.

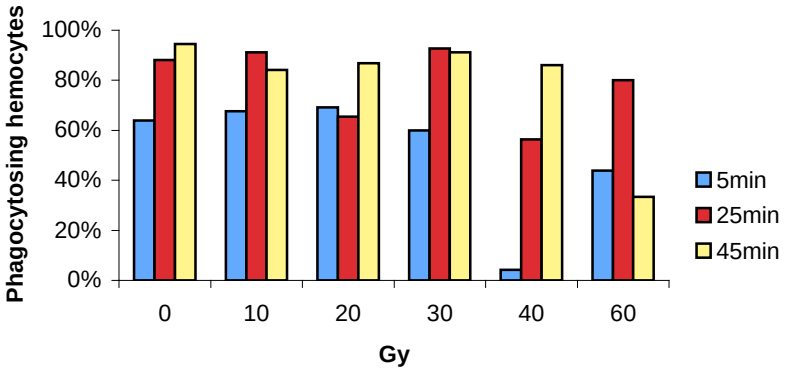


***Figure 4.26.*** 14 hour time point. Number of hemocytes containing engulfed bacteria 14 hours after irradiation exposure. For each irradiation level 0, 10, 20, 30, 40 and 60 Gy, the hemocytes were inoculated with E.coli bacteria for 5, 25 and 45 min resulting in an increasing level of "engulfing" hemocytes, showing the time course of the reaction to a bacterial infection.

35 hours after irradiation, again, hemocytes from 3$^{rd}$ instar larvae were extracted and inoculated with bacteria. It is important to point out that these animals were irradiated as 2$^{nd}$ instar larvae and then allowed to develop, so they would be 3$^{rd}$ instar larvae for this time point (Tab. 3.1., Irradiation preparation and setup). The difference in age and therefore developmental stage could possibly influence the receptivity to irradiation treatment and the rate of recovery to irradiation damage. Fig. 4.27. shows that the overall phagocytosis rate after 45 min of inoculation with bacteria remains constant in all irradiation levels, besides a slight decrease at 60 Gy. However, after 25 min of inoculation larvae exposed to 20 Gy show a decrease in hemocyte activity. As well, the 5 min inoculation shows decrease in activity at 10, 20 and 30 Gy. Taken together this data suggests a negative effect on hemocytes at the low irradiation levels, but also a recovery and possible activation effect at the 40 and 60 Gy level.

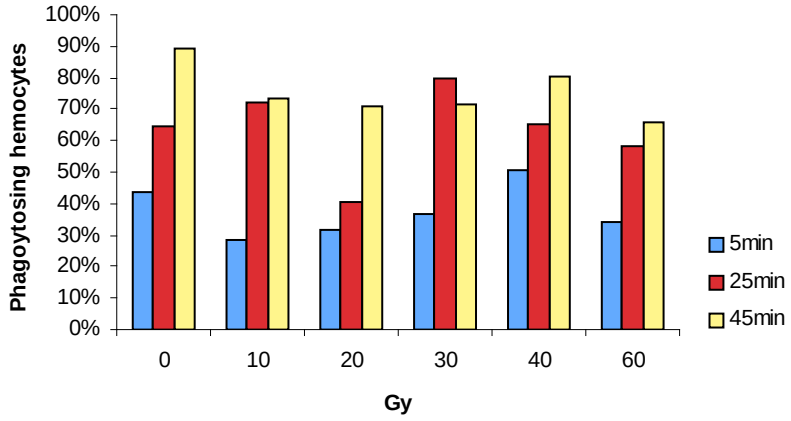


***Figure 4.27***. 35 hour time point. Number of hemocytes containing engulfed bacteria 35 hours after irradiation exposure. For each irradiation level 0, 10, 20, 30, 40 and 60 Gy, the hemocytes were inoculated with E.coli bacteria for 5, 25 and 45 min resulting in an increasing level of "engulfing" hemocytes, showing the time course of the reaction to a bacterial infection.

As previously explained for the 35 hour time point, also the larvae used for the 57 hour time point could show an altered receptivity to proton irradiation due to their developmental stage during exposure. For this time point 1$^{st}$ instar larvae were irradiated and subsequently allowed to develop to 3$^{rd}$ instar larvae, which took about 57 hours (Tab. 3.1.). At 45 min of inoculation a decrease in activity at 20 and 30 Gy is observed but also a recovery effect at 40 and 60 Gy. At 25 min inoculation the decreased activity is at its lowest point at 30 Gy but also recovers completely at 40 and 60 Gy. Similar trends are recognisable for the 5 min inoculation time points.

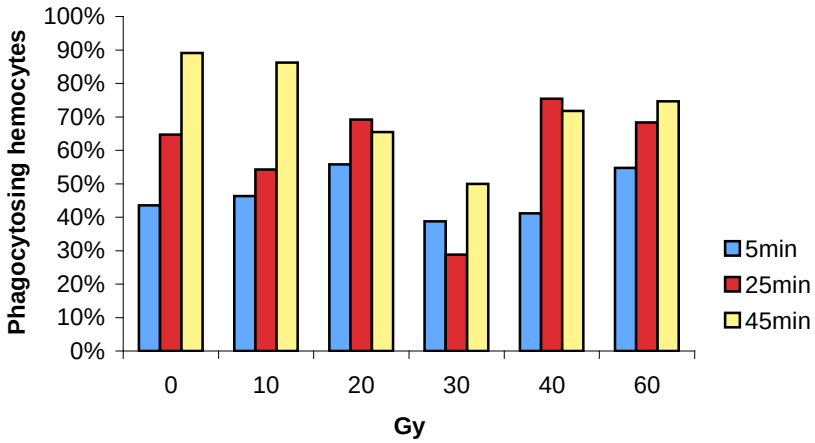


***Figure 4.28***. 57 hour time point. Number of hemocytes containing engulfed bacteria 57 hours after irradiation exposure. For each irradiation level 0, 10, 20, 30, 40 and 60 Gy, the hemocytes were inoculated with E.coli bacteria for 5, 25 and 45 min resulting in an increasing level of "engulfing" hemocytes, showing the time course of the reaction to a bacterial infection.

Two interesting trends have been observed in this first experiment set up. First, ionising radiation can negatively influences hemocyte activity, but is depending on the developmental stage of the embryo during irradiation as well as the recovery time of the embryo afterwards. For instance, the 2 hour time point (Fig. 4.25.) shows only a slight decrease in activity at 10 Gy exposure, while the 14 hour time point (Fig. 4.26.) shows a significant decrease at 40 and 60 Gy. Second, a positive recovery effect of decreased hemocyte activity was observed at the high exposure levels in the 35 and 57 hours measurements after irradiation (Fig.4.27; 4.28.). It could be possible that a certain level of proton treatment elevates the production or activity of hemocytes. As well, it might be possible that the damaged cells produced by 40 and 60 Gy treatments in the 35 and 57 hours samples, trigger hemocytes to remove these damaged cells from the system. This activated state subsequently helps in the faster removal of bacterial cells from the system.

The second question addressed with the Phagocytosis Assay was: *How many bacteria were engulfed per individual hemocyte cell with respect to irradiation level as well as hours after exposure?* This analysis should investigate, if single hemocyte cells are damaged by proton irradiation in way, which would limit their ability to function as efficiently as in untreated controls. Even though the overall number of phagocytosing cells might not change in a sample (investigated in the first part of the experiment = first question), the activity of single cells could still be decreased, showing additional effects of irradiation on the hemocytes. For this analysis, each single GFP-labelled hemocyte cell imaged with a fluorescence microscope was examined and the number of engulfed bacteria per cell was determined. Since bacterial cells tend to accumulate closely together in a hemocyte, an average bacterial size of 0,624 $\mu m^2$ was estimated to calculate an absolute number of bacteria per hemocyte cell.

2 hours after irradiation exposure the hemocytes of 3rd instar larvae were inoculated with E.coli bacteria for 5, 25 and 45 min (Fig. 4.29.). As explained above, due to a technical failure of the facility the 40 and 60 Gy levels could not be conducted for the 2 hour time point. After 45 min of inoculation the number of bacteria per hemocyte seems to be constant. As well, after 5 min of inoculation fluctuation is minor. Nevertheless the 25 min inoculation time point shows an elevated number of E.coli cells per hemocyte at 10 and 20 Gy, indicating a possible activating effect of low level proton irradiation. At 30 Gy this effect is not observed anymore.

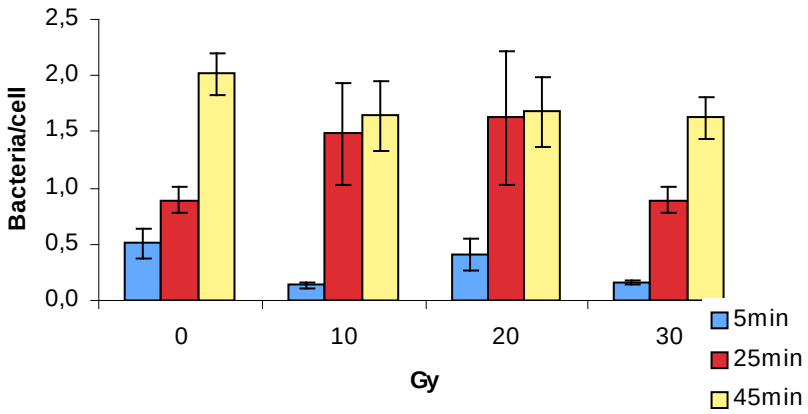


***Figure 4.29.*** 2 hour time point. Number of bacteria per hemocyte 2 hours after irradiation exposure. For each irradiation level 0, 10, 20, 30, 40 and 60 Gy, the hemocytes were inoculated with E.coli bacteria for 5, 25 and 45 min resulting in an increasing level of engulfed bacteria per hemocyte, showing the time course of the reaction to a bacterial infection.

At 14 hours after irradiation bacteria per hemocyte numbers were also calculated for 40 and 60 Gy levels. The 45 min inoculation (Fig. 4.30.) time point shows fluctuation with increasing irradiation exposure. A decreased activity at 10 Gy is recovered with an increased activity at 20 Gy. At 30 and 40 Gy the levels back at the 0 Gy control but the 60 Gy time point shows a strong decreased ability of hemocytes to take up bacterial cells. As well, the 25 min inoculation shows fluctuations with an activating trend for low and a decreasing trend towards the high irradiation levels. Similar results are observed for the 5 min inoculation. These larvae were at the early 3rd instar stage when exposed to proton irradiation.

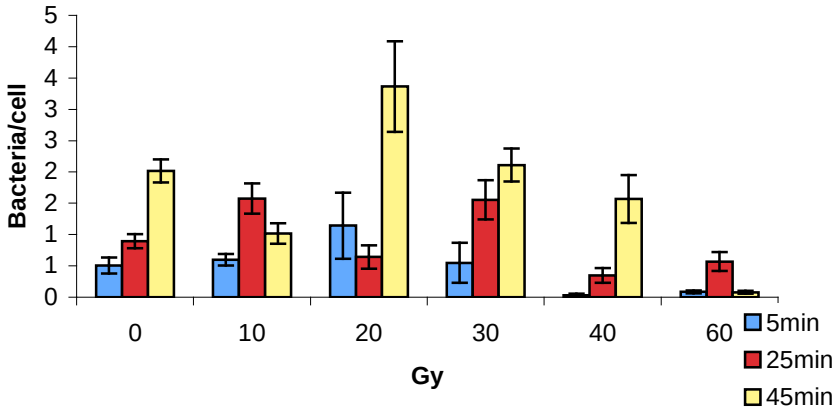


***Figure 4.30***. 14 hour time point. Number of bacteria per hemocyte 14 hours after irradiation exposure. For each irradiation level 0, 10, 20, 30, 40 and 60 Gy, the hemocytes were inoculated with E.coli bacteria for 5, 25 and 45 min resulting in an increasing level of engulfed bacteria per hemocyte, showing the time course of the reaction to a bacterial infection.

The hemocyte activity 35 hours after irradiation is presented in Fig. 4.31. At 0, 10 and 20 Gy the number of bacterial cells per hemocyte seems to be relatively constant. At 30 Gy an activating effect after 25 min of inoculation is observed but not seen after 45 min of inoculation. Both the 40 and 60 Gy exposed larvae show and elevated number of bacterial cells per hemocytes in all their inoculation time points compared to the 0 Gy control. As already explained before, the larval developmental stage could influence the results and need to be considered for interpretation. The 3$^{rd}$ instar larvae used for the 35 hour time point, were 2$^{nd}$ instar larvae during irradiation treatment (Tab. 3.1.).

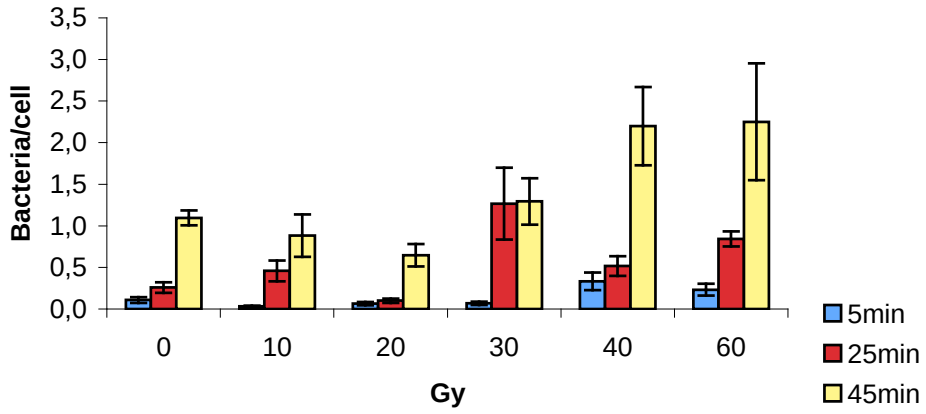


***Figure 4.31.*** 35 hour time point Number of bacteria per hemocyte 35 hours after irradiation exposure. For each irradiation level 0, 10, 20, 30, 40 and 60 Gy, the hemocytes were inoculated with E.coli bacteria for 5, 25 and 45 min resulting in an increasing level of engulfed bacteria per hemocyte, showing the time course of the reaction to a bacterial infection.

57 hours after only the 10 Gy time point overall resembles the results of the 0 Gy control (Fig. 4.32.). The 45 min inoculation time point shows a decreasing trend at the low irradiation levels until 30 Gy. At 40 and 60 Gy hemocytes are able again to take up the same number of bacteria per hemocyte as in the 0 Gy control and even more. After 25 min inoculation the activating trend is also observed in the 40 and 60 Gy levels as in the 45 min inoculation. Only the 20 Gy time point shows additional activation. Already 5 min inoculation resembles this overall activating trend at 40 and 60 Gy. As explained for the 35 hour time point the larvae needed to be at an earlier developmental stage during irradiation, so they would be at the 3$^{rd}$ instar larvae stage 57 hours after the treatment. Therefore, these larvae were exposed at 1$^{st}$ instar stage.

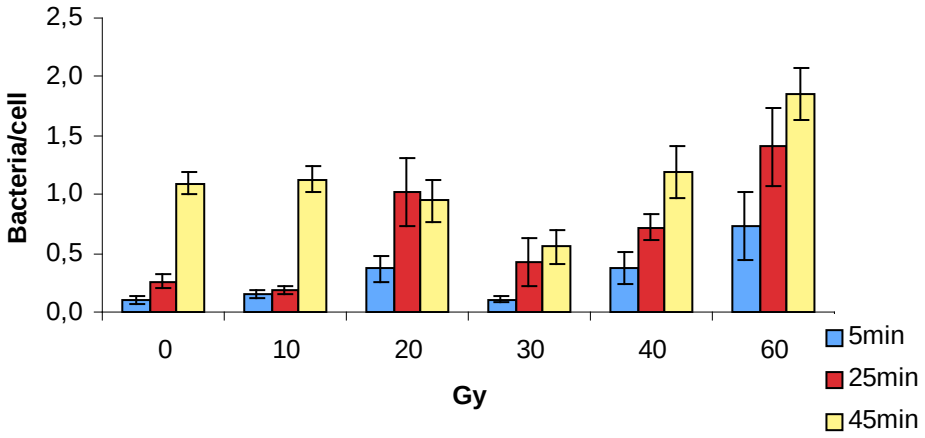


***Figure 4.32.*** 57 hour time point. Number of bacteria per hemocyte 57 hours after irradiation exposure. For each irradiation level 0, 10, 20, 30, 40 and 60 Gy, the hemocytes were inoculated with E.coli bacteria for 5, 25 and 45 min resulting in an increasing level of engulfed bacteria per hemocyte, showing the time course of the reaction to a bacterial infection.

The decreasing, recovering and activating effects of proton irradiation on hemocytes was observed again, similar to the results seen in the first part of the experiment. In contrast, the 2 and 14 hour time points (Fig. 4.29.; 4.30.) show increased trends in hemocyte activity at low levels of exposure. Still, at 40 and 60 Gy at the 14 hour time point the activity decreases. Comparable to the results of the first analysis is the recovery effect observed in the 35 and 57 hour time point (Fig. 4.31.; 4.32.). Both show an elevated activity at 40 and 60 Gy exposure. It has to be pointed out again that these effects could either result from the developmental stage of the larvae during irradiation or for instance to effects like hemocyte activation in response to the removal of radiation damaged cells.

### 4.4.2. Cellsize

As part of the image analysis for the Phagocytosis Assay the cell size of all hemocytes was measured following flight and irradiation. Only the irradiation data is presented herein (Fig. 4.33.), since the flight data has been collected by a colleague and has not been published to date. At 4 time points, 2, 14, 35, and 57 hours after proton irradiation exposure, the diameter of the hemocytes extracted from 3$^{rd}$ instar larvae was measured, averaged and plotted together with the standard error. Due to a technical failure of the irradiation facility the 40 and 60 Gy exposure for the first time point could not be conducted. The hemocyte size two hours after irradiations

remains constant throughout the exposure levels. 14 hours after irradiation the hemocyte size slightly increases at 40 and 60 Gy compared to the lower exposure levels. A similar trend as being observed in the previous time point is also represented in the 35 hour examination. In here, the increase of hemocyte size already starts at 20 and 30 Gy. The last time point, 57 hours after irradiation, shows an increasing trend in cell size, which decreases at 40 and 60 Gy.

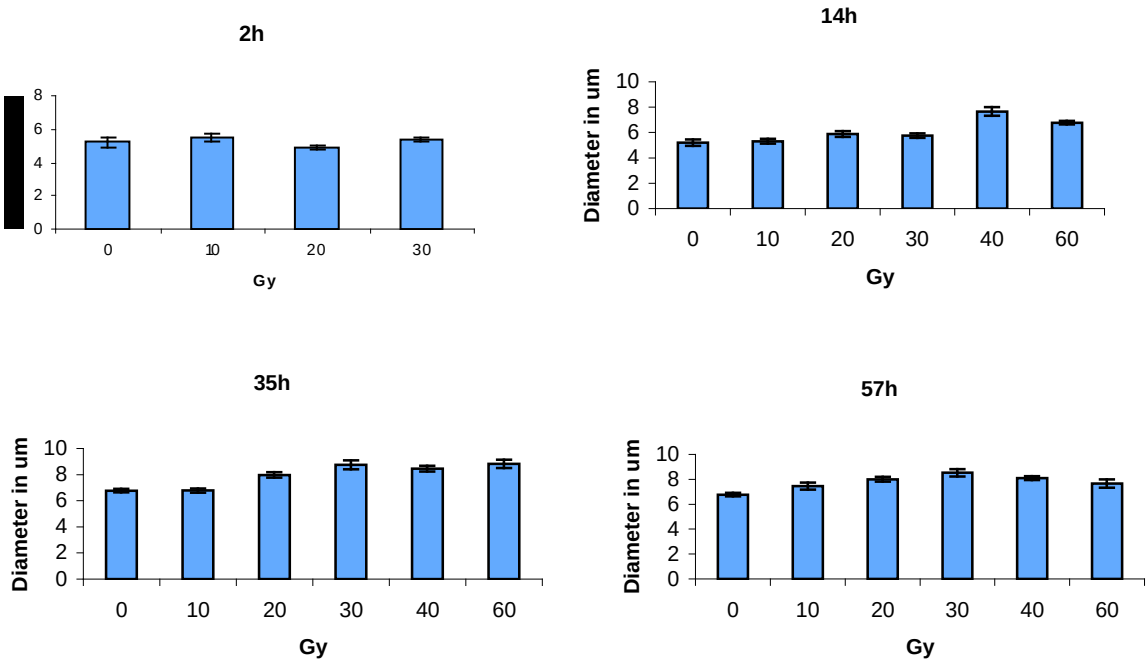


***Figure 4.33.*** Mean hemocyte cell-diameter at different time points following irradiation. At 4 time points, 2, 14, 35 and 57 hours after proton irradiation exposure, the diameter of the hemocytes extracted from 3rd instar larvae was measured, averaged and plotted with the respective standard errors.

It is possible that the observed changes in cell size could influence the activity of hemocytes phagocytosing bacteria, but further investigations will be necessary to confirm this theory.

### 4.4.3. The Clearance assay

To further investigate phagocytosis changes the Clearance Assay (Fahlen et al., 2001; Klein et al., 2000; Penheiter et al., 1997; Brandt et al., 2004) was performed as described in 3.7.3. Adult female flies that were irradiated as 3$^{rd}$ instar larvae were infected with a specific number of streptomycin resistant *E.coli* HB101 bacteria using the picospritzer system. At three time points after infection, 3, 24, and 72 hours, the remaining number of vital bacteria was evaluated by homogenizing

three flies and plating the diluted extract on streptomycin containing agar plates. The number of colonies was counted the following day and the efficiency of the immunological reaction was visualized and calculated (Fig. 4.34.). Since this assay has never been performed following proton irradiation, a first test run (Fig. 4.34. left) containing only low sample numbers was conducted, showing no standard error. A later repeat with lower irradiation intensities and higher animal numbers (Fig. 4.34. right) was successful as well, but showed fluctuations resulting in high error bars.

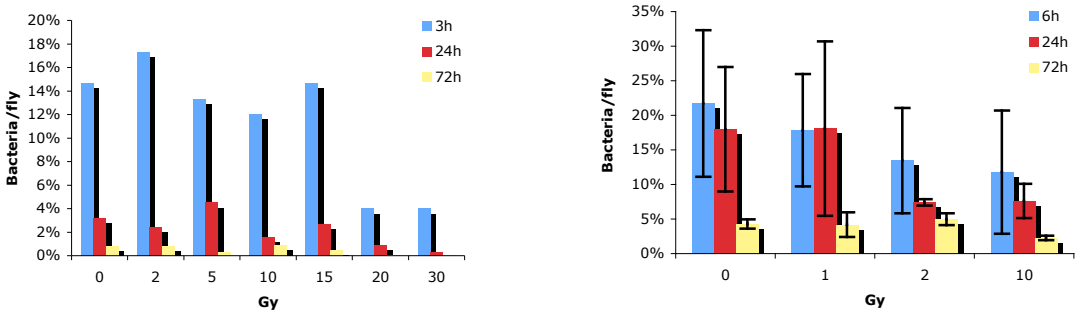


***Figure 4.34.*** Clearance Assay data from first (left) and second (right) run. Adult flies were infected with E.coli bacteria and incubated. After 3 (blue), 24 (red) and 72 (yellow) hours after infection the number of vital bacteria was determined and plotted as bacteria per fly. The X-axis shows the different Gy levels of the exposed animals.

The flies survived the irradiation and infection treatment for the necessary amount of time period and showed a decreasing trend in number of surviving bacterial cells with respect to increased intensities of irradiation. Still, further repeats will need to be conducted to reduce the high standard errors (Fig. 3.34. right). If this trend is confirm, the effect could consequently mean that the immune system clears the bacterial cells faster out of the system, indicating an increased activation due to the irradiation treatment.

# V. DISCUSSION

There is evidence that space flight related factors affect cells, tissues and organs in astronauts resulting in muscle atropy, bone loss, immune system changes and other medical issues (Moore and Oser, 1996; Nicogosian, et al., 1994). As well, behaviour and virulence factors of microorganisms adapt to space flight conditions and may cause severe medical issues upon an infection in space. Manned long duration space missions leading away from Earth have to be prepared carefully by studying all potential threats using model organisms and other available research methods (Borchers et al., 2002; Sonnenfeld 2003).

To study the effects of space flight and space radiation on organisms, several hundred *Drosophila melanogaster* flies were sent to space as part of the FIT experiment onboard a 12-day Space Shuttle mission. Due to strictly limited astronaut time, the only human involvement comprised of switching the food trays between the loaded and empty containers three days into the mission. This act separated the developing eggs that had been laid during the first days of the mission from their parental generation, resulting in new flies that partly or completely developed in space. Due to further technical restrictions none of the adult flies, larvae or embryos were fixed in space, but returned to Earth, and depending on the respective assay, either were treated immediately or kept until developed to the desired stage.

Another part of the FIT experiment included ground based irradiation studies, simulating space radiation exposure. *Drosophila melanogaster* flies were exposed to proton radiation at the Loma Linda University Medical Center in California. Radiation intensities ranged from 1 to 60 Gy covering the amount of exposure that would be experienced by astronauts on long duration missions to the Moon, Mars and beyond, as well as during solar flares or other increased radiation events.

The work conducted for this diploma thesis focused on apoptosis and immune system functions in either space flown or proton irradiated *Drosophila* flies. The link between these two important cellular mechanisms consists of the need to remove dead apoptotic cells by phagocytosis. This final step in the cell-death programme protects tissues from exposure to the toxic contents of dying cells and also serves to prevent further tissue damage by stimulating production of anti-inflammatory cytokines and chemokines. The clearance of apoptotic-cell corpses is important for normal development during embryogenesis, the maintenance of normal tissue

integrity and function, and the resolution of inflammation (deCathelineau and Henson, 2003).

Gene expression studies conducted with microarrays and quantitative Real-Time-PCR showed changes in three genes, – Minibrain, Wengen and Morgue – which are involved in the apoptotic mechanism and immune system, following space flight and bacterial infection. Minibrain is involved in a variety of cellular processes ranging from regulating key developmental and cellular processes such as neurogenesis (Fischbach and Heisenberg, 1984) to cell proliferation, cytokinesis, and induction of apoptosis. Wengen, a member of the tumor necrosis factor (TNF) receptor family binds Eiger, which is expressed during embryogenesis and in response to genotoxic stress. Downstream, Wengen physically interacts with dTraf2, which is involved in activation of Dif and Relish, but also activates the JNK pathway resulting in initiation of apoptosis (Kauppila et al., 2003). Morgue serves as an enhancer of grim-reaper-induced apoptosis by regulating ubiquitiniation processes. (Wing et al., 2002).

To date only the first set of flight and ground samples was tested for Minibrain, Wengen and Morgue gene expression changes. This first set of data still gives an idea about potential difference in gene expression between flight and ground samples triggered by the bacterial infection. Only day 1 flies (Fig. 4.2.) of the flight sample show a decrease in Minibrain expression, which has also been observed in the apoptosis positive controls of Minibrain (Fig. 4.1.). The ground control maintains a positive expression showing a clear difference between flight and ground. Except for the day 1 in the flight sample there is no obvious gene expression change pointing towards an apoptosis initiation, as seen in the positive controls. Since Minibrain is involved in a variety of cellular processes (2.2.2.), a change in expression might not primarily be due to an altered apoptosis rate or reaction to the infection but also be triggered by other stress factors. This mix of inputs might be responsible that no clear pattern can be seen in Minibrain gene expression. Similar problems for data interpretation occurred with the genes Morgue and Wengen. Time points 0 at day 1 (Fig. 4.6.) and day 3 (Fig.4.7.) of Morgue show the only strong evidence for Morgue reacting in combination with apoptosis. Wengen shows a positive fold change in the flight sample of day 1 at time point 0 (Fig. 4.10.) indicating a possible involvement of apoptosis elements. Further repeats of the experiment are necessary for better interpretation of the data.

Another factor to be considered causing difficulties to interpret the data is the different developmental stages of the larvae after the space flight. Only day one flies completely developed in space, but they were of mixed age, which further influenced gene expression. Same aged day three and five flies spent parts or complete time of their pupae stage on Earth, which means a drastic change in environmental conditions during development. These variations might explain different receptivity of *Drosophila* to external factors depending on their developmental stage.

The possibly elevated apoptosis activation level was differently investigated by measuring caspase cleavage speed in 3$^{rd}$ instar larvae. The initiator caspase Dronc contains a CARD domain able to digest a substrate at the VDVAD protein sequence. As well, substrates for initiator caspase Dredd and executioner caspases Drice and Dcp-1 were cleaved faster than ground control samples (Fig. 4.18.; Tab. 4.4.). As explained in 2.2.1., caspase activation in *Drosophila* involves initiator caspases, which activate the executioner caspases. The latter group of proteases are the actual effector enzymes responsible for the break down process. Since substrates for initiator (Dronc, Dredd) and effector caspases (Drice, Dcp-1) were digested at an elevated level in the flight compared to the ground sample, it is reasonable to assume that the apoptotic machinery seems to be in an activated state due to the space flight conditions. In Fig. 5.1., the apoptotic pathways and their effector proteins in *C.elegans*, *D. melanogaster* and Mammals are being compared.

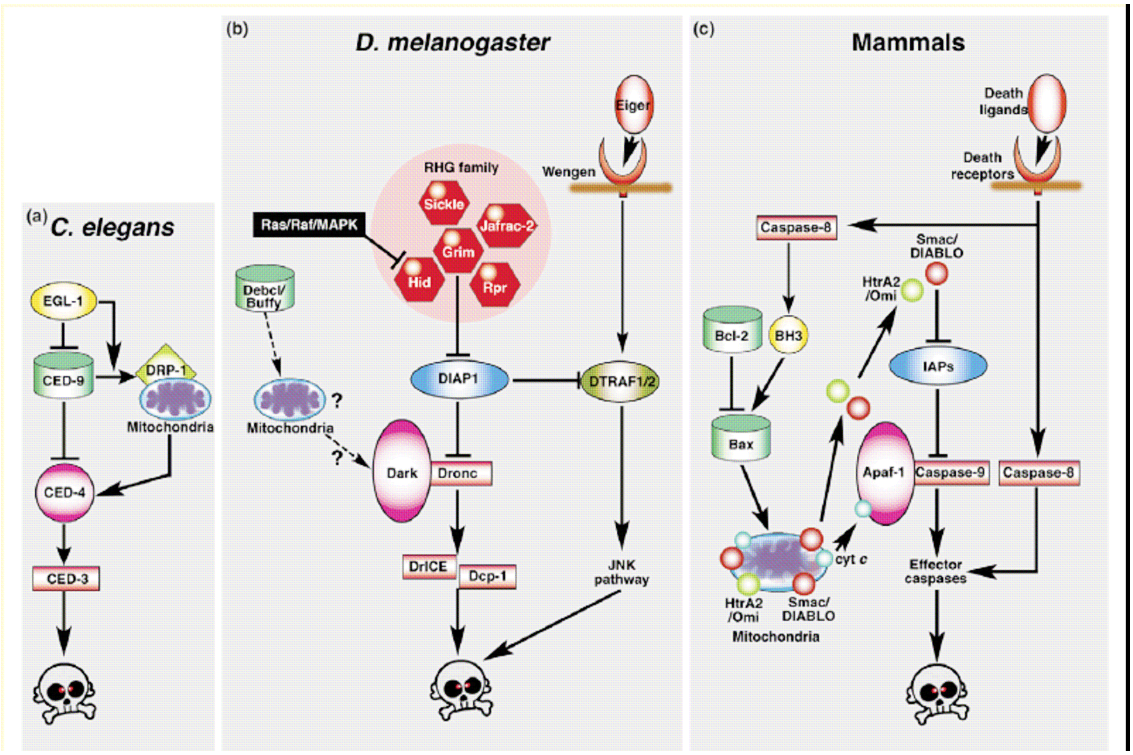


***Figure 5.1..*** Comparison of the apoptotic pathways of C.elegans (a), D.melanogaster (b) and Mammals (c).Functional homologues are represented by the same colour. CED, cell-death abnormal; EGL, egg-laying defective; DRP, dynamin-related protein; Rpr, Reaper; Hid, Head involution defective; dTraf1/2, Drosophila tumor-necrosis-factor-receptor-associated-factors 1 or 2; BH3, Bcl-2 homology-3 (Kuranaga 2007).

Space radiation is the most dangerous environmental threat causing many of the observed problems for long duration space flight (Ohnishi and Ohnishi, 2004). So far, mechanical shielding of space ships is only effective to a certain level. Medical countermeasures may consist of medications to prevent or lessen the effects of space radiation damage for instance with dietary adaptations (Kennedy and Todd, 2003). In case these countermeasures are not successful, it is important to develop therapies to treat the negative effects caused by radiation exposure.

Ground based studies of the FIT experiment included proton irradiation studies of *Drosophila* examining the effects on the animals' health, organs and cells. In this thesis irradiation effects on apoptosis and immune system function in particular were studied. First, a behavioural assay, the Climbing Assay, developed in our laboratory, indicated successful proton exposure by showing decreased motility of flies being irradiated (Fig. 4.4.3.). These effects might be caused by progressive degeneration of neuronal and/or neuromuscular junctions. Application of the Tunel assay (4.3.2.) on developing 3$^{rd}$ instar larvae illustrated an elevated level of DNA degradation with respect to increased proton exposure and developmental stage of the animals indicating an elevated level of apoptosis (Fig.4.22). The expression of

the tumor suppressor protein p53 in proton irradiated *Drosophila* embryos was also shown in accordance with previously publishes p53 expression in response to γ-radiation exposure (Sogame et al., 2003). A p53 radiation responsive element in front of a GFP protein (Brodsky et al. 2000) triggered the expression of GFP with increasing irradiation levels (Fig. 4.23.).

Next, phagocytosis activity of *Drosophila* blood cells, or hemocytes, in response to proton irradiation was measured in 3$^{rd}$ instar larvae with the Alexa Fluor E.coli Phagocytosis Assay (4.4.1.) and in adults with the Clearance Assay (4.4.3.). The Alexa Fluor E.coli Phagocytosis Assay analysis, first, comprised of a comparison of the number of hemocytes containing engulfed bacteria with “empty” cells. Second, the number of engulfed bacteria per single hemocyte cell was investigated. The first analysis showed a decreased activity at a certain irradiation level in all samples. Interestingly, in the 35 and 57 hour time point these decreases were reverted to normal and increased activity, respectively, at the highest exposure levels (Fig. 4.25; 4.26.; 4.27; 4.28.). These effects might be caused by the increasing level of DNA damage, which subsequently triggers the immune systems’ phagocytosing cells. At low level irradiation this pre-infectious activation was not strong enough, but at high intensities might result in the observed recovery effect. Investigations of the phagocytosis capacity of single hemocytes confirmed this possible triggering effect by apoptotic cells. At 35 and 57 hours after irradiation hemocytes were capable of taking up more bacteria when irradiated with 40 and 60 Gy compared to non-exposed animals (Fig. 4.29.; 4.30.; 4.31.; 4.32.). The various profiles of hemocyte activity and efficiency following irradiation treatment are very likely influenced by the developmental stage of the larvae during irradiation. As explained in 3.3., the larvae for the later time points must be younger, 2$^{nd}$ or 1$^{st}$ larvae, so they are 3$^{rd}$ larvae at the time when the assay is performed.

As part of the Alexa Fluor E.coli Phagocytosis Assay the cell diameter of hemocytes was determined (4.4.2.). An increasing trend with higher irradiation exposure was observed, but it is not known to date if difference in cell size has any influence on hemocyte functionality or efficiency (Fig. 4.33.).

The Clearance Assay (4.4.3.) showed a trend towards an elevated level of immune system activity following radiation exposure, and bacterial cells were removed faster than in the untreated control sample (Fig. 4.34.). Although these results were consistent with the trends observed in the Alexa Fluor E.coli

Phagocytosis Assay, further investigations are needed to understand the details and connection between these results. Firstly, the Clearance Assay showed elevated immune system activity at lower irradiation exposure than the Alexa Fluor E.coli Phagocytosis Assay and secondly, the exposed animals were at different developmental stages, adults for the Clearance Assay and 3$^{rd}$ instar larvae for the Alexa Fluor E.coli Phagocytosis Assay.

In conclusion, an increased trend in apoptotic activity was observed after space flight and in flies exposed to proton irradiation. It is hypothesized that the increased apoptosis in flight is caused by an elevated radiation level in space compared to the Earth's surface. Ground based irradiation experiments also showed that immune system function was altered compared to untreated samples, which might be linked to an elevated number of apoptotic cells, which need to be removed from the system by activated hemocytes. Receptivity to proton radiation varied with respect to the developmental stage of the flies during exposure.

Similar space flown experiments to study the effects of the space environment on organisms were conducted with *Caenorhabditis elegans*, for instance during the ICE FIRST mission (Selch et al., submitted). Apoptosis in returned worms was not altered (Higashitani et al., 2005) and immune system functions are still being analysed. Still, several observed gene expression changes were regulated by Insulin-mediated pathways, which are known to interact with autophagy indicating possible undetected changes of programmed cell death.

Future experiments onboard the almost finished International Space Station will involve astronauts with backgrounds in molecular biology and similar areas of expertise capable of conducting more sophisticated research in space to pinpoint, which space environment property causes what response in organisms and consequently humans. So far, no life threatening medical incidents have occurred, that would have led to an abortion of an ISS or Space Shuttle mission, but the fact that future mission to the Moon and Mars will not be able to be aborted easily needs the development of respective countermeasures against infections or other diseases, so astronauts will be able to cure themselves without threatening mission success.

# VI. REFERENCES


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# VIII. Curriculum vitae

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### *Research Experience*

- **NASA Ames Research Center**, USA (August 2006 - present): Post-flight analysis of a Space Shuttle experiment, FIT, examining *Drosophila melanogaster*. Applied methods include fluorescence microscopy, RT-PCR, proton and gamma irradiation, TUNEL, infection, p53 and enzymatic assays.
  Supervisor: Dr. Sharmila Bhattacharya
- **Austrian Space Forum – University Innsbruck**, Austria (Fall 2005 - Summer 2006): Participation in the biology research team, cultivation of desert microbes, development of planetary protection assays for an analogue Mars mission AustroMars at the Mars Desert Research Station in Utah.
  Supervisor: Dr. Brigit Sattler
- **University Leiden**, Netherlands (Fall 2005): Cultivation of a halophilic archaea, *Natronorubrum* HG-1, isolated in Siberia. Examination after simulated Martian conditions, irradiation and desiccation.
  Supervisor: Dr. Pascale Ehrenfreund
- **Australian Centre for Astrobiology**, Australia (Summer 2005): Analysis of extremophilic microbes isolated at a volcanic island and Mt. Hood, USA. Methods performed include DNA extraction optimization, culturing uncultivable microbes and 16S DNA analysis.
  Supervisor: Dr. Roberto Anitori
- **University Vienna** (June 2005): Analysis of cytoskeletal-linker protein plectin. Techniques performed include general DNA analysis methods (PCR, gel electrophoresis, restriction enzyme assays) and operation of gene gun.
  Supervisor: Prof. Gerhard Wiche
- **German Aerospace Centre**, Germany (Spring 2005): Worked on planetary protection focused on optimization of sampling techniques and subsequent DNA extraction for microbial analysis.
  Supervisor: Dr. Petra Rettberg
- **NASA Ames Research Center**, USA (Summer 2004): Post-flight experiments of space flown *Caenorhabditis elegans* worms (ICE-FIRST mission) consisting mainly of micro-array analysis. RNA extraction and sample preparation were the main focus of this work.
  Supervisor: Dr. Catharine Conley
- **University Vienna**, Austria (Spring 2004): Cultivation and protein-isolation in cyanobacteria, HPLC and columns chromatography.
  Supervisor: Prof. Georg Schmetterer

### *Project Management*

**Austrian Space Forum**, Austria (Fall 2005 – Summer 2006: Participated in the main project lead team of the analogue Mars mission, AustroMars. Collective involvement included preparation and execution of a research project, organizing and holding press conferences, managing mission operation schedules and insight into funding structure.

### *Conferences*

- **International Astronautical Congress,** Spain (October 2006): Selection and sponsoring by the European Space Agency included talk and poster about ICE-FIRST.
- **Next Generation Exploration Conference**, NASA Ames (August 2006)

- **15th IAA Humans in Space Symposium**, Austria (May 2005)
- **4th European Astro/Exobiology Network Association** (EANA), UK (November 2004)

### *Education*

University of Vienna (Fall 2001 – September 2007 (anticipated))
Magister (Master of Science), Molecular Biology

### *Publications*

*Presentations by students:*

Selch, F., Szewczyk N. J., Conley, C.A. Worms in space: biological response during a 10-day roundtrip to the International Space Station. Platform Talk: 57th International Astronautical Congress (October 2006).

Selch, F., Szewczyk N. J., Conley, C.A. Worms in space: biological response during a 10-day roundtrip to the International Space Station. Poster: 8th European Space Agency Student Participation Program at the 57th International Astronautical Congress (October 2006).

Selch, F. "Worms in Space"- Molecular Biology on the International Space Station. Invited Seminar: Australian Centre for Astrobiology, Macquarie University (September 2005).

*Presentations by others:*

Zhao, Y., Youds, J., Tarailo, M., Tarailo, S., Rose, A., Selch, F., Szewczyk, N., Conley, C.A. Mutational and genomic response of the nematode *Caenorhabditis elegans* to a 10-day spaceflight. Platform Talk: Mutagenic Consequences of The Space Environment COSPAR Colloquium (2006).

### *Additional Information*

Twelve months of military service for the Austrian Army, which included officers training incorporating technical leadership training and interpersonal seminars.

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