PALACKY UNIVERSITY

Faculty of science

Laboratory of growth regulators

A study of stress tolerance in Tardigrades

Diploma thesis

Author: **Otomar Peško**

Study program: B1501 Biology

Field of study: Experimental biology of plants

Form of study: Present

Supervisor: **Mgr. Jiří Voller, Ph.D.**

The year of presentation: 2020

Bibliografická identifikace

Jméno a příjmení autora	Otomar Peško
Název práce	Studium tolerance želvušek ke stresu
Typ práce	Diplomová
Pracoviště	Laboratoř růstových regulátorů
Vedoucí práce	Mgr. Jiří Voller, Ph.D.
Rok obhajoby práce	2020
Abstrakt	Tardigrada (želvušky) patří k nejodolnějším mnohobuněčným organismům na planetě Zemi. Díky tomu se staly centrem zájmu pro studium odolnosti vůči různým typům stresů. Aby se vyrovnaly s extrémními podmínkami okolního prostředí, vytvořily si rozličné obranné mechanismy v závislosti na typu působícího stresu. Tyto mechanismy ovšem zůstávají do značné míry neobjasněné. Oxidativní stress je častým a potenciálně i fatálním narušením homeostázy. Může vzniknout jak z vnitřních (v rámci metabolismu) i vnějších příčin (např. vystavení radiačnímu záření). Tato práce je jednou z prvních studií metabolické odpovědi želvušek na oxidativní stres. *Hypsibius exemplaris* byl vystaven působení peroxidu vodíku a semipolární metabolity byly analyzovány pomocí UHPLC MS/MS v pozitivním i negativním módu. Je prezentován návrh možného elementárního složení kandidátních antioxidantů.
Klíčová slova	Tardigrada, tolerance ke stresu, oxidativní stres, peroxid vodíku, UHPLC
Počet stran	69
Počet příloh	0
Jazyk	Anglický

Bibliographical identification

Author's first name and surname	Otomar Peško
Title of thesis	Study of stress tolerance in Tardigrades
Type of thesis	Masters
Department	Laboratory of Growth Regulators
Supervisor	Mgr. Jiří Voller, Ph.D.
The year of presentation	2020
Abstract	Tardigrades, one of the most resilient multicellular organisms on the Earth, have been a focus of interest in stress tolerance research. In order to deal with extreme environments, Tardigrades have developed diverse protective mechanisms that remain largely unelucidated. Oxidative stress is a frequent and potentially fatal disturbance of homeostasis. It may arise from both internal (metabolism) and external causes (ionizing radiation). Here we present one of the first studies of the metabolic response of a Tardigrade to oxidative stress. *Hypsibius exemplaris* was exposed to hydrogen peroxide and the semipolar metabolites were analyzed by UHPLC MS/MS operating in both positive and negative modes. Possible molecular formulas of candidate antioxidants are reported.
Keywords	Tardigrada, stress tolerance, oxidative stress, hydrogen peroxide, UHPLC
Number of pages	69
Number of appendices	0
Language	English

I declare I have written this master's thesis by myself with the use of cited literature.

In Olomouc

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ABBREVIATIONS

e.g.	exempli gratia
ESA	European Space Agency
ESI	electrospray ionization
FC	fold change
LD_{50}	median lethal dose
MS	mass spectrometry
RH	relative humidity
ROS	reactive oxygen species
RT	retention time
sp.	species pluralis
UHPLC	Ultra-High Performance Liquid Chromatography
UVR	ultraviolet radiation

1 INTRODUCTION

Tardigrades show remarkable resilience to stress as they can survive extreme temperatures, high salinity, high doses of radiation, desiccation, and vacuum (Møbjerg et al. 2011; Jönsson 2019). To this end, Tardigrades employ diverse strategies and produce a wide range of protective substances (Goldstein 2018; Gabriel et al. 2007). The detailed knowledge of the underlying mechanisms is missing. Moreover, it is highly probable that many protective mechanisms are unknown. With the use of new omics technologies, we can get a better picture of the molecular processes of Tardigrade special abilities.

This thesis's main objective was to analyze metabolic changes in *Hypsibius exemplaris* exposed to hydrogen peroxide. To our knowledge, this is one of the first metabolomics studies of tolerance of *H. exemplaris* to oxidative stress.

2 REVIEW OF THE LITERATURE

2.1 DISPERSAL AND TAXONOMY

The superphylum Panarthropoda consists of Arthropoda, Onychophora, and Tardigrada. This superphylum exhibits a wide range of body plans and differences in morphology (Smith et al. 2016). The phylum Tardigrada is allied with Arthropods and contains more than 900 species. Most of them are limno-terrestrial, living in a terrestrial environment in a thin layer of water on lichens, moss, and algae. Fewer species are purely aquatic, living all their lives in the water supported by algae and bryophytes. *H. exemplaris*, the study object of this thesis, is a freshwater species. Some of the limno-terrestrial species can be found in water, whereas no aquatic species have been found outside marine habitats (Garey, McInnes, and Nichols 2008; Bartels et al. 2016).

Tardigrades are divided into two main classes Heterotardigrada (armored) and Eutardigrada (unarmored). The class Eutardigrada contains superfamily Hypsibioidea with *H. exemplaris* used in this study (Bertolani et al. 2014; Glimel 2013). *Hypsibius dujardini* (Doyère, 1840) [Richters, 1910; *Macrobiotus ursellus* Della Valle, 1915; *Hypsibius murrayi Macrobiotus palustris* Dujardin, 1851; *Macrobiotus lacustris* Dujardin, 1851; *Macrobiotus tetradactylus* Lance, 1896; *Macrobiotus murrayi* Richters, 1907; *Macrobiotus samoanus Richters*, 1908; *Macrobiotus breckneri* Marcus, 1929] was recently redefined as *H. exemplaris.* (Gasiorek et al. 2018; Guidetti and Bertolani 2005).

The tardigrades occur all around the world in very diverse habitats, from hot springs to glaciers. Some of the species are mostly endemic, while others are spread worldwide. The distribution has been supported by geological events forming the terrestrial part of the globe as we know it today. The research has been conducted in all biogeographical regions, and therefore the number of newly discovered species has risen. Thus, we can get a better picture of their dispersal. Overall data suggest that the dispersal of terrestrial tardigrades resulted from passive distribution after the Pangea division (Pilato and Binda 2001).

2.2 ANATOMY

The well-conserved body plan of tardigrades consists of a head segment and four trunk segments with legs (Smith and Goldstein 2017). Tardigrade's body size is less than 1 mm (around 0.1 mm). The body is either translucent or covered with colored cuticle (brown, orange, yellow, green, pink, red). In general, species living in water show lower population density but higher diversity. The body shape differs in aquatic species more than terrestrial (Nelson 2002).

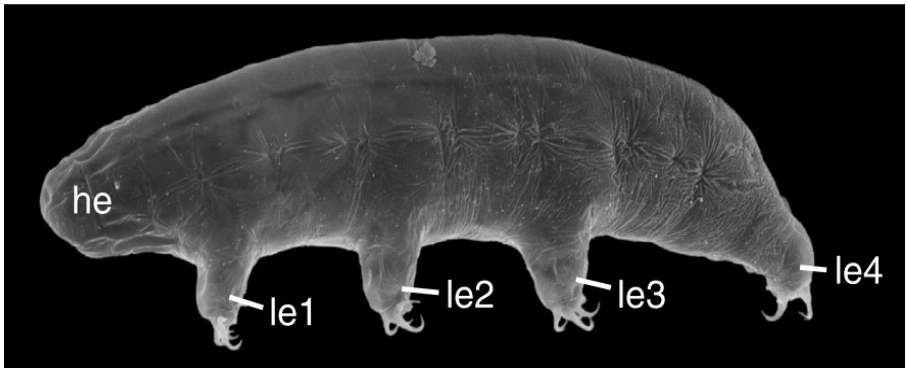

Fig. 1. Scanning electron micrograph of an adult *H. exemplaris* with marked head segment (he) and four legs (le1- le4). Source: (Gross et al. 2019).

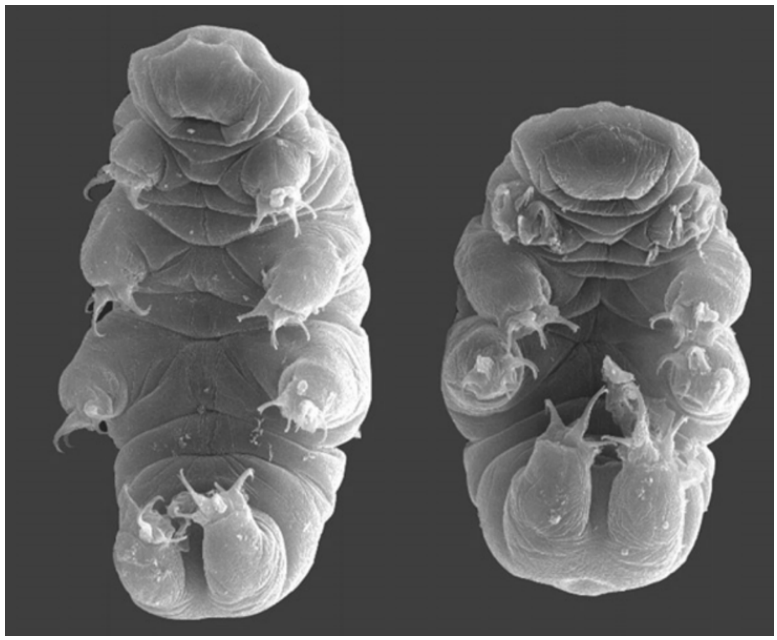

Fig. 2. Scanning electron microscopy image of two adults of species *H. exemplaris*. Source: (Gabriel et al. 2007).

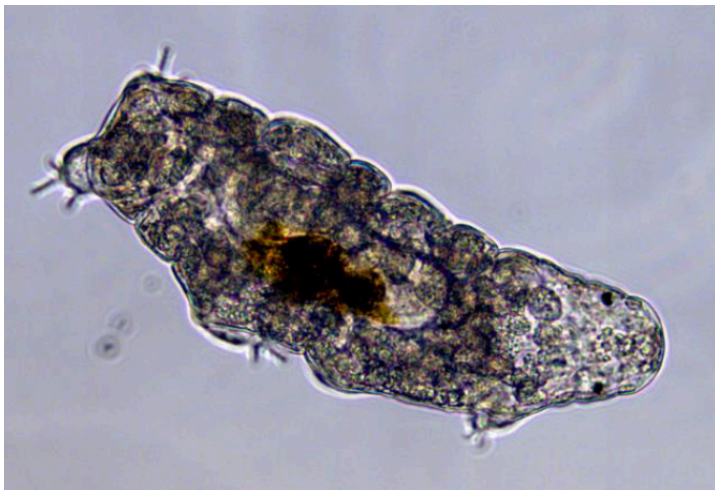

Fig. 3. Image of *H.* exemplaris under light microscope. Source: (Jönsson 2019).

Their movement is ensured by musculature composed of independent elongated cells in the body wall containing slim and thick myofilaments. The muscle cells do not create a compact muscle tissue but can be divided into four muscle groups (dorsal, ventral, dorsoventral, and lateral). The leg muscles vary in number and positioning of muscles. The last pair of legs differs from the others having fewer muscles. In relaxed muscle, zones A, I and H can be recognized. The contractile apparatus of somatic muscles is antagonized by the internal body pressure and is similar to the smooth and striated muscles of some invertebrates (Walz 1974; Halberg et al. 2009).

Due to the development of new imagining techniques (Gross et al., 2019) was able to look inside the body of *H. exemplaris*. With the use of non-invasive X-ray computed tomography (CT), the scientists were able to produce 3D images with high resolution enabling them to measure sizes and volumes of the internal organs. In the studied population, the body size was 152 μm long (without the last pair of legs, adults up to 230 μm) and 32 μm wide with the total volume of 0.14 nl including skin and cuticle. The body incorporates all vital systems present in other groups of tardigrades. The nervous system consists of a brain and trunk ganglions on the ventral side of the body. The digestive system opens with a buccal tube connected to the pharynx, which is accompanied by two salivary glands. Some tardigrades species are carnivorous and therefore, their buccal tube is a bit wider than the buccal tube in herbivore species. *H. exemplaris* can become a prey of bigger species like *Marcobiotus sp.* or *Milnesium sp.* and is a preferable host of parasitic fungus *Ballocephala pedicellata*. However, both carnivorous and

herbivorous species have developed a stylet system which allows them to penetrate the cell of the plant or animal prey (Nelson, Guidetti, and Rebecchi 2015; Glime 2017). Different tardigrade species show different food preferences (Schill et al. 2011). The preferred food of *H. exemplaris* is immobile unicellular algae *Chlorococcus* (Gabriel et al. 2007). The digestive system continues to the esophagus. The esophagus is attaching midgut to the pharynx and it is the most prominent part of the digestive system connected to foregut in the front and hindgut at the back of the body. The excretion apparatus is represented by Malpighian tubules connected to the hindgut. The digestive system is terminated with a cloaca between the third and fourth pair of legs. The reproductive system is represented by and ovary located dorsally of the midgut. (Gross et al. 2019; Nelson 2002). A pair of eyes on the head segment is more complicated than previously thought. The eye itself has two main parts: a single cup cell (pigmented), microvillous cell, and one or two modified ciliary cells. The response to light differs from the conditions used in measuring (e.g. the intensity of light), age or even species (Greven 2007). As a result of their size, there is no need for a unique respiratory system and respiration takes place through the cuticle. The distribution of gasses over the body is ensured by movements of body fluid and coelomocytes (storage cells) in the moving body (Nelson, Guidetti, and Rebecchi 2015). Over one hundred storage cells can be found distributed throughout their body concentrated especially in large areas of open body cavity filled with hemocoel-type of fluid (Gross et al. 2019).

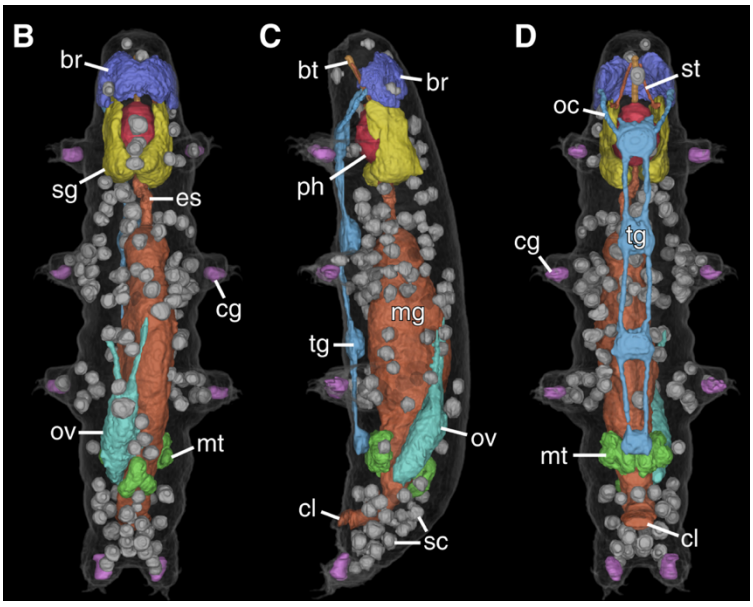

Fig. 4. X-ray nanoCT data 3D image of whole body of adult *H. exemplaris* in dorsal (B), lateral (C) and ventral (D) view. Abbreviations: br (brain), bt (buccal tube), st (stylet), oc (outer connectives), sg (salivary glands), es (esophagus), ph (pharynx), tg (trunk ganglion), cg (claw glands), mg (midgut), ov (ovary), mt (Malpighian tubes), sc (storage cells), cl (cloaca). Source: (Gross et al. 2019).

2.3 LIFE CYCLE

The life cycle of *H. exemplaris* is usually around two weeks but can be prolonged by entering latent life phases. Multiple reproductive strategies occur in the phylum of Tardigrada, depending on their natural habitat. Marine and limno-terrestrial species are usually gonochorists with the exceptional occurrence of hermaphroditism. In non-marine tardigrades, the most common reproductive model is thelytokous parthenogenesis. Although *H. exemplaris* reproduces by this model, (Bertolani 2001) implies that there is a possible occurrence of hermaphrodites in the *Hypsibius* genus. In thelytokous parthenogenesis females lay diploid eggs that undergo meiosis followed by reduplication of the chromosomes to keep a diploid set of chromosomes. As soon as the female exits the exuvia with one-cell embryos left inside, the meiosis takes place. Due to that, a single individual is needed to reproduce and facilitate the colonization of new tardigrade habitats. (Gabriel et al. 2007; Nelson 2002; Bertolani 2001).

The cuticle of tardigrades is flexible but doesn't grow with their body. Therefore, molting is necessary for body growth. The same process is typical in their relatives Arthropoda. Molting itself takes between five to ten days and occurs

several times during tardigrade's life (four up to twelve times). With every molting body length and size changes. The body grows faster in the early stages of life and continues to grow even after reaching sexual maturity. The growth rate highly depends on nutrition. When molting, not only the outer surface of the body is detached, but also the entire lining of foregut, hindgut, stylets, buccal tube, and claws. During this period, they cannot feed due to the lack of sclerified part in their digestive apparatus. The exuvia (old "skin") not only served previously as protection of body but many tardigrade species use the exuvia as a cover for up to 40 laid eggs (Nelson 2002; Nelson, Guidetti, and Rebecchi 2015; Glime 2017). The eggs hatch about four days after the exuvia separation (Goldstein 2018).

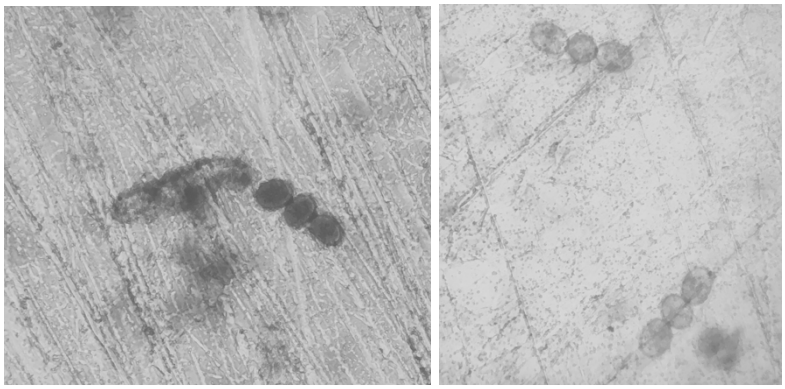

Fig. 5. Images from light microscope of an adult *H. exemplaris* laying 3 eggs (left image) and 2 transparent exiuvias with 3 eggs inside (right image). Source: author of this thesis.

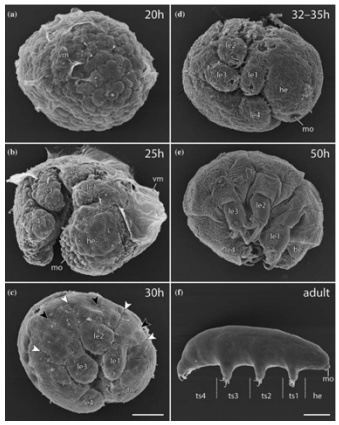

Fig. 6. Development of *H. exemplaris* from an embryo to an adult. Abbreviations: he (head segment), le1–le4 (leg 1–4), mo (mouth opening), ts1–ts4 (trunk segments 1 to 4), vm (vitelline membrane). Scale bars: c (for a–e), 10 mm; f, 30 mm. Source: (Gross, Minich, and Mayer 2017).

2.4 CRYPTOBIOSIS

Limited mobility and life in aqueous microhabitats require adaptations to be rapid and extreme changes in the surrounding environment. Four pairs of legs with claws and microscopic size enable tardigrades to live on the surface of small leaves of bryophytes, liverworts, in the interstitial spaces of terrestrial algae, soil or leaf litter. Although they can live on land, tardigrades are aquatic animals and still need a thin layer of water to survive (Glimel 2013; Wright 2001). Due to lichens, liverworts and other plants' ability to endure almost complete desiccation and then return to normal state (when water is abundant), the organisms living in these habitats had to adapt to such conditions.

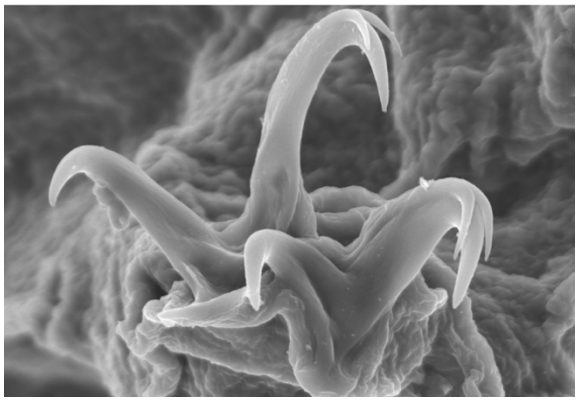

Fig. 7. Scanning electron microscope image of claws of *H. exemplaris*.
Source: (Gasiorek et al. 2018).

Tardigrades have developed strategies to deal with such unfavorable conditions by entering so-called quiescence or diapause. Returning to the normal state is possible at any stage of their life. Surviving extreme conditions is closely linked with cryptobiosis. Anton van Leeuwenhoek first used this term in the 17th century. Thanks to the development of experimental methods and understanding of physiology nowadays, the description of cryptobiosis has slightly changed. The term refers to entering a reversible latent ametabolic state caused by desiccation (anhydrobiosis), osmotic stress (osmobiosis), freezing (cryobiosis), anoxia (anoxybiosis) or high concentrations of toxic substances (chemobiosis) (Wright 2001; Bertolani et al. 2004; Neves et al. 2020). Phylogenetic studies suggest that cryptobiosis developed independently twice in tardigrades during evolution. The absence of this ability in certain species is probably a result of subsequent loss

due to specific selective pressure rather than the original trait. (Guidetti, Altiero, and Rebecchi 2011).

Nowadays we understand that cryptobiosis depends on the level of liquid body water. Although the body of tardigrades is covered with the protective cuticle, they are dependent on water. Tardigrades adapted to these situations by forming a unique life stage called a tun. A tun is a tardigrade latent state with significant metabolic depression made to overcome unfavorable or stress conditions. By forming a tun tardigrades create plenty of folds to decrease the body surface. That helps to slow down water loss in preliminary desiccation (it makes the water loss slower) and protects the internal organs, followed by an extreme decline in cuticle permeability during dehydration. The most desiccation-tolerant species infold the most significant parts of their body (Wright 2001; 1989).

Tardigrades enter anhydrobiosis as soon as the surrounding water evaporates and form a tun to protect internal organs and secure survival. The main movements in the tun formation are longitudinal contraction and invagination of legs and intersegmental cuticle. Cuticle works as a primary barrier of transpiration during initial drying and differs in thickness between the segments. The decline in cuticle permeability and decrease of the surface (hiding the thinner and more flexible cuticle parts inside) supports the hypothesis of tun-formation as a process improving the stress-tolerance of tardigrades. The tun formation is an active process and not just an effect of changes in body volume caused by water loss. (Wright 2001). The musculature moderates structural changes which are crucial for successful tun formation. Remaining systemic integrity is the key to returning to the active state (Halberg, Jørgensen, and Møbjerg 2013). When tardigrades undergo desiccation in anoxia, the body collapses and flattens and cannot return to the normal state when rehydrated. There is a correlation between tolerance to desiccation and tun formation (Wright 2001).

Fig. 8. Scanning electron microscopy image of a tun of *Richtersius coronifer*. Source: (Persson et al. 2011).

There are 3 most important factors for successful cryptobiosis: loss of most free and bound water, suspension of metabolism, and synthesis of protectants. By reducing the body surface and forming a tun, tardigrades can survive for decades (Bertolani et al. 2004).

To test a long-term survival of tardigrades, various tardigrade species samples were assembled from museum and private collections of mosses and lichens aged from 9 to 138 years old. After rehydration, none of the adults showed any signs of life right after or in 5 days following rehydration. Their internal organs were indistinguishable, and their cuticle unusually stretched almost resembling the shape of an adult. From eggs of all tested species in this study (*Ramazzottius oberhaeuseri*, *Richtersius coronifer*, *Macrobiotus hufelandi* group and *Milnesium tardigradum*) only 4 eggs of *Ramazzottius oberhaeuseri* hatched after 9 years in anhydrobiosis. No eggs from other species revived. The author implies a connection between tolerance to long term anhydrobiosis and tolerance to rapid desiccation. The leading cause of high mortality after long term desiccation is probably oxidation. Oxidation can damage DNA and gives rise to toxins. The vitality decreases with more extended time spent in anhydrobiosis. In avoiding damage during anhydrobiosis, reactive oxygen species (ROS) scavenging enzymes play an essential role (Guidetti and Jönsson 2002; Erdmann and Kaczmarek 2017).

Observing bdelloid rotifers coming back to life from dehydrated moss samples led Anton van Leeuwenhoek in the 17th century to define anhydrobiosis, a state caused by desiccation (Wright 2001). Water bears are not the only

anhydrobiotic organisms. This ability also occurs in brine shrimp *Artemia salina* (its' encysted gastrulae), rotifers, yeast, and bacteria as well as in "resurrection" plants (e.g. *Selaginella lepidophylla* or *Craterostigma plantagineum,* some ferns, seeds, and lichens) (Crowe 2002). Most of higher plants contain or create specific tissues adapted to overcome dehydration (e.g., seeds, pollen). However, vegetative tissues of resurrection plants can survive almost full dehydration and return to active life within 48 hours. This outstanding ability is granted by the accumulation of high doses of sucrose, which protects enzymes and cellular structures in a dehydrated state (Scott 2000).

2.5 THE EFFECT OF VARIOUS TYPES OF STRESS ON TARDIGRADES

Tardigrades have been recommended for biological research as a model organism, same as *Caenorhabditis elegans* or *Drosophila* sp. *H. exemplaris* was recommended as a model organism for the research of the evolution of developmental mechanisms because of its' suitable features. Its´ small and compact genome (haploid genome size of around 75 Mb, 5 pairs of chromosomes). The possibility to keep them at room temperature or freeze them and bring them back to life when needed. The fact that embryos are easy to observe in the microscope (the embryos are optically clear) and eggs are laid in small groups in a clear molt (Goldstein 2018; Gabriel et al. 2007).

2.5.1 HYDROSTATIC PRESSURE

One of the very challenging physical conditions is high hydrostatic pressure. For most organisms, the upper limit of hydrostatic pressure is 0.3 GPa. To establish much higher pressure, the scientists used a two-stage cubic anvil high-pressure apparatus to generate a pressure of 7.5 GPa. This hydrostatic pressure equals the value of pressure 180 km below the Earth's surface. Tested groups of wild adults of *Milnesium tardigradum* underwent an effect of 7.5 GPa in different exposure times. At first, 20 minutes at 7.5 GPa did not seem to have any dramatic impact on viability as 18 out of 20 tested individuals were alive. In the following experiments, the exposure times were prolonged to 3, 6, 12, and 24 hours. After 3 hours of exposure, all specimens were alive right after exposure. However, mortality rose faster than in control. After 6 hours of exposure, all individuals were alive. Nonetheless, they could not move since their legs were damaged, and all tested

individuals died within 6 days after exposure. After a 12hour exposure, only a few individuals were found alive. Only after 24hour exposure, all individuals were found dead. Based on these results, a survival limit was estimated to 13 hours of exposure at 7.5 GPa (Ono et al. 2008). This result is outstanding when compared to normal atmospheric pressure of 101.325 Pa.

2.5.2 TEMPERATURE

Thermo-tolerance of tardigrades is exceptional. The tolerance of various tardigrade species to low temperatures was tested, for example, in space or in Antarctica. The survival rates of individual species alter. In freeze-tolerance it is essential to control or inhibit intercellular freezing and prevent cellular damage. Tun formation is not crucial for survival in the cold. Fast freezing, followed by light supercooling, can be lethal for cells (risk of cytoplasmatic freezing). The strategy of freeze-tolerance varies between tardigrade species. Some rely on the synthesis of protein cryoprotectants, some on saccharides or other cryoprotectants which determines the cryo-tolerance limit (Wright 2001).

For example, the survival rates of *Richtersius coronifer* in anhydrobiosis after exposure to liquid nitrogen (approximately -196°C) for 30 minutes and 120 minutes did not differ from control. Only after exposure of 20 hours, the survival rates started to decrease. For hydrated state (active animals), the initial survival rates after the 30 minutes exposure were around 30 % lower and after 120 minutes exposure time around 50 % lower compared to the dehydrated state (Persson et al. 2011).

Sømme et al., tested cold tolerance of *Echiniscus jenningsi*, *Macrobiotus furgifer* and *Diphascon chilenense.* In hydrated state low mortality rate was reported in all tested groups after exposure to -22 °C for 600 days and in a dehydrated state for 3040 days. After exposure to -80 °C from 7 to 150 days, hydrated specimens of *Echiniscus jenningsi* showed higher mortality, but samples of *Macrobiotus furgifer* and *Diphascon chilenense* did not show any change in mortality in the same conditions. Whereas the temperature of -180 °C killed all hydrated specimens, the dehydrated animals of all three tested species showed good survival after 14 days at -180 °C. These results confirm that tardigrades can survive extremely low temperatures for extended periods. However, tardigrades in

anhydrobiotic state can deal with freezing better than in hydrated (active) state (Sømme, L. , Meier 1995).

Results of the survival of tardigrades in high temperatures differ among many studies. In early 19th century studies report the highest temperature with recorded survival of *Macrobiotus hufelandi* was 151 °C for 15 minutes of tardigrades in anhydrobiosis (Rebecchi, Altiero, and Guidetti 2007).

A different study explored the effects of a wide range of temperatures (from 60 °C to 110 °C, one hour exposure) on multiple species of heterotardigrades and eutardigrades (*Milnesium tardigradum*, *Paramacrobiotus richtersi*, *Macrobiotus tonollii*, *Macrobiotus sapiens, Echiniscus granulatus, Echiniscus testudo*). A small and significant decrease in survival was reported after the exposure to 60 °C in heterotardigrades *Echiniscus granulatus* and *Echiniscus testudo* and the eutardigrades *Paramacrobiotus richtersi* and *Macrobiotus tonollii*. Increasing the testing temperature to 75 °C and 80 °C resulted in a subsequent decrease in survival in all tested species. Interestingly, the survival rates at 90 °C dropped in most of the tested species except for *Echiniscus granulatus* with the survival of 54 % and *Milnesium tardigradum* with a survival rate of 96,7 %. Only three species survived the exposure to 95 °C, *Echiniscus granulatus,* and *Echiniscus testudo,* both with survival around 3 % and *Milnesium tardigradum* with survival rates of 90 % after an hour in 95 °C and 91.7 % after an hour in 100 °C. Above 100 °C, the survival rates dropped, but even after 102 °C, some individuals of *Milnesium tardigradum* survived (1.7 % survival rate) (Hengherr et al. 2009).

In a different study, high thermo-tolerance (up to 70 °C) was observed in *Richtersius coronifer*. This species showed no change in mortality after exposure to 70 °C for 60 minutes. Above 70 °C, the survival rate decreased quickly (to 20 % at 80°C), and no animal survived exposure to 100 °C (Ramløv and Westh 2001).

The results of experiments with active and latent states of *Ramazzottius varieornatus* (freshwater species) suggest that some tardigrade species have an upper limit of thermo-tolerance. The desiccated specimens reached 50 % mortality after exposure to 82.7 °C for an hour and a rapid decrease of the lethal temperature to 63.1 °C for 24 hours of exposure time. In comparison to active non-acclimated animals, desiccated specimens were more tolerant. Active non-acclimated tardigrades reached 50 % mortality after exposure to 37.1 °C after 24

hours exposure, and acclimated animals reached the same level of mortality at 37.6 °C in the same conditions. In general, without a doubt, the individuals in anhydrobiosis can tolerate higher temperatures than active animals. However, a short period of acclimation can increase the tolerance of active animals slightly to high temperatures (Neves et al. 2020).

2.5.3 OXIDATIVE STRESS

Oxidative stress caused by an imbalance between producing reactive forms of oxygen (ROS) and antioxidant protection has multiple adverse effects on living organisms. Free reactive oxygen radicals possess one or more unpaired electrons, which make them extremely reactive. ROS can damage macromolecules like DNA, lipids, and proteins, majorly affect any organism, but some ROS can work as a signal molecule (Liguori et al. 2018). Exposure to radiation (e.g., UVC or ionizing radiation) also causes DNA damage (e.g. thymine dimers formation). However, tardigrades are equipped with mechanisms capable of repairment of this damage. The possible mechanism of their DNA repair ability is based on the occurrence of a photo-dependent repair enzyme, working only in the presence of light (Horikawa et al. 2013).

2.5.4 OSMOTIC STRESS

Tardigrades have to cope with osmotic stress living in aquatic habitats or in a thin layer of water. There are two ways how to deal with osmotic stress: entering a dormant state (osmobiosis) or keeping a high metabolic rate. Marine tardigrades in an active state react to changes in salinity with changes in their body volume. Limno-terrestrial cryptobiotic eutardigrades keep their body fluids hypertonic to the surroundings by excreting hypotonic fluid and are less tolerant to high salinity than aquatic tardigrades. In high salinity, cryptobiotic eutardigrades in an active state become less active and later die. The upper lethal limit of salt for *Richtersius coronifer* was set at 500 mOsm/kg. Further analysis of body fluids will be needed to decode and fully understand the mechanisms of osmoregulation in tardigrades (Møbjerg et al. 2011).

2.5.5 RADIATION

2.5.5.1 IONIZING RADIATION

Ionizing radiation is a challenge for all living organisms. Several studies reported that tardigrades belong to most radiation-tolerant organisms. Ionizing radiation is dangerous because it can ionize atoms and create ions which cause consequential damage. Radiation-tolerance depends on intracellular radio-sensitivity and the affected stage of life. Genetic information is essential for the survival of any living organism, and therefore, it is damage can be fatal. The effect of radiation can be either indirect, when water reacts to radiation resulting in radiolysis and creation of dangerous and very reactive free radicals able to damage DNA. Another possibility is a direct effect on DNA resulting in ionization of atoms in DNA itself. Radiation can cause single-strand breaks, double-strand breaks or base damage of DNA. There are DNA repair mechanisms which can minimize the damage but have limited power due to complexity of the damage. Besides tardigrades among radiation-tolerant species are for example brine shrimps, larvae of chironomids or bdelloid rotifers. Whether radiation-tolerance is based on the level of water content is or not entirely clear. A common explanation is an association with anhydrobiosis. Adaptation to natural stress, desiccation, in this case, includes activation of mechanism protective against water loss. Low water content possibly lowers the chance of indirect DNA damage and relates to a higher concentration of protectants. On the contrary, (Jönsson, Harms-Ringdahl, and Torudd 2005) suggest that radio-tolerance is not based on biochemical protectants but relies on more effective DNA repair mechanisms (Schill et al. 2018; Rebecchi, Altiero, and Guidetti 2007; Jönsson, Harms-Ringdahl, and Torudd 2005). In more contemporary study, new omics technologies propose possible involvement of avoidance of DNA damage as well as mechanisms of DNA repair (Jönsson 2019).

(E. Beltrán-Pardo et al. 2015) tested the effect of gamma radiation on various life stages of *H. exemplaris*. $LD_{50/48h}$ of adult individuals was around 4 180 Gy. Doses higher than 100 Gy reduced the hatchability of laid eggs and lowered the fertility of adults exposed. They reported a difference in reaction in embryos in the early and late embryonic development stages. Late embryos did not display any developmental damage, while exposure to doses of 50, 200, and 500 Gy on

early embryos reduced their hatchability. Survival of those juveniles able to hatch from exposed eggs was profoundly affected by 500 Gy exposure. However, irradiation by 50 Gy and 200 Gy did not have such destructive power over juveniles (they were able to grow to adult stage and produce offspring), but the fertility was lower compared to controls. (Beltrán-Pardo et al. 2015) implies that the reason for the higher damage extent in earlier developmental stages is higher mitotic cell activity.

(Fernandez et al. 2016) reported that the bystander effect is also increasing mortality in experiments with *H. exemplaris*. Individuals were irradiated directly and indirectly by gamma radiation of 3 000 Gy and 5 000 Gy. From each directly irradiated group (3 000 Gy and 5 000 Gy), one individual was put to a non-irradiated group of 14 individuals. After that, the survival rates were recorded immediately and 2 hours after exposure. Then the survival of all individuals was recorded until death. Both direct and indirect irradiation decreased the survival compared to control. Individuals exposed to 3000 Gy had higher survival rates than those exposed to 5 000 Gy. On the other hand, no difference was found between different doses in bystander groups.

Because mitotic cells are more radiosensitive and tardigrades are eutelic organisms (with a “fixed” number of cells in adult life), the risk of cell damage is lower. *H. exemplaris* is not the most radiation-tolerant species among other tardigrades. In tardigrades the limno-terrestrial tardigrade species are the most desiccation and radiation-tolerant. Therefore, the higher tolerance in adult stages in *H. exemplaris* implies other defense mechanisms than connection between defense to desiccation and radiation-tolerance. Interestingly tardigrades show similar mortality rates after exposure to radiation in a hydrated and dehydrated state. Damage of DNA is the main problem caused by radiation. To compare the extent of radiation’s damage, for humans, it is LD_{50} of ionizing radiation 4 Gy, mice 7 Gy, rat 6 Gy, rabbit 8 Gy, monkey 4,5 Gy, goldfish 7,5 Gy, cockroach 50 Gy. (Hashimoto and Kunieda 2017; Bolus 2001).

2.5.5.2 UV RADIATION (UVR)

The impact of UVR depends on the UV wavelengths organism was exposed to. Short-waved UVC and shorter waved part of UVB damage DNA directly, causing possible crosslinking. Longer waved UVA, and longer waved part of UVB have an indirect impact on DNA by creating reactive oxygen species (ROS) that generate consequential damage in DNA, proteins, etc. Since UVR is present on the Earth, organisms have developed mechanisms on how to protect themselves from UV caused damage. The possible protection can be antioxidants, DNA repair proteins, or pigmentation.

(Horikawa et al. 2013) tested DNA damage in hydrated and dehydrated *Ramazzottius varieornatus* and hydrated *H. exemplaris*. The assessment of DNA damage was carried out based on the content of UVC-induced thymine dimers after the exposure to UVC as well as DNA repair mechanisms. In hydrated state exposure to UVC irradiation of 2.5 kJ/m^2 and more caused death in *H. exemplaris* while in *R. varieornatus,* around 80 % of exposed individuals were active 5 days after irradiation. None of *R. varieornatus* survived a dose 10 kJ/m^2. In contrast, dehydrated states showed higher tolerance to UVC radiation. Individuals in anhydrobiosis survived doses of 2.5 kJ/m^2, 5 kJ/m^{2,} 10 kJ/m^2 and 20 kJ/m^2. In dehydrated state, individuals after exposure to all doses tested produced eggs after rehydration (211 eggs in total after 2.5 kJ/m^2). *R. varieornatus* laid 162 eggs in total after exposure to 2.5 kJ/m^2 but any after a higher dose of UVC. No significant correlation was found between hatchability and exposure to UVC. *R. varieornatus* is a terrestrial species and therefore is supposed to be more radiation-tolerant than aqueous *H. exemplaris* due to adaptation to his rapidly changing natural habitats. *R. varieornatus* in anhydrobiosis accumulated much fewer thymine dimers caused by exposure to UVC than in a hydrated state. Therefore authors imply that anhydrobiosis helps to prevent DNA damage caused by UVC radiation (Horikawa et al. 2013).

2.6 SPACE CONDITIONS

Stress-tolerance of tardigrades inspired the creation of numerous creative experiments to test their survival in a wide range of extreme environments. Because of tardigrade high radiation-tolerance, they were suggested as a suitable model organism for space research (Erdmann and Kaczmarek 2017; Horikawa 2008; Jönsson 2007). In cooperation with European Space Agency (ESA), tardigrades were sent to space and were exposed to open space radiation, solar ionizing radiation, UV solar radiation, and multiple types of stresses combined. In space, the most challenging conditions are radiation, absence of oxygen, very low temperatures, and desiccation. Therefore, any organism able to overcome these conditions must be well adapted. Among the organisms able to survive in such environment are some species of Bacteria, Rotifera, Archea, Nematoda, Arthropoda, and Tardigrada. The result of the space studies show that tardigrades can survive space conditions. However, any additional stress (UV, ionizing radiation or cosmic radiation) significantly reduces their survival rate (Erdmann and Kaczmarek 2017).

2.7 PROTECTIVE MECHANISMS

In general, any organism can either protect itself from damage or develop a repair mechanism for originated damage. (Schill et al. 2018) Most animals that can overcome desiccation are those who are most radiation tolerant. That implies a possible link between adaptation to desiccation and tolerance to other types of stresses (as mentioned above). Therefore, the mechanisms of how organisms cope with this type of stress is essential.

(Wright 1989) tested desiccation tolerance of a strain of *H. exemplaris*. He reported that the preconditioning of animals increases the survival in lower relative humidity (RH). *H. exemplaris* need 150- 200 minutes dehydration in 85 % RH to survive in RH around 20 % (Wright 1989). (Kondo et al. 2015), examined the effect of different preconditioning periods to survival in low RH in *H. exemplaris*. Without any preconditioning, *H. exemplaris* did not survive in 10 % RH. Exposing animals to 95 % RH for a day increased the survival to almost 100 %. Longer preconditioning (up to 4 days) did not differ the survival. When varying the RH in preconditioning, groups preconditioned in 33.5 % RH and 62 % RH had very low survival. In contrast, animals preconditioned in 85 % RH had almost the same

survival as those preconditioned in 95 % RH. As a part of this experiment, they used inhibitor of transcription α-amanitin, which subsequently reduced recovery rates in animals exposed to severe desiccation. To confirm that used inhibitor is not just toxic for tardigrades, the same inhibitor was applied to groups exposed only to 95 % RH, and the survival rates did not change. These results imply that *H. exemplaris* needs some time during preconditioning for the production of protectants for successful anhydrobiosis, and these products desire de novo transcription and translation (Kondo, Kubo, and Kunieda 2015)

To successfully undergo desiccation various organisms, produce numerous types of protectants. Some organisms like yeast and metazoans (animals) produce disaccharides (trehalose or sucrose) or glycerol substituting the free water loss. (Boothby et al., 2017) reported that *H. exemplaris* does not produce trehalose since it does not contain trehalose phosphatase, enzyme required for trehalose synthesis. Despite that, *H. exemplaris* shows high desiccation tolerance (Boothby et al. 2017a). Trehalose protects nucleic acids, lipid membranes, and proteins. It creates hydrogen bonds between cellular membranes and maintains the native structures unharmed. Though alternative explanation postulates that hydrophilic molecules enter a glassy state which suppresses any possible damage. Some scientists report that not all tardigrades produce trehalose, and those who do, do not make the same quantity (Wright 2001; Erdmann and Kaczmarek 2017; Wełnicz et al. 2011; Boothby et al. 2017a).

In research, scientists focused on the synthesis of trehalose, and its' possible practical use in a fight with pathogens. Trehalose is important for virulence of a deadly insect pathogen *Beauveria bassiana*. Experiments with genetic manipulation (deletion) of trehalose phosphatase genes led to decreased virulence, decreased conidial thermotolerance, decreased tolerance to UVB, and increased sensitivity of hyphs to chemical stress which has possible implications in development of new fungicides (Qiu et al. 2020).

Another example is human yeast pathogen *Candida albicans*. Accumulation of trehalose shields yeast cells from macrophages. Strains of *Candida albicans* unable to synthesize trehalose were less tolerant to macrophage attack because of lower tolerance to oxidative stress inside the phagolysosomes as a reaction to defective trehalose synthesis (Martínez-Esparza et al. 2011).

On the contrary, in *Aspergillus fumigatus,* the absence of TpsA and TpsB gene activity of trehalose-6-phosphate synthase higher the virulence in mice model. *Aspergillus fumigatus* is an opportunistic human pathogen. In an immunosensitive patients (leukemic patients, people with transplants), *Aspergillus fumigatus* can infect lungs and cause a severe infection called Aspergillosis with possible fatal outcome. In the experiment a double mutant of genes of trehalose synthase caused hypervirulence possibly due to modified cell wall synthesis. Hyphae and conidias of a double mutant tpsAB lost electron-dense outer layer resulting in bypassing the host immune reaction. Double mutant conidia were also less tolerant to oxidative stress caused by hydrogen peroxide (100 mM) compared to single mutants and wild type implying an important role of trehalose as an antioxidant (Al-Bader et al. 2010).

Antioxidant activity of trehalose was also reported in experiments with oxidative stress using hydrogen peroxide. Exposure to oxygen radicals stimulates trehalose accumulation in *Saccharomyces cerevisiae,* in leading to increased survival rate. Not being able to produce trehalose, mutants of *S. cerevisiae*, contained a much higher concentration of damaged proteins even after a short exposure to hydrogen peroxide (Benaroudj, Lee, and Goldberg 2001).

During desiccation, the DNA damage usually occurs, but tardigrades developed an effective system how to protect themselves. In recent studies, (Hashimoto et al., 2017) reported Dsup (tardigrade-unique DNA-associating protein) as a possible protectant. This protein was isolated from chromatin of tardigrades, which implies a link between Dsup and DNA protection (Hashimoto and Kunieda 2017).

Tardigrades also use other types of cell protectants like heat shock proteins (HSP), molecular chaperons that take part in protein folding and prevention of protein aggregation. (Jönsson and Schill 2007) report induction of HSP70 protein synthesis after exposure to gamma radiation (500 Gy). They quantified this induction after stress exposure in tardigrade *Richtersius coronifer* (radiation, desiccation, heat). HSP70 can act as a molecular chaperone, guarding and preventing protein damage or as a stabilizer of DNA preventing chromosomal aberrations, telomere instability, or induction of apoptosis.

LEA proteins (late embryogenesis abundant) have similar function as trehalose, and thus can perform as hydration buffer and prevent protein

aggregation (Erdmann and Kaczmarek 2017; Yamaguchi et al. 2012). What is unique about LEA proteins is their ability to withstand heat and maintain their solubility. LEA proteins contain Cytoplasmic Abundant Heat Soluble (CAHS), Secretory Abundant Heat Soluble (SAHS), and Mitochondrial Abundant Heat Soluble (MAHS) creating a molecular shield in a waterless environment. These are unique heat soluble proteins found only in tardigrades. According to (Yamaguchi et al. 2012), CAHS and SAHS are the major heat soluble proteins in tardigrades. Both change structure to α-helix so as LEA proteins do. These results suggest that CAHS and SAHS have a similar function as LEA proteins. They tested desiccation tolerant tardigrade *Ramazzottius varieornatus* in which LEA proteins were less expressed. (Yamaguchi et al. 2012), also implies that CAHS proteins protect the cytoplasm and nucleus while SAHS proteins protect extracellular components due to their occurrence. Similarity in gene sequences of CAHS a SAHS was reported between studied *Ramazzottius varieornatus* and *H. exemplaris* and suggests a high abundance of these proteins in *H. exemplaris*. (Erdmann and Kaczmarek 2017; Tanaka et al. 2015; Yamaguchi et al. 2012).

A different protectant can be found among intrinsically disordered proteins. This is an interesting class of proteins lacking tertiary structure playing different roles in transcription, organization of cells, or stress tolerance. (Boothby et al. 2017b) reported tardigrade-specific intrinsically disordered proteins (TDPs) as crucial for desiccation-tolerance in tardigrades. They tested whether *H. exemplaris* produces a protectant that would increase the survival in rapid desiccation after previous slow drying (preconditioning). Using two other tardigrade species *Paramacrobiotus richtersi* (species that cannot deal with rapid desiccation) and *Milnesium tardigradum* (species that requires much less preconditioning than *H. exemplaris*) they were able to show differences in reaction to rapid desiccation. *Milnesium tardigradum* expresses constitutively high levels of TDPs and did not upregulate the expression of TDPs as a response to drying. On the other hand, *Paramacrobiotus richtersi* and *H. exemplaris* increased the expression of many TDPs. With a use of RNAi to disturb the function of CAHs and SAHs genes (Boothby et al. 2017b), showed survival of *H. exemplaris* in a hydrated and dehydrated state. In a hydrated state, no significant difference occurred. Interestingly, in both cases where RNAi targeted either SAHs or CAHs, tardigrade-specific intrinsically disordered proteins significantly lowered survival after

desiccation compared to controls. These results imply the importance of some highly expressed proteins during desiccation in desiccation tolerance mechanisms of tardigrades.

A different possible mechanism was reported by (E. A. Beltrán-Pardo et al. 2013) with the use of Rad51 protein. Rad51 is an enzyme recombinase used in homologous recombination in DNA exchange activity. Its role is to help repair damaged strands by using a non-damaged strand as a template. One of the species tested was *H. exemplaris* (among *Milnesium cf. tardigradum* and *Macrobiotus cf. harmsworthi*). The result shows that Rad51 highly induced in *Milnesium cf. tardigradum* after 3 hours of exposure to gamma radiation in comparison to control. When (Beltrán-Pardo et al. 2013) looked closer at the gene and amino acid sequences of Rad51 protein, they found similarities with other species across the animal kingdom. For example, HdRad51 from *H. exemplaris* showed high resemblance with Janapanese firebelly newt (*Cyanops pyrrhogaster*), MtRad51 from *Milnesium cf. tardigradum* had high resemblance with nematode *Trichinella spiralis*. All HdRad51, MtRad51, and MhRad51 (from *Macrobiotus cf. harmsworthi*) were structurally similar to Rad51 H352Y from *Saccharomyces cerevisiae*. (Beltrán-Pardo et al. 2013) also tested sequence alignment based on the structure of tardigrades with other animals. It showed conserved motifs usually contained in recombinases implying DNA binding and ATPase activity of Rad51.

3 MATERIALS AND METHODS

3.1. CULTIVATION OF *HYPSIBIUS EXEMPLARIS*

The *H. exemplaris* strain was obtained from Sciento company (Manchester, UK). The animals were maintained in Volvic mineral water at 18 °C and fed by a strain of the immobile green algae *Chlorella vulgaris* IPPAS C-1. The cultures were kept in flat plastic 1000 ml tissue culture cultivation flasks with the bottom inner surface roughened by a sandpaper to facilitate movement of the animals. This modification also makes the media change and isolation of the animals easier in comparison with filtering as the medium can be poured out of the flasks carefully and most of the active tardigrades would stay attached on the flask surface. The procedure was usually repeated several times with the supernatants to increase the yield of the animals.

About 15 to 20 cultures in 250 ml of medium were maintained. The feeding regimen was modified several times during the population expansion phase of the study. At the beginning, the tardigrades were fed every two or three weeks usually with the change of the culture medium. Sometimes this regimen resulted in low activity of the animals and low egg production. Rather than feeding on the flask surface, they were floating in the medium freely or attached to algae clusters without visibly moving and feeding. The ideal feeding period was found to be around a week. Algae layer should cover the bottom of the culture flask, surrounding tardigrades, so they do not have to look for it. Under such conditions usually about 10-30% of adults produced eggs within 14 days. The media was changed in 2-3 weeks periods.

3.2 HARVESTING TARDIGRADES FOR EXPERIMENTS.

The tardigrades were separated from the algae by repeating steps including vigorous shaking of the culture flasks, allowing tardigrades to reattach to the scratched surface, pouring the supernatant from the flask and resuspension of tardigrades in fresh Volvic water.

3.3 EVALUATION OF HYDROGEN PEROXIDE TOXICITY

The tardigrades were harvested into fresh Volvic water as described above and 60 µl of the suspension was dispensed into 384-well plate Cell Carrier Ultra. Sytox Green Nucleic Acid stain was the added in 20 µl of Volvic water. After that concentration row of hydrogen peroxide in Volvic in another 20 µl was added. Final concentration of Sytox Green was 5 µM. Sytox green is not toxic for tardigrades and therefore suitable for staining of dead individuals (Richaud and Galas 2018). Our pilot experiments show that tardigrades tolerate the stain for at least a week. The final concentrations of hydrogen peroxide were 0.02-200 mM (2, 3, and 4 times dilution row starting at 200 mM). The plates were incubated at room temperature. Images were obtained at time 0, 4, 8, 16, 24 and 48 h by the automated microscope by Cell Voyager 7000S (Yokogawa Electric Corporation, Tokio, Japan). Based on the acquired data a suitable stress (hydrogen peroxide in concentration of 40 and 400 uM) was selected for the metabolomic study. The first concentration did not have any significant effect on survival after 24 hour exposition, the higher concentration had signifficant effect on survival of tardigrades (reduction of viability about 10 % in several repeated experiments).

3.4 PREPARATION OF THE SAMPLES FOR THE METABOLOMIC STUDY

18 cultures by about 1 million of tardigrades in 5ml fresh Volvic water were prepared in plastic Petri dishes. 6 of them were treated with hydrogen peroxide to the final concentration of 40 µM (hydrogen peroxide low – PL) and other 6 with hydrogen peroxide to the final concentration of 400 µM (hydrogen peroxide high – PH). The last 6 samples were used as a control. The cultures were incubated for 6 hours at room temperature. Thereafter, the samples were transferred to 15 centrifuge tubes, 10 ml of ice-cold Volvic water was added and the samples were centrifuged for 5 min at 1000 g in the centrifuge cooled to 4 °C. The supernatant was removed, and the pellet was resuspended in 15 ml of ice cold Volvic water. The washing procedure was repeated 2 times. The pellets were flesh frozen in liquid nitrogen and stored at -80 °C.

3.5 EXTRACTION

1 ml of extraction solution (80% methanol and 0.1% formic acid) was added to each microtube with the frozen tardigrade pellet. The samples were homogenized for 10 minutes using Verder MM 400 ball oscillating mill (Retsch, Haan, Germany) with metal balls at 27 Hz. The following step was sonication on a ultrasonic bath (VWR International, Radnor, USA) for 10 min. The extracts were centrifuged in a 5424 centrifuge (Eppendorf, Hamburg, Germany) at 20238 g for 10 min at room temperature. After that, the supernatants were evaporated on a TurboVap LV nitrogen evaporator (Caliper LifeScience, Hopkinton, USA), and the remaining material was dissolved in 20% methanol with 0.1% formic acid to a concentration of 100 mg/ml. From each extract, an aliquot was filtered on a 0.2 [mu] m Micro Spin Filter microfilter (Ciro Manufacturing Corp., Deerfield Beach, USA) from recycled cellulose using a 5424 centrifuge (Eppendorf) at 4136 g for 5 min. The filtrates were transferred to vials for UHPLC-MS / MS analysis.

3.6 UHPLC-MS/MS

The extracts were separated on a reverse-phase column on an Acquity UPLC BEH C18 1.7 µm column (Waters, Milford, USA), the column dimensions were 150 x 2.1 mm. The UHPLC system consisted of a PDA detector (Acquity Ultra Performance), Sample manager (FTN Acquity UPLC), and a pump (Quaternary Solvent manager Acquity UPLC Class H) (all Waters). The column temperature was set at 30 °C. The sample temperature in the Sample Manager was set to 4 °C. The injection volume of the sample was 5 µl. Mobile phases used were ≥99.9% acetonitrile (A) and 5 mM formic acid in deionized water (B). Deionized water was prepared in a Simplicity 185 (Millipore Corp., Billerica, USA).

Detection of the separated analytes was performed on a Synapt G2-Si mass spectrometer (Waters). ESI served as the ion source, and the analyzers were quadrupole and TOF in a tandem arrangement (QqTOF). The samples were measured in both positive and negative modes. Nitrogen was used as the misting gas and the gas at the inlet slit. Leucine-enkephalin (5 ng / µl) was used as a lock spray. Further parameters of the mass spectrometer settings are listed in Table 1. Data collection took place in DDA (Data Dependent Acquisition) mode, the collision energy at MS^2 was 20 eV. Data were recorded in centroid format. The total analysis time of one sample was 22 minutes.

Table 1. Settings of the mass spectrometer for detection.

Parameter	Settings
Range of measured weights	50-1500 Da
Capillary tension	2 kV
Ion source temperature	120 °C
Cone voltage	15 V
Desolvation temperature	500 °C
Cone gas flow	30 l/h
Desolvation gas flow	600 l/h

To evaluate the data program MassLynx, ver. 4.0 (Micromass, Manchester, UK) was used.

3.7 DATA ANALYSIS

Analysis of the metabolomic data was done in R. It is described and explained in the section RESULTS.

4 RESULTS

4.1 OXIDATIVE TOLERANCE OF *HYPSIBIUS EXEMPLARIS*

A toxicity of a wide range of hydrogen peroxide concentrations (0.02-200 mM) was evaluated using light and fluorescent (Sytox Green staining) microscopy. The concentrations of 40 and 400 μM were selected for the metabolic study. The first concentration did not have any significant effect on survival after 24 hour exposition, the second one killed about 10 % tardigrades in 3 independent experiments.

The concentrations of hydrogen peroxide were selected based on pilot experiments at 400 and 40 μM. The first concentration did not have any significant effect on survival after 24 hour exposition, the highest concentration had signifficant effect on survival of tardigrades (reduction of viability about 10 % in several repeated experiments).

Images below demonstrate three morphological types observed in the experiment.

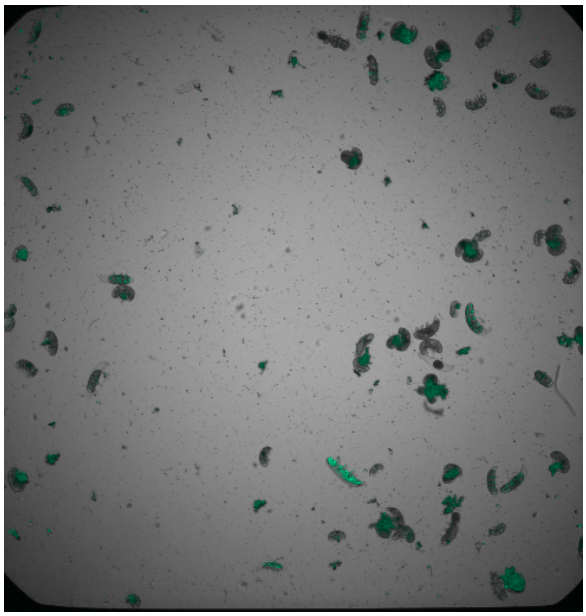

Fig. 9. Image of the control individuals of *H. exemplaris* with the typical oval and bent body shape (without green fluorescence).

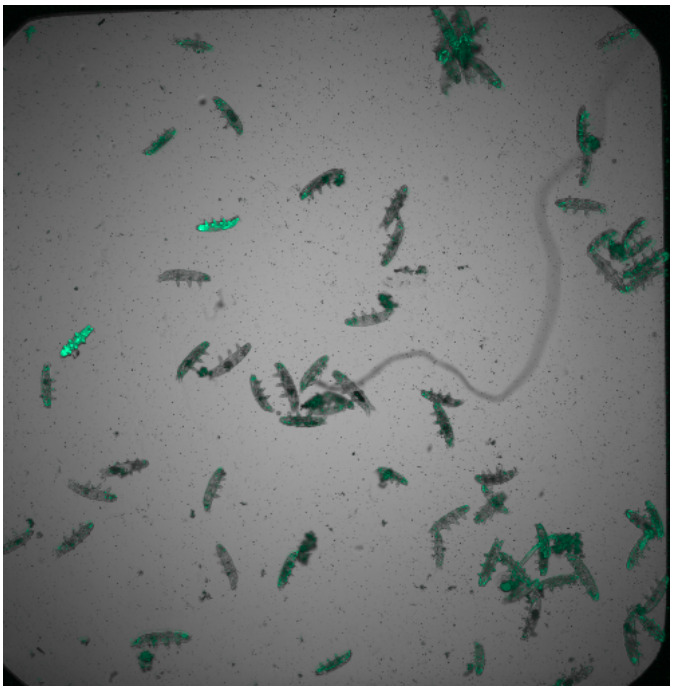

Fig. 10. Image of individuals of *H. exemplaris* in latent state with typical stretched body and legs. Individuals stained with Sytox Green are dead.

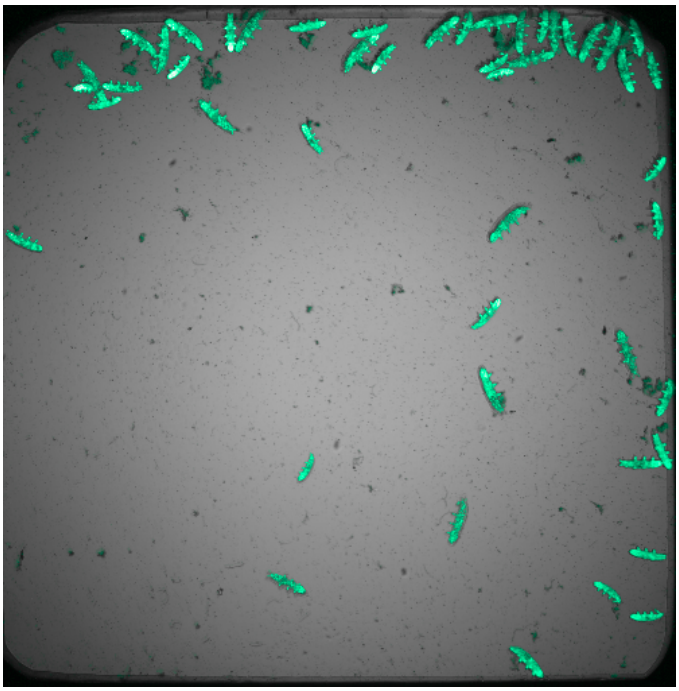

Fig. 11. Image of dead individuals of *H. exemplaris* with typical stretched body and legs stained with Sytox Green.

4.2 METABOLOMIC DATA ANALYSIS

The control samples are designated C (C1-C6), the samples treated with the high concentration of hydrogen peroxide PH (PH1-PH6) and with the low concentration PL (PL1-PL6).

4.2.1 QUALITY CONTROL

A quality control was carried out after filtering out the features with the retention time (RT [min]) lower than 2 min and higher than 16 min in all the samples. After this step, 1404 out of 4797 (29.3 %) features remained.

The peak area distribution in the individual samples and inter-sample pair correlation were further considered. The histograms (Fig. 12) of the feature peak area clearly show that C1 is an outlier with an overall low intensity of the signal. Overall, a rather high level of noise across the samples is obvious – there is a high proportion of data points with area below 200 where precise value determination is unreliable.

Next, we compared the pair plots of the feature peak area distribution within and between the individual sample groups (Fig. 13-16). The tighter the clustering along a line with a non-zero slope, the more similar the samples are. If a point lies on the line with the slope = 1, the feature has the same concentration in both samples. Both technical and biological variability can cause a deviation from the diagonal line.

The following patterns emerged:

1) The samples except for C1 show a clear correlation for the majority of the features.

2) The variability is higher within the control group.

3) In some pair-wise comparisons, the points cluster along a second diagonal, which is indicative of a systemic effect distorting the measurement. This is particularly problematic in the case of the within-group comparison.

Those observations further support the decision to remove C1 as an outlier. If more control samples were available, removal of the other control samples (C2, C3) where the clustering of the points along the second diagonal occurs would be appropriate. In our case, we decided to keep those samples and later control if the metabolites identified in the analysis are affected by the artifact.

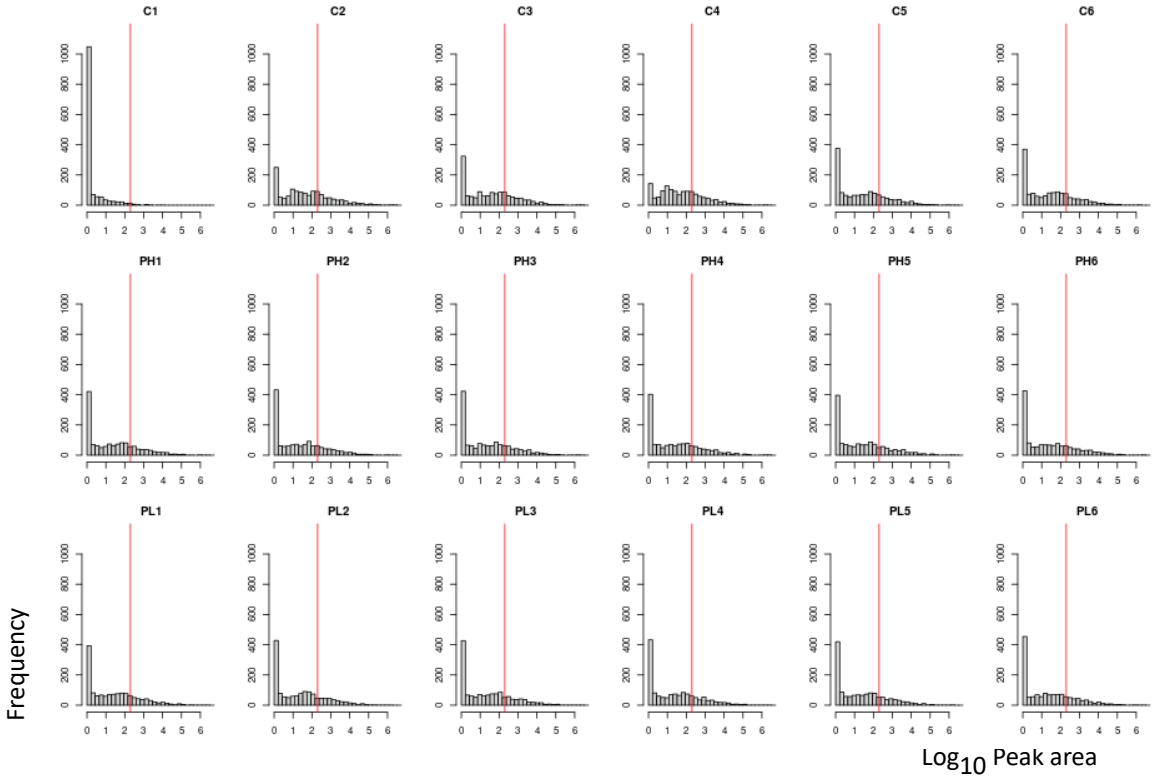

Fig. 12. The histograms of the peak area after the features with 2 < RT <16 min were filtered out. The red horizontal line designates peak area = 200.

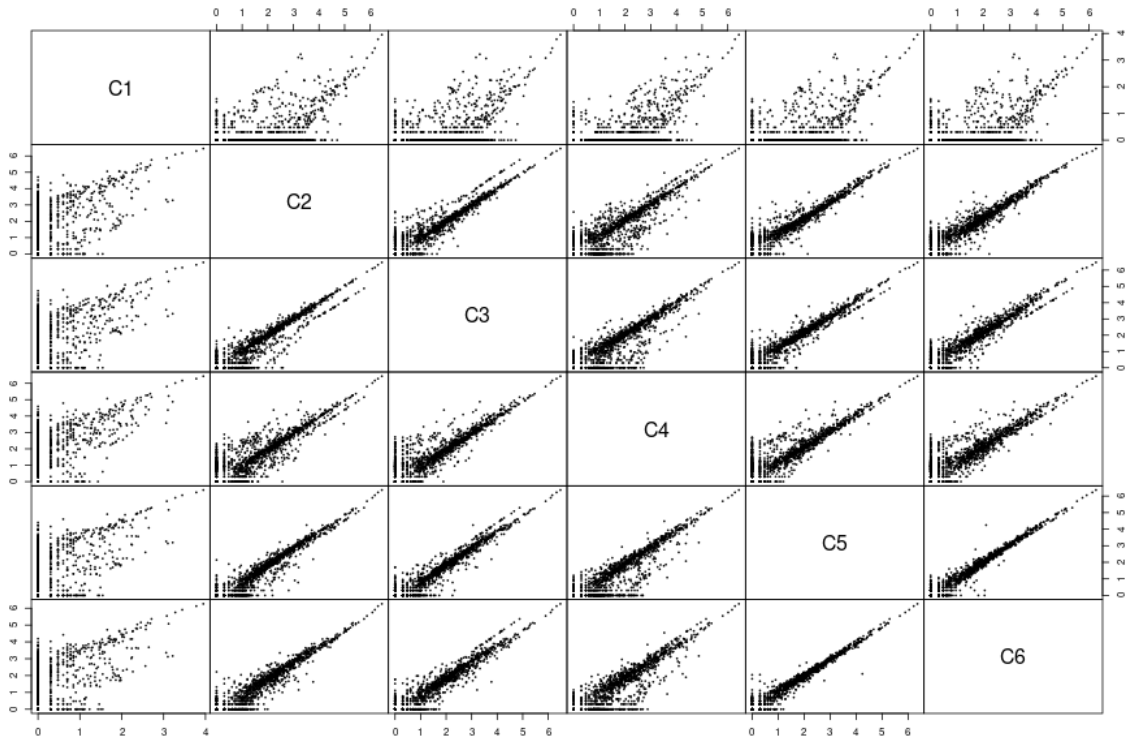

Fig. 13. The pairwise comparisons of the peak area distribution for the control samples. The axes are logarithmic (log10).

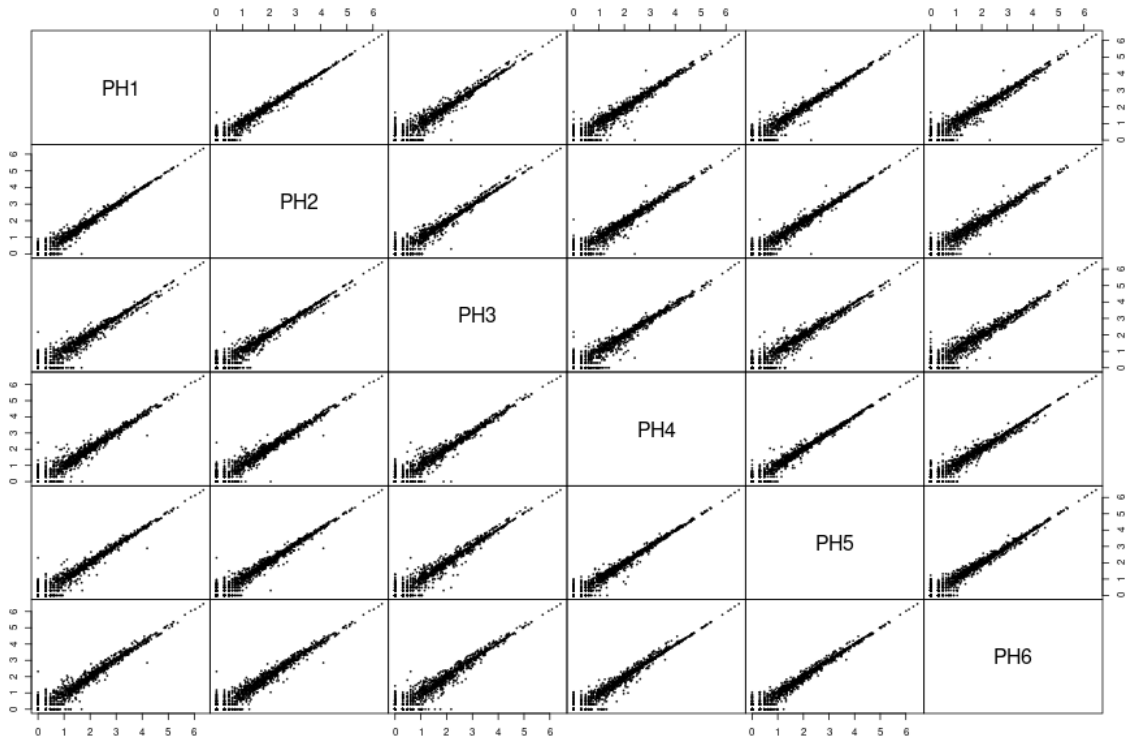

Fig. 14. The pairwise comparisons of the peak area distribution for the PH samples. The axes are logarithmic (log10).

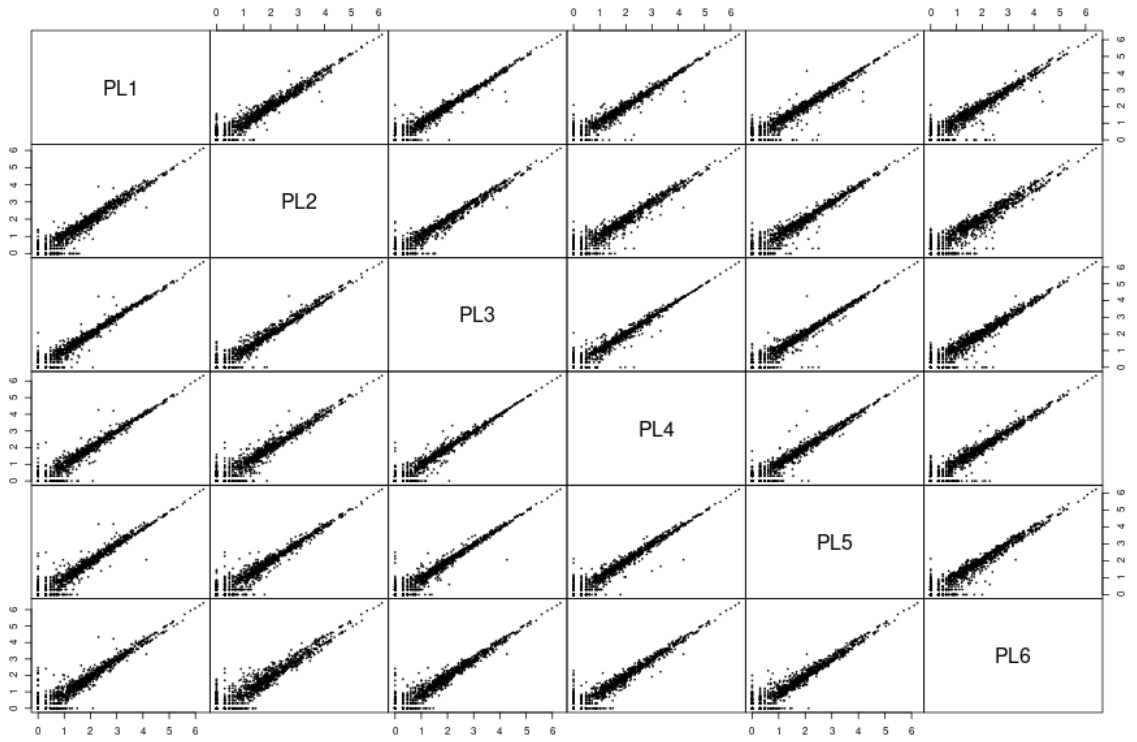

Fig. 15. The pairwise comparisons of the peak area distribution for the PL samples. The axes are logarithmic (log10).

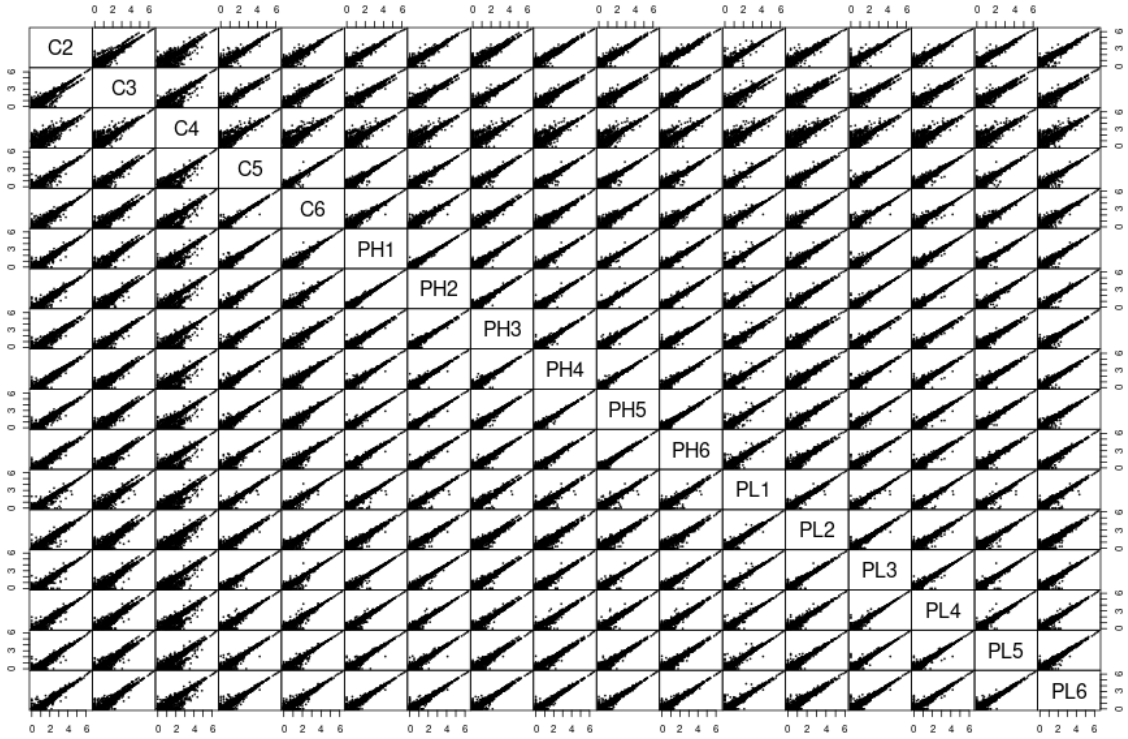

Fig. 16. The pairwise comparisons of the feature peak area distribution for all the samples. The axes are logarithmic (log10).

4.2.2 FILTERING OF THE FEATURES WITH A LOW PEAK AREA

The experience suggests that the area of the peaks with the value below 200 is imprecise and their removal improves the analysis.

In the next filtering step, only features with the peak area > 200 in at least 4 samples within any treatment group were selected. Further, the minimum maximum feature peak area > 500 was required. An overview data filtering procedure is shown in the Table 2. The resulting data are shown in Fig. 17 a 18 and the descriptive statistics is given in the Table 3.

Table 2. Overview of filtering steps.

Original data	4797	(100 %)
Any (2 min < RT < 16 min)	1404	(29.3 %)
peak area > 200 in at least 4 samples within any treatment group	356	(7.4 %)
minimum maximum feature peak area > 500	314	(6.5 %)

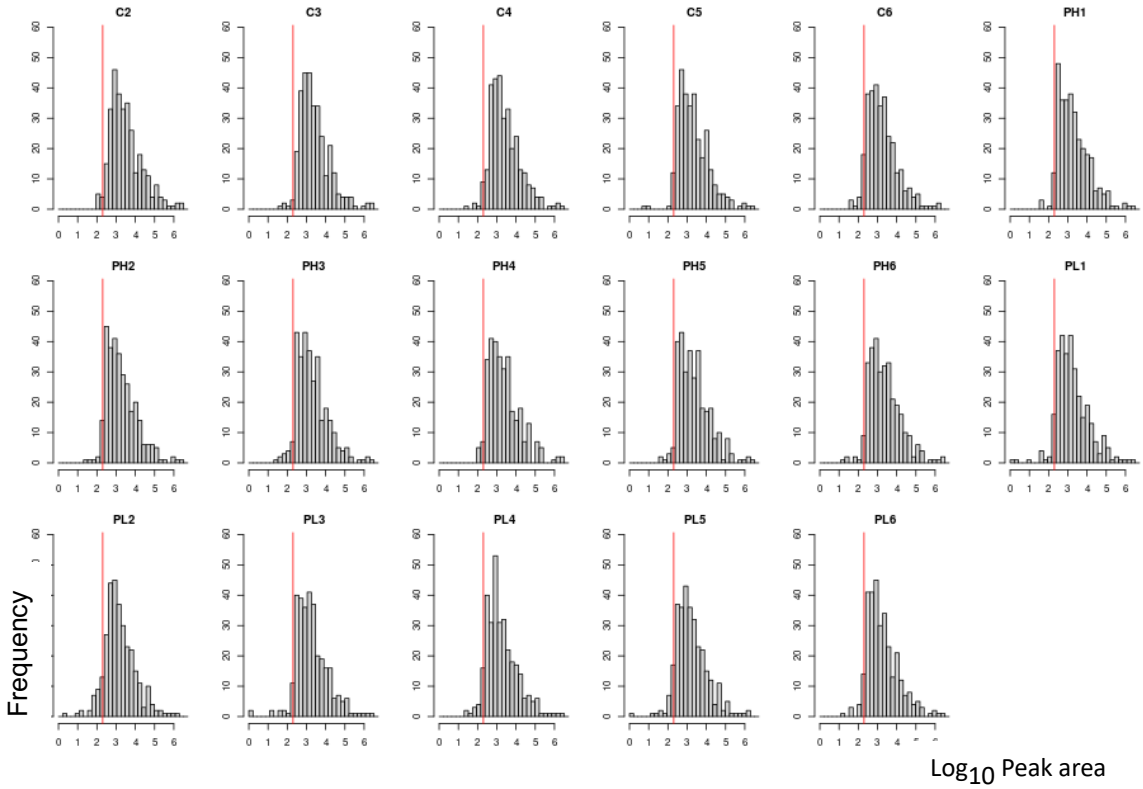

Fig. 17. The histograms of the feature peak area of the individual samples after the features after the peak area filtering steps. The red horizontal line designates peak area = 200.

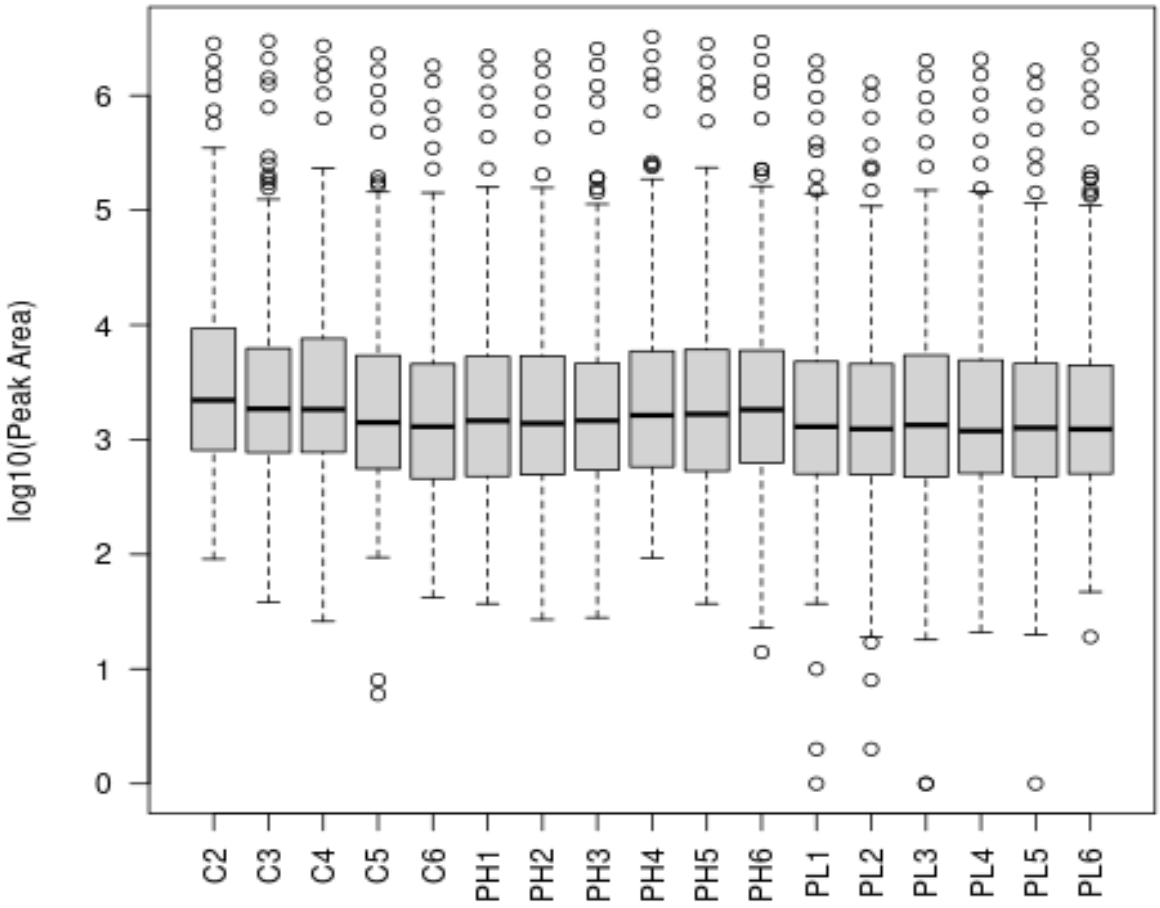

Fig. 18. The boxplot of the feature peak area in the individual samples after the peak area-based filtering.

Table 3. The descriptive statistics of the feature peak area in the individual samples. Q – quantile.

sample	min	Q 25%	median	Q 75%	max	mad	mean	sd	sum
C2	91	814 .0	2207 .5	8977 .5	2840702	2475 .2	43319 .6	231497 .4	13602347
C3	38	772 .0	1853 .5	6209 .5	2983946	2019 .3	38217 .3	236431 .6	12000237
C4	26	775 .3	1833 .0	7530 .0	2709793	2056 .4	35549 .1	215248 .9	11162416
C5	6	557 .5	1410 .0	5383 .5	2293294	1593 .8	28907 .3	178722 .3	9076881
C6	42	456 .8	1298 .5	4575 .8	1816180	1445 .5	23177 .3	140388 .7	7277660
PH1	37	478 .3	1459 .5	5252 .0	2210469	1673 .1	28356 .7	173354 .6	8903995
PH2	27	495 .3	1383 .5	5341 .0	2190776	1590 .1	27811 .7	172163 .8	8732885
PH3	28	546 .8	1462 .0	4635 .0	2562325	1673 .9	30910 .6	199459 .8	9705913
PH4	93	578 .3	1629 .5	5887 .5	3249237	1879 .2	40370 .5	251847 .2	12676328
PH5	37	531 .5	1666 .5	6061 .8	2825533	1974 .1	35278 .7	218520 .8	11077505
PH6	14	629 .3	1825 .0	5967 .0	2947556	2183 .9	36179 .8	226772 .1	11360440
PL1	1	503 .0	1296 .0	4800 .5	1994616	1436 .6	26693 .9	156727 .5	8381902
PL2	2	498 .3	1239 .5	4575 .5	1306458	1437 .4	18145 .2	104022 .4	5697595
PL3	1	472 .8	1339 .0	5390 .3	2020939	1575 .3	26151 .1	157889 .1	8211453
PL4	21	514 .8	1191 .0	4919 .5	2073147	1343 .9	26826 .3	162722 .5	8423456
PL5	1	476 .3	1265 .0	4569 .3	1664698	1469 .3	22057 .3	131543 .1	6926006
PL6	19	506 .3	1234 .5	4439 .0	2532148	1399 .6	31453 .5	196969 .8	9876386

4.2.3 DATA NORMALIZATION

The descriptive statistics and the plots of the filtered data (Table 3 and Fig. 18) show that the individual samples differ markedly in the mean, median, and cumulative sum of the feature areas. This suggests that the individual samples have different total concentrations of the analytes. However, no clear difference among the sample groups that would indicate a suppressing effect of the treatment on the metabolism in general or a widespread loss of analytes due to oxidation is evident. Normalization of the sample signals should therefore be considered. In metabolomic data analysis, standardization of the total feature area is often used. We decided for this strategy as well.

The results of the normalization are shown in Fig. 19, 20. The corresponding dendrogram and heatmap is Fig 21.

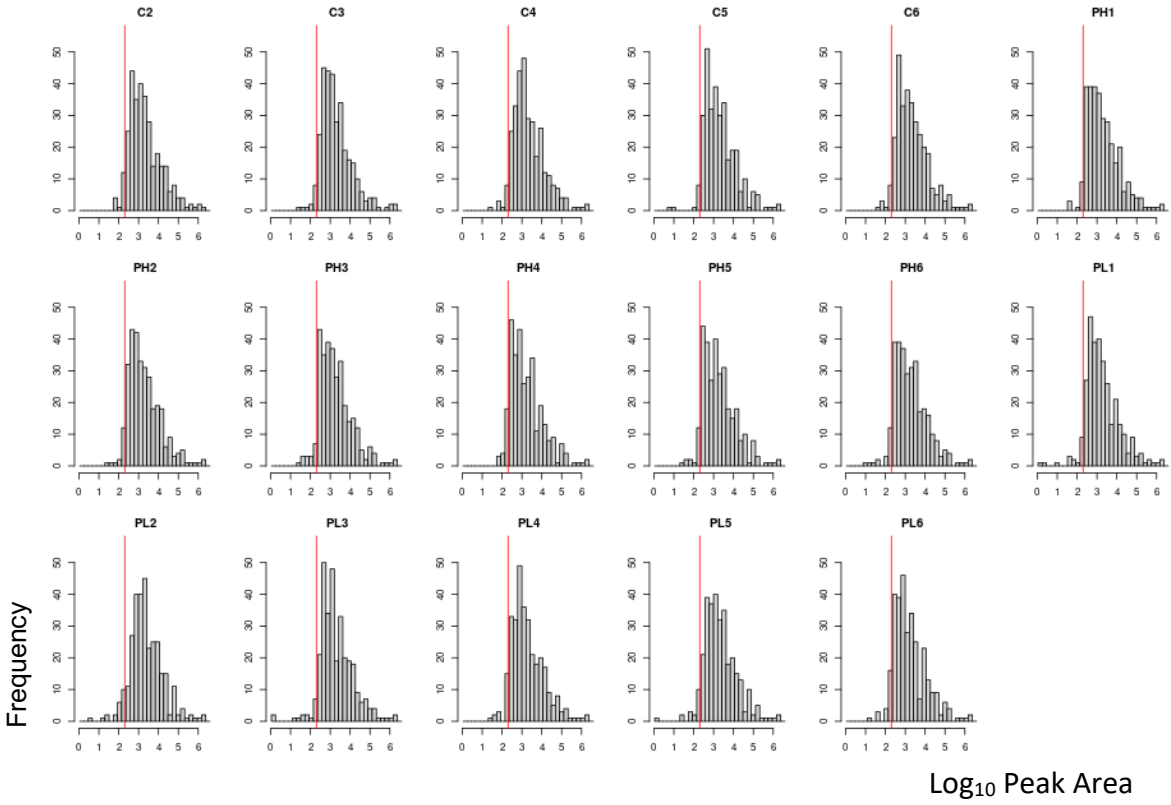

Fig. 19. Histograms of feature peak area of the individual samples after the peak area filtering steps and normalization. The red horizontal line designates peak area = 20.

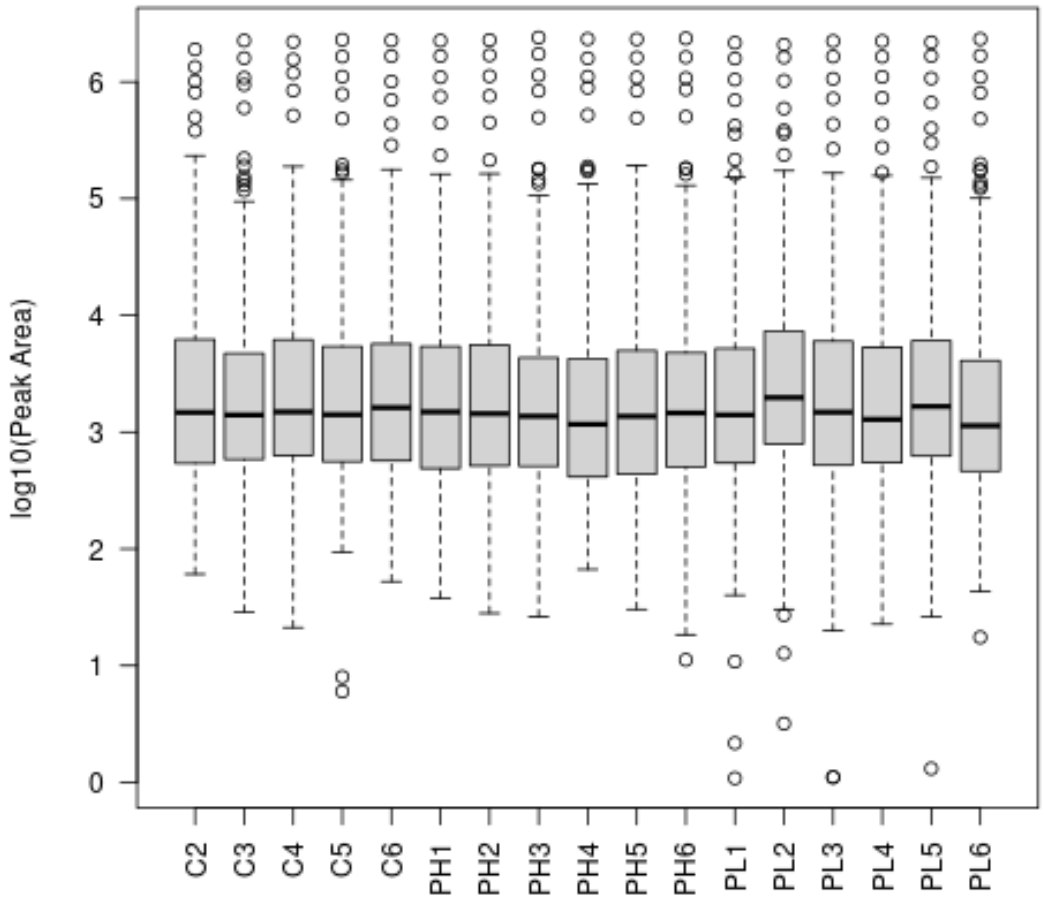

Fig. 20. The boxplot of feature peak area in the individual samples after the peak area filtering steps and normalization for the total peak sum.

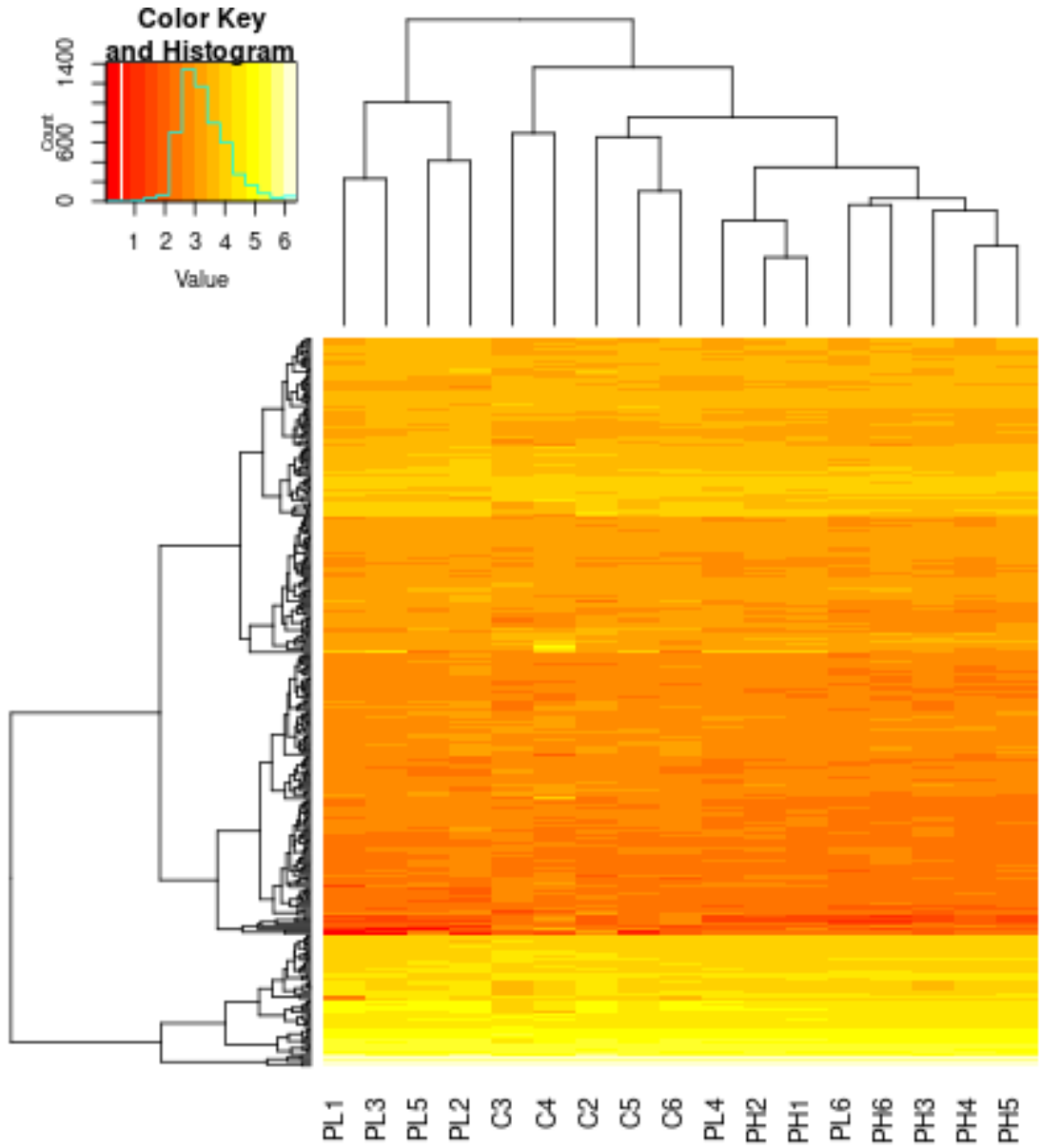

Fig. 21. The heatmap and dendrogram of the normalized data (Euclidean distance, complete linkage).

4.3 IDENTIFICATION OF METABOLITES WITH DIFFERENTIAL CONCENTRATIONS AFTER HYDROGEN PEROXIDE TREATMENT

We decided to focus on the comparison of C and PH groups because the experiments with various filtering (minimal maximum peak area across the samples) and normalization procedures (sum, median, variance stabilizing) showed that C and PL samples tend to cluster together. An analysis of the differences between C and combined PH and PL samples was carried subsequently for a comparison. The robust and conservative Wilcoxon test was used in order to minimize the effects of extreme data points, including the unreliable values below 200 threshold. P-values were corrected by the Benjamini-Hochberg procedure as implemented in p.adjust function in R. The results of the analysis are shown as volcano plots. Fold change (FC) is a ratio of the group medians. Metabolites with differential concentrations are given in the Table 4.

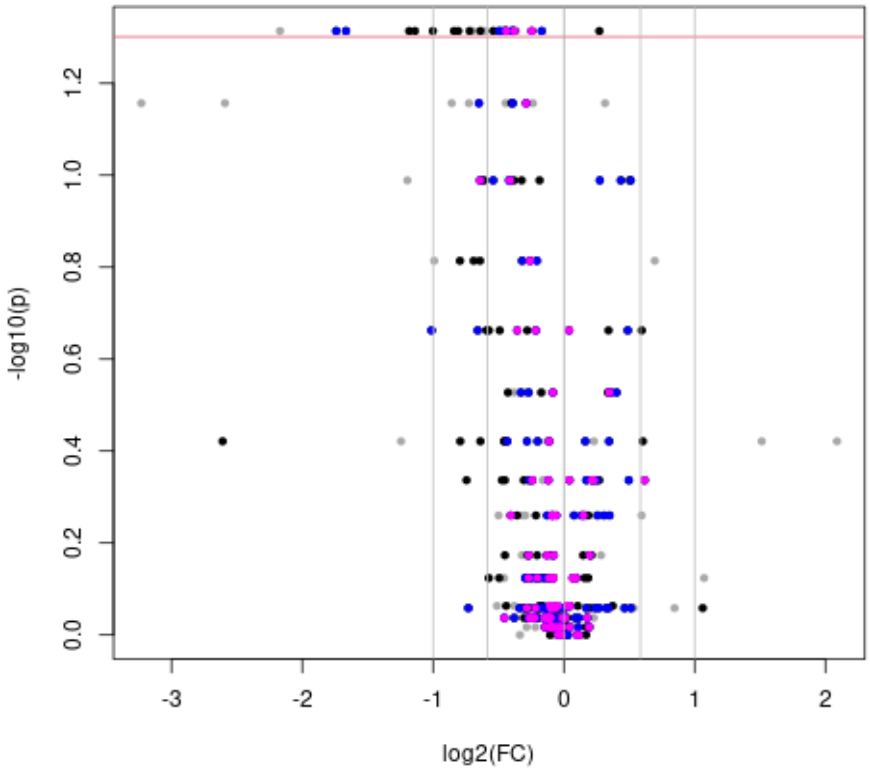

Fig. 22. The volcano plot for the comparison of PH vs. C samples. The color coding according to the median peak area in the group with the higher signal: gray <= 500, black > 500, blue > 1500 and magenta > 5000. The red horizontal line corresponds to the corrected p-value 0.05. Gray vertical lines indicate FC of 0.5, 0.66, 1.5 and 2.

Table 4. Metabolites with log2 (FC) < -1, corrected p-value <0.05 and both median and mean peak area of the group with the higher signal were selected for identification.

Feature no.	000678	000818	000819	001260	001725
m/z	291 .113	327 .3477	327 .3477	455 .3245	591 .4716
RT [min]	7 .243	15 .607	15 .718	6 .922	10 .681
Control median	528 .8	2817 .0	2011 .8	566 .0	1335 .0
PH median	264 .1	843 .1	634 .1	257 .0	588 .3
PL_median	388 .4	1079 .1	791 .4	488 .0	1312 .7
PL+PH_median	298 .2	952 .2	698 .5	339 .0	769 .5
control_mean	529 .7	3197 .1	2298 .9	561 .0	1358 .7
PH_mean	264 .7	919 .2	665 .7	267 .2	603 .5
PL_mean	331 .4	1430 .4	1046 .2	515 .2	1343 .1
PL+PH_mean	298 .0	1174 .8	856 .0	391 .2	973 .3
control_mad	39 .1	522 .3	21 .7	25 .5	275 .8
PH_mad	99 .8	192 .4	130 .9	77 .4	122 .7
PL_mad	54 .4	318 .0	158 .5	30 .1	42 .4
PL+PH_mad	147 .0	346 .4	263 .2	190 .9	391 .4
control_sd	32 .0	803 .9	448 .3	31 .1	233 .3
PH_sd	82 .1	343 .4	223 .2	63 .0	98 .9
PL_sd	118 .1	1030 .9	758 .8	163 .4	378 .1
PL+PH_sd	103 .0	779 .7	569 .1	175 .2	467 .6

4.4 DATA VALIDATION

In order to recognize possible measurement and analysis artifacts, the peak area distributions of the selected features in both normalized and original data were plotted. Further, we examined the position of the feature data points in the pair plots. Here we show figures only for 2 features (000818 and 000819) for which the elementary composition was identified (see below). The features are highlighted in the volcano plots 23 and 24 The figures 25-28 show that both treatment samples separate well from the controls in both original and normalized data. Pair plots for the comparisons within the groups show that the points corresponding to those features do not lie on the weaker aberrant diagonal. Those observations increase our confidence that the observed differences in the peak areas among groups are not artefacts due to a measurement error or suboptimal normalization.

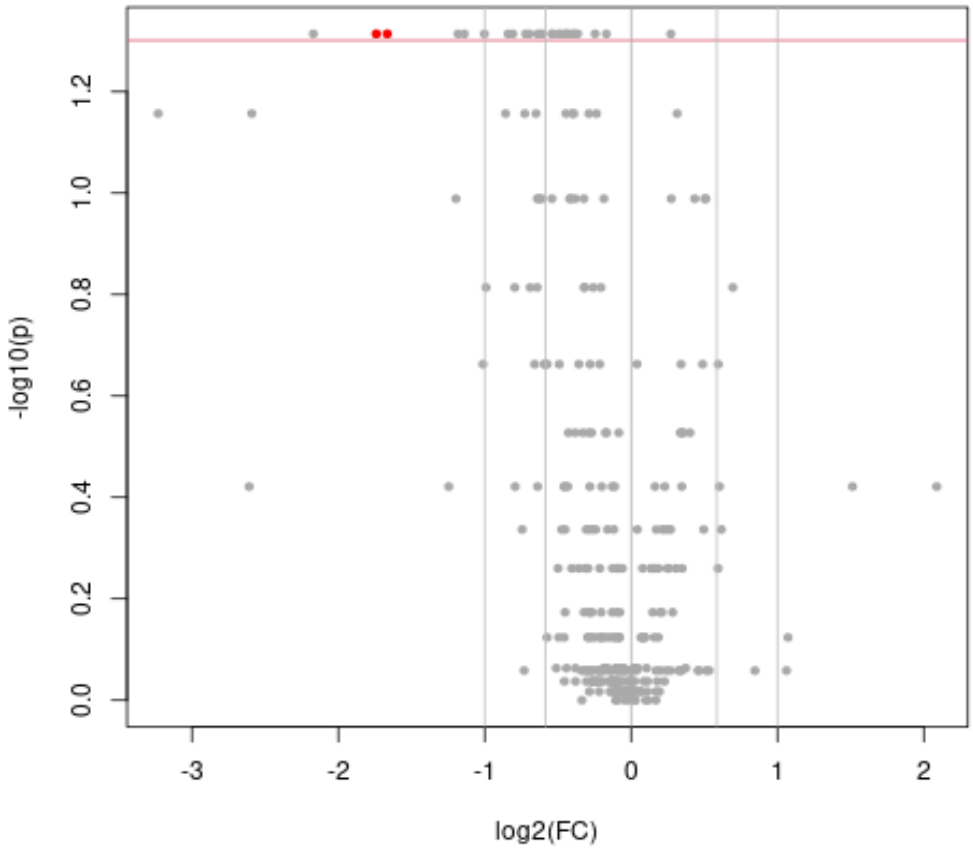

Fig. 23. The volcano plot for comparison of PH vs. C samples. The red dots designate features 000818 and 000819.

The red horizontal line corresponds to the corrected p-value 0.05. The gray vertical lines designate FC of the medians 0.5, 0.66, 1.5, and 2.

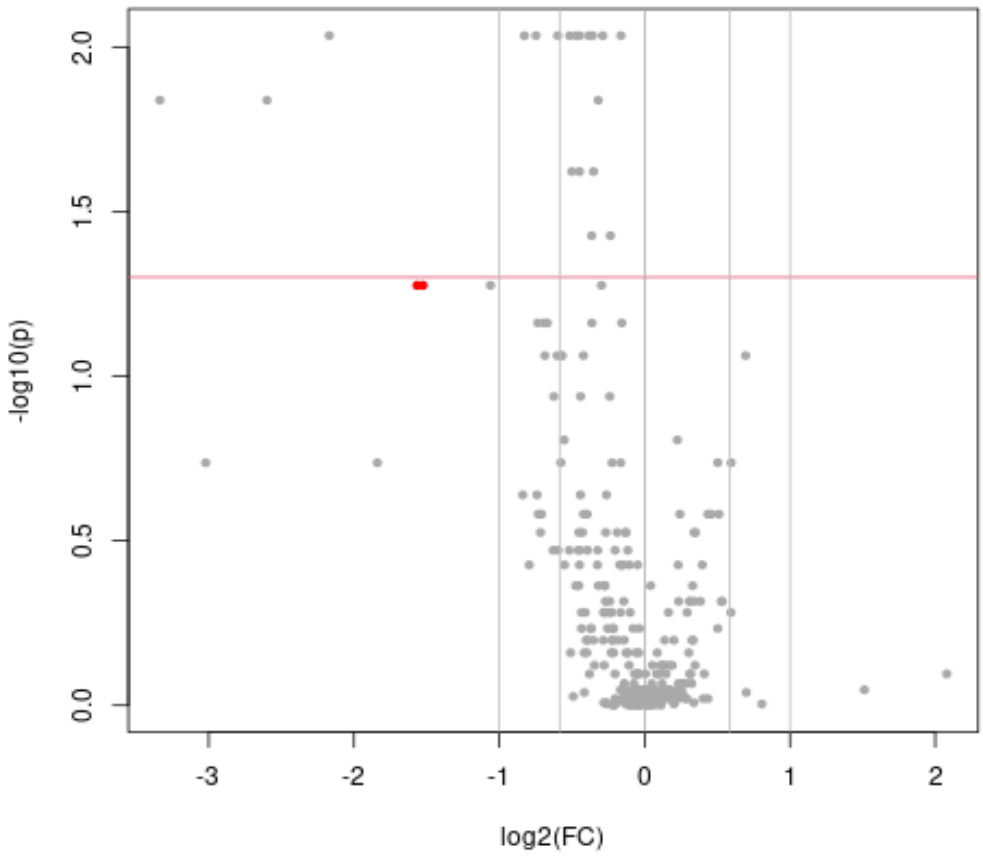

Fig. 24. The volcano plot for comparison of PH + PL vs. C samples. The red dots designate features 000818 and 000819.The red horizontal line corresponds to the corrected p-value 0.05. The gray vertical lines designate FC of the medians 0.5, 0.66, 1.5, and 2.

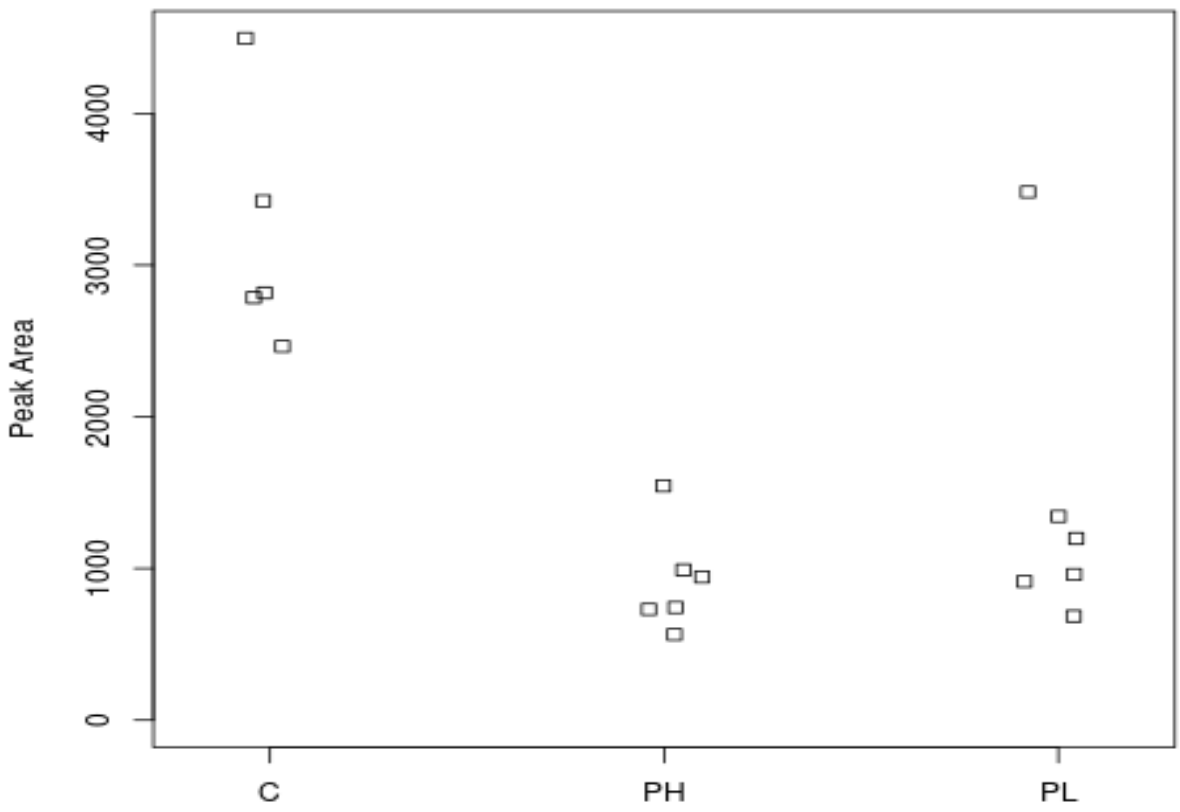

Fig. 25. The distribution of the normalized peak area for the feature 000818.

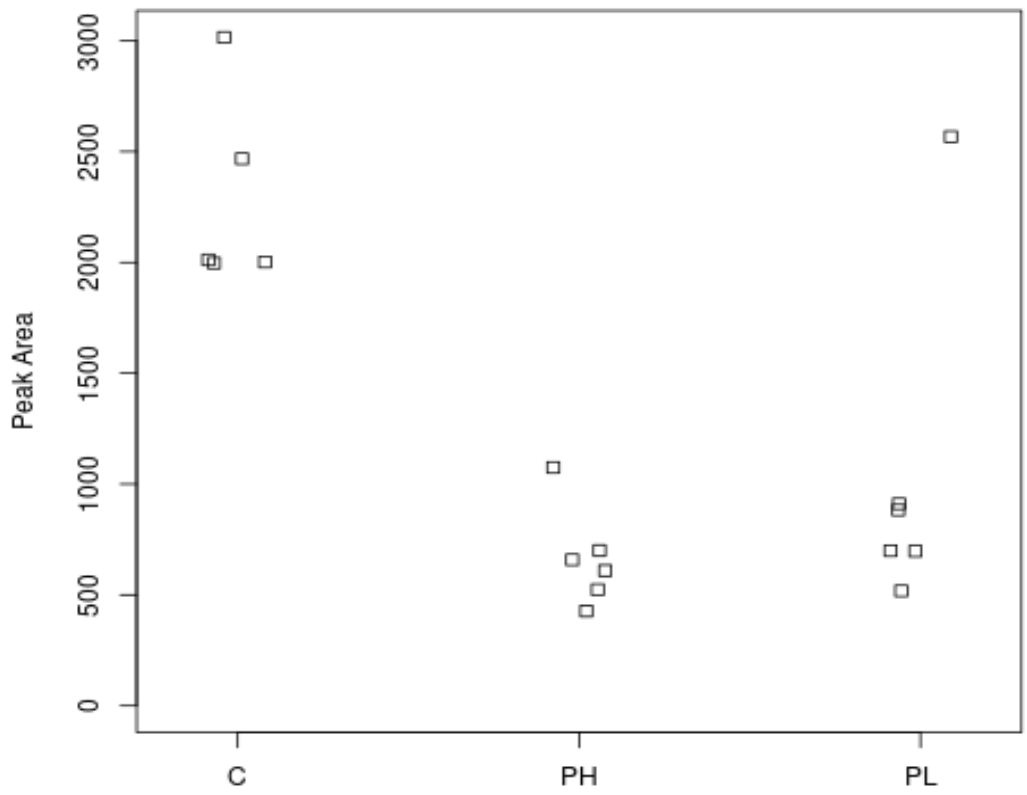

Fig. 26. The distribution of the normalized peak area for the feature 000819.

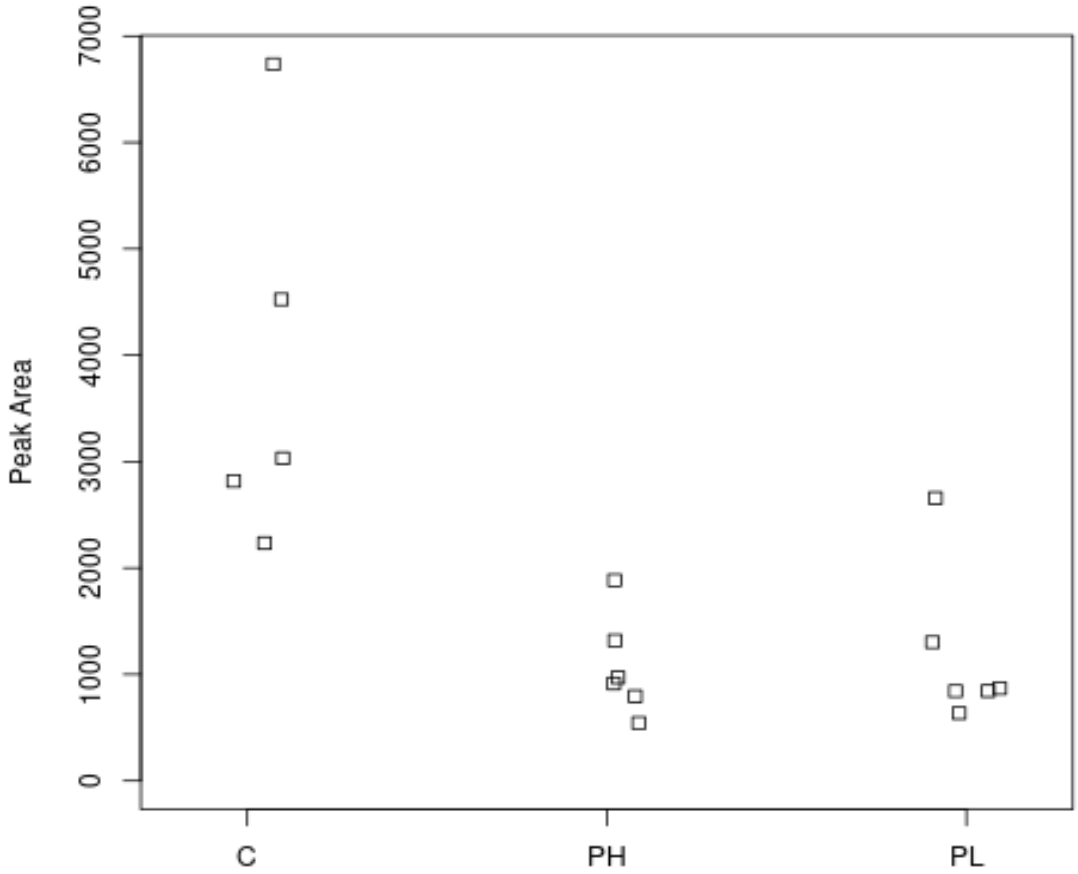

Fig. 27. The distribution of the peak area for the feature 000818 without normalization.

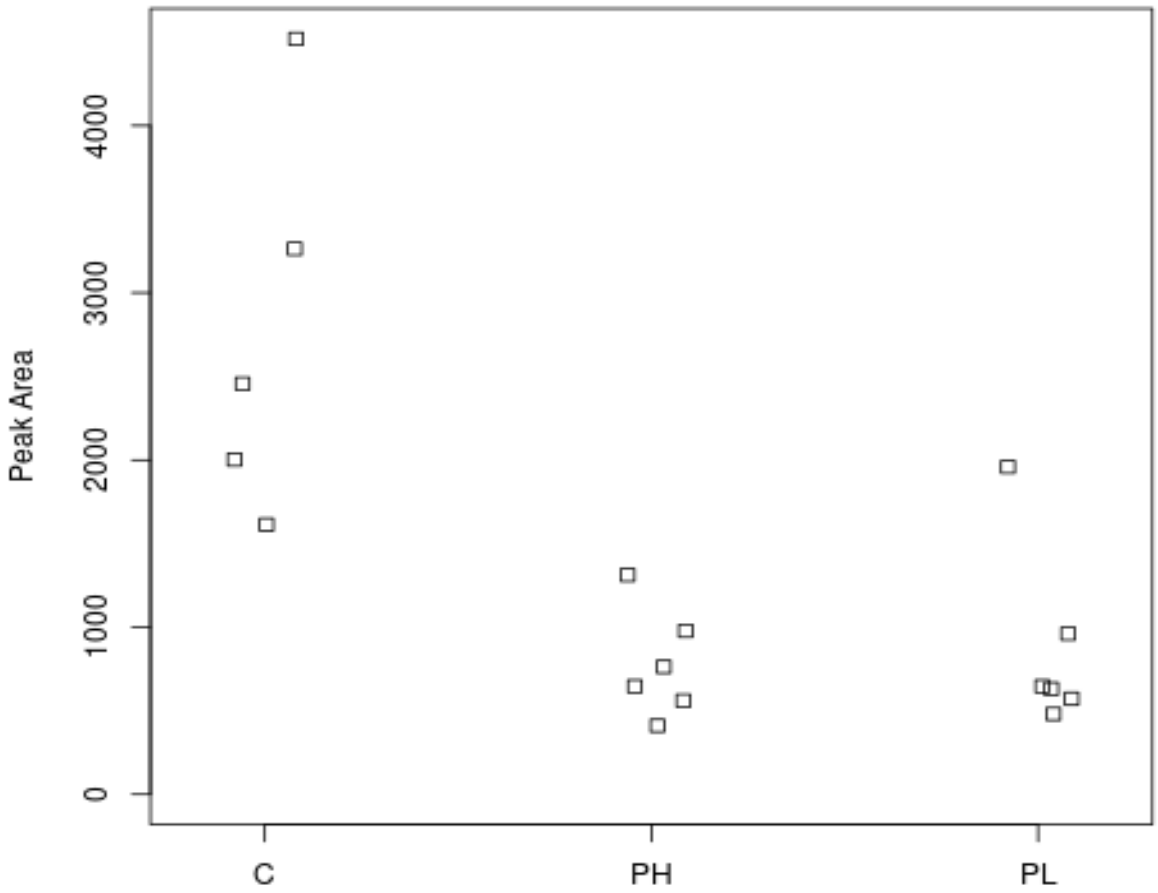

Fig. 28. The distribution of the peak area for the feature 000819 without normalization.

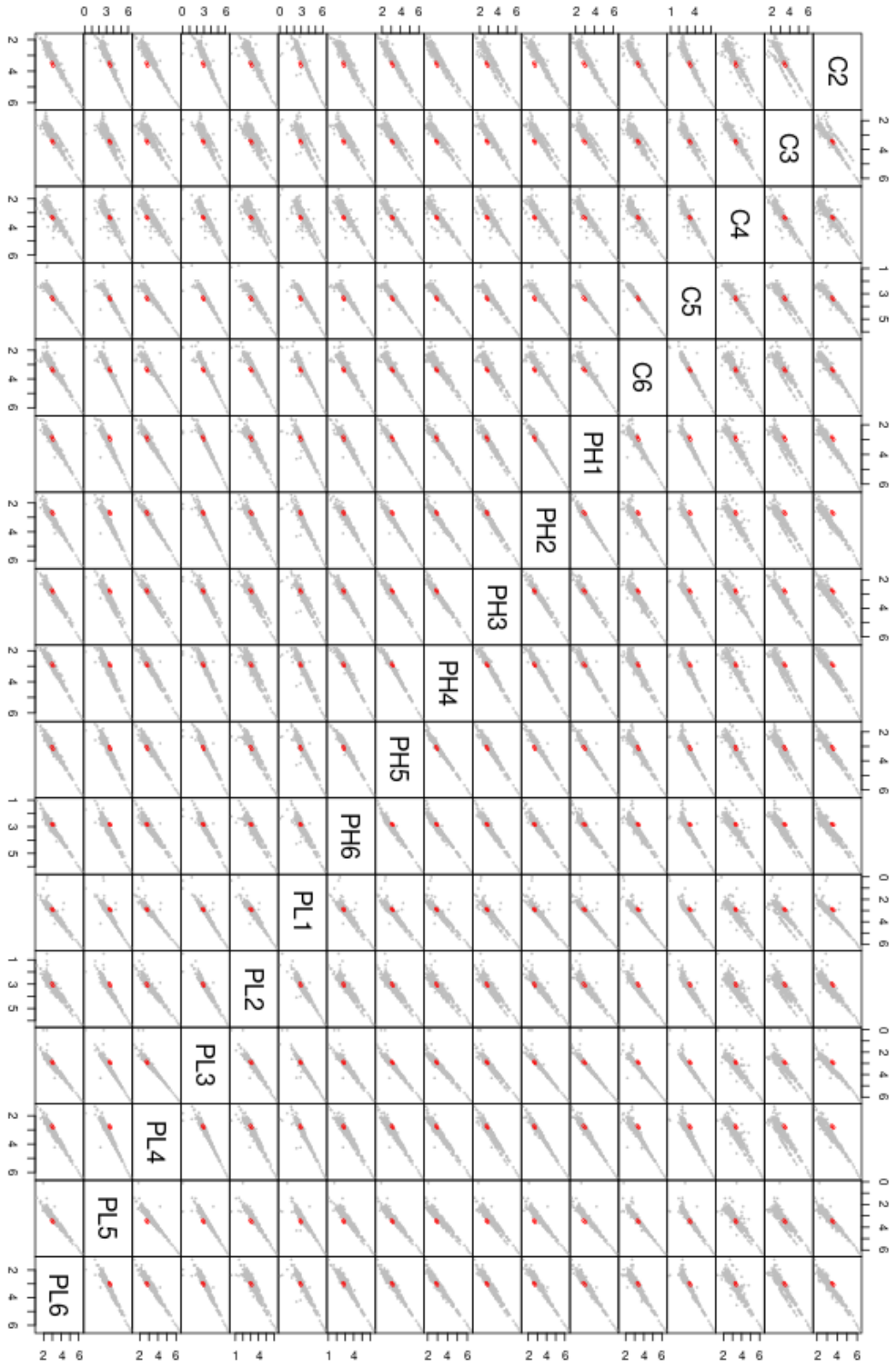

Fig. 29. The pair plot of the feature peak area after the filtration step with the features000818 and 000819 highlighted in red.

4.5 IDENTIFICATION OF THE FEATURES

We tried to estimate the summary formula of the features that were identified as sensitive to the hydrogen peroxide. MassLynx software was used for this purpose. We succeeded in the case of features 00818 and 00819 with m/z 327.3477 and RT [min] of 15.607 and 15.718 respectively for which the distinct and well defined peaks were present and mass was measured. These features are probably isomers. This notion is further supported by the observation that their peak area is highly correlated across all 18 samples (Pearson correlation coefficient > 0.99). Interestingly, another feature (00817) with the same m/z and RT of 16.49 min was identified in the chromatogram (Fig. 31). It was not included in the original analysis of the sensitivity to the hydrogen peroxide treatment because it did not pass RT filter (RT < 16 min). Interestingly, post-hoc analysis showed that it may be also sensitive to hydrogen peroxide, although to the higher concentration only (Wilcoxon test p=0.0043).

Candidate molecular formulas are given in the Table 5.

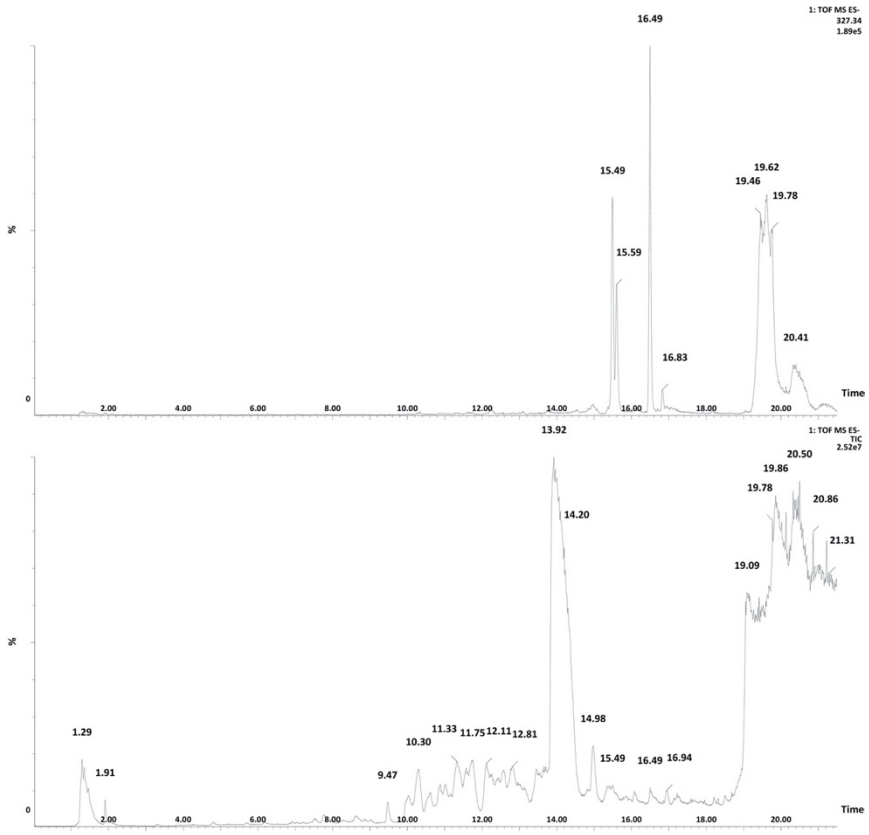

Fig. 30. Chromatograms of the potential isomers 00818 (RT=15.49), 00819 (RT=15.59) and 00817 with m/z 327.34. X axis is the retention time [minutes], Y axis is the signal intensity.

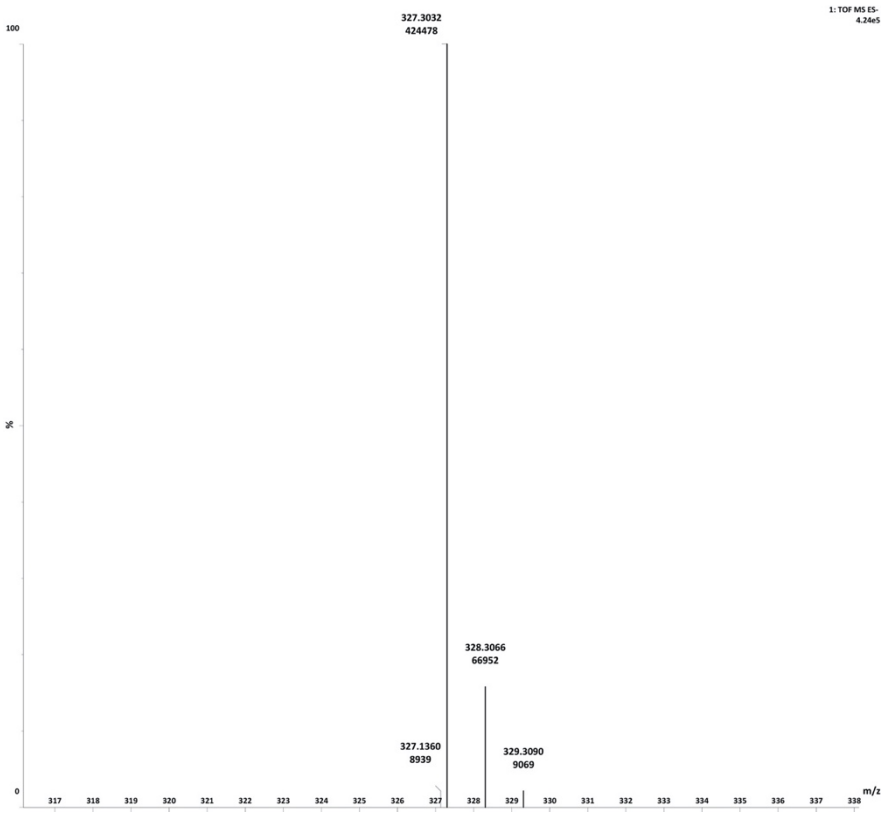

Fig. 31. Chromatogram from mass spectrometry of selected feature 00819 (RT=15.59) with m/z of 327.3032. X axis is the retention time [minutes], Y axis is the signal intensity.

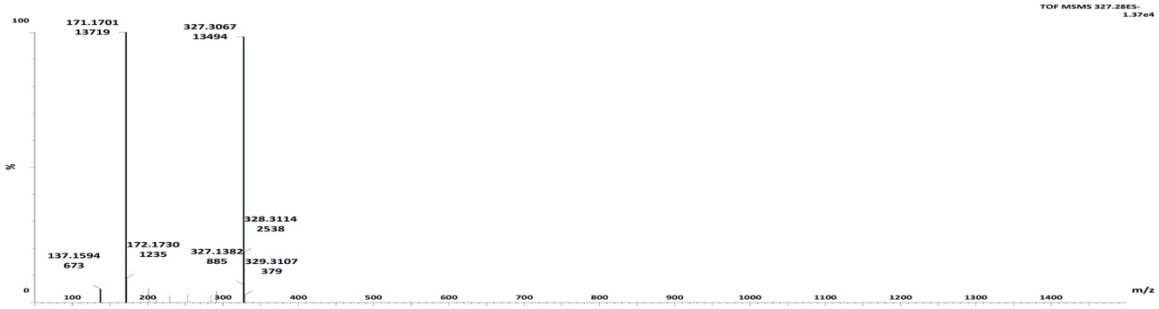

Fig 32. Chromatograms of MS/MS spectrum of selected feature 00819 (RT=15.59) with m/z of 327.3032. X axis is the retention time [minutes], Y axis is the signal intensity.

Table 5. Possible elemental composition of selected features with measured mass of 327. 3477, error of 1.5 PPM and calculated mass.

Calculated mass	Possible formula
327.3012	C19 H39 N2 O2
327.3124	C18 H39 N4 O
327.2899	C20 H39 03

5 DISCUSSION

Tardigrades are extremophiles tolerant to various types of stress including radiation, oxidative stress, osmotic stress, high temperature, and freezing. Our understanding of the underlying mechanisms is very limited as only a few molecular studies have been carried out up to date. Here we present a study of semipolar metabolom of *H. exemplaris* exposed to hydrogen peroxide, a first study of this type in Tardigrades to our knowledge. The higher concentration of 400 μM induced death in about 10 % of population after 24 hours in the pilot experiments. The lower one (40 μM) did not have any negative effect on viability. The samples were harvested after 6 hours. We hypothesized that at those concentrations a successful stress response could be observed.

The samples (hexaplicate for each condition) were extracted and subjected to UHPLC-MS/MS analysis. The analysis of the 1404 identified features included quality control, feature filtering, identification of features probably influenced by the treatment, and metabolite identification. The quality control revealed that the number of the features with high quality signal is rather low. One of the control samples was excluded from the analysis altogether. After removing the features outside of the retention time window 2-16 minutes, 1404 (29.3%) remaining features were filtered based on their peak area. The first filtering step requiring a peak area over 200 in at least 4 samples within any treatment group yielded 356 (7.4%) features. The second one – a minimum maximum feature peak area across the samples over 500 - yielded 314 (6.5%) features. Restricting the peak area to more than 200 was based on the observation that the estimation of the area is unreliable below this threshold. Both steps reduce the noise and the number of comparisons in statistical tests.

The plots of the pairs of the feature peak area distributions within the treatment groups revealed that some samples are affected by a systematic error – a part of the features lies on a second diagonal. Unfortunately, the most affected samples were those from the control group. We therefore decided to keep them in the analysis. Significant differences in the feature peak area medians and sums suggest that the samples differ in the overall metabolite content probably due to different degree of dilution. We therefore normalized the peak area values by rescaling the sums in each sample to a constant value before the group

comparison. We are aware that normalization steps should be performed with as many reliable data points as possible – i.e., before the (most of) filtering steps. In our case, however, this was not possible due to the large number of low concentration features across the samples. Also, the systemic errors in the samples revealed by the pair analysis may contribute to a suboptimal normalization.

During the identification of differential concentrations of the metabolites between the sample groups, we decided to focus on the comparison control vs. high hydrogen peroxide concentration groups because the experiments with various filtering (minimal peak area across the samples) and normalization procedures (no, sum, median, variance stabilizing) showed that the control and low concentration hydrogen peroxide samples often cluster together. A comparison of the control vs. all hydrogen peroxide-treated samples was carried out for comparison subsequently. The robust but conservative Wilcoxon test was used in order to minimize the effects of the extreme data points including remaining values below 200 threshold. An application of the more sensitive parametric (regularized) t-test would probably require variance transformation step for the optimal performance. On the other hand, Wilcoxon test can be used with the normalized values without any transformation.

5 metabolites with a decreased concentration in the samples treated with the high concentration of hydrogen peroxide were selected for further analysis. The selection criteria were a fold change between treatment and control less than 0.5, a corrected p-value less than 0.05 and both the median and mean peak area in the control group above 500. No upregulated metabolites fulfilling the corresponding criteria were identified. Out of those 5 features, a candidate molecular formula was found for two with the same M/z of 327.3477 differing in the retention time. It is highly probable, that they are isomers as their peak under the area is highly correlated across all samples (Pearson correlation > 0.99). The observed decrease of the concentration after the hydrogen peroxide treatment suggests that those compounds could possibly act as antioxidants. Notably inspection of the chromatograms and MS spectra revealed a third candidate isomer with retention time over the 16 minutes threshold used for feature filtering. Concentration of this feature was also markedly decreased after 400 µM hydgoren peroxide treatment.

Follow up experiments should confirm the role of the identified metabolites in stress response of tardigrades. The following questions will be addressed in near future hopefully. Do the concentrations of the partially identified metabolites change in response to other oxidative stress inducing treatments like ionizing radiation? Is the observed concentration change limited to the states with a disturbed redox balance, or is it a part of general stress response activated by exposure to other stress modalities like osmotic and heat shock? Could those compounds or extracts containing them protect human cells against (oxidative) stress?

6 CONCLUSION AND OUTLOOK

We carried out the first metabolomics study of the effect of hydrogen peroxide on a tardigrade *H. exemplaris*. The semipolar metabolites were analyzed by UHPLC MS/MS operating in both positive and negative modes. 314 features passing filtering based on retention time and peak area intensity were compared. 5 metabolites were downregulated after the hydrogen peroxide treatment. For two of them - likely isomers - a candidate molecular formula was identified.

We hope that follow-up experiments not only confirm the role of those metabolites in stress response of *H. dujardini* but also answer the following questions. Do the concentrations of those metabolites change in response to other oxidative stress inducing treatments like ionizing radiation? Is the observed concentration change limited to the states with a disturbed redox balance, or is it a part of general stress response activated by exposure to other stress modalities like osmotic and heat shock? Are those metabolites specific for *H. dujardini* or Tardigrades in general. And finally, could those compounds or extracts containing them protect human cells against (oxidative) stress?

7 ACKNOWLEDGMENTS

I would like to thank my parents for their eternal support, faith, and kindness throughout all my academic life. Also, I would like to thank my supervisor Dr. Jiří Voller for his help and advice and to Dr. Jan Červený from CzechGlobe - Global Change Research Institute, Czech Academy of Sciences for the reliable supply of *Chlorella vulgaris*.

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