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Tardigrade Cell Culturing

- A study in viability of storage cells in *Richtersius coronifer*

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Picture on the front page is from: **http://www.artbytom.com/shop-for-art/The-Tardigrade-Queen.html**, [12/18/2015 – 3:15 pm]

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Abstract

Much of the research into the mechanisms of cryptobiosis at the cellular level in tardigrades have been constrained by the need to extract and gather enough cells for any given experiment. Developing a cell culture of tardigrade cells would provide a powerful tool for future research in this area, but has not been achieved yet. Our project aims to collect information about tardigrade storage cells and develop methods for working with them, so that they may be used to establish a cell culture. Specifically, we are using the storage cells of the species *Richtersius coronifer*. Using Grace's Insect Medium and glucose solutions, we determined that the tardigrades responded to osmolalities above 250 mOsm/kg as hostile environments and that storage cells could not survive in Grace's Insect medium for two days. Using a Trypan Blue viability test, we examined the viability of storage cells over a time span of 2.5 hours when extracted in 9 different medias. Three of those media were different dilutions of Grace's Insect Medium with osmolalities of 129, 226 and 363 mOsm/kg respectively. Three of them were glucose solutions with osmolalities of 196, 246 and 404 mOsm/kg and three of them were Frog Ringer solutions with pH-values of 6.5, 7.5 and 8.5 and an osmolality of 295 mOsm/kg. Statistical analysis showed that many of the results are inconclusive, but statistically significant results suggest that the viability of the cells is highest when kept in media reflecting the internal osmolality of the tardigrade. We suggest that it may be interesting to use embryonic tardigrade cells in future attempts at creating a tardigrade cell culture.

Abstrakt

Meget af forskningen i de underliggende mekanismer for kryptobiose på det cellulære niveau i tardigrader har været begrænset på grund af nødvendigheden af at ekstrahere og samle tilstrækkeligt mange celler til et givent eksperiment. En cellekultur af celler fra tardigrader vil være et nyttigt redskab for fremtidig forskning på området, men sådan en cellekultur er endnu ikke blevet oprettet. Vores projekt har til formål at indsamle information om tardigradernes oplagringsceller (på engelsk: storage cells) og udvikle metoder til at arbejde med dem, således at de kan bruges til skabe sådan en cellekultur. I vores projekt bruger vi arten *Richtersius coronifer*. Ved brug af Grace's Insect Medium og glukoseopløsninger observerede vi, at tardigraderne reagerede på osmolaliteter over 250 mOsm/kg som fjendtlige miljøer og at oplagringsceller ikke kunne overleve i to dage. Ved brug af en Trypan Blå viabilitetstest undersøgte vi viabiliteten af oplagringsceller over en periode på 2,5 timer, når de blev ekstraheret i et af 9 forskellige medier. Tre af disse medier var forskellige fortyndinger af Grace's Insect Medium med osmolaliteter på henholdsvis 129, 226 og 363 mOsm/kg. Tre af dem var glukoseopløsninger med osmolaliteter på 196, 246 og 404 mOsm/kg og tre af dem var Frog Ringer opløsninger med pH-værdier på 6,5, 7,5 og 8,5 og en osmolalitet på 295 mOsm/kg. Statistisk analyse viste at mange af vores resultater er utilstrækkelige til at drage konklusioner, men dem der er statistisk signifikante viser at viabiliteten af cellerne er højest, når mediets osmolalitet afspejler tardigradens interne osmolalitet. Vi foreslår at det måske vil være at foretrække, at bruge embryonske celler fra tardigraden i fremtidige forsøg på at skabe en tardigrade cellekultur, for at undgå problemer med osmoregulering.

1. Introduction

One of the most fundamental ways in which we, as humans, have been able to transcend our physical limitations as well as advance our scientific understanding of the world in general has been by studying animals. Especially studying evolutionarily beneficial traits in animals that we can imitate to a fashion (flight, night-vision, under-water breathing, etc.).

One group of animals that have developed an incredibly interesting and potentially powerful ability are the tardigrades, which can enter a state known as *cryptobiosis*. In addition to simply being an interesting phenomena that we don't understand and so might learn something from studying, it is also of both philosophical interest (getting us closer to understanding what life really is) and of course of practical interest as well (just look at all the rich people paying to be frozen in hope of waking up in the future or for that matter all the stasis-pods in Sci-Fi, as well as the current attempts at organ freezing for transplants).

Basically, when these animals are faced with an unfavorable environment, they can suspend seemingly *all* metabolic activity. In this state, the tardigrades do not require any nutrition, they can 'survive' extreme levels of normally damaging stress factors like radiation, heat, cold, dehydration and, more. In addition, it practically reduces aging to zero; the tardigrade is, in effect, dead. Until it becomes active again.The reason that this would be evolutionarily favorable is that despite having no metabolic activity and technically being a dead organism for decades, all it takes is a cue to signal a more friendly environment, like a drop of water, and the metabolic activity will restart and the animal will wake up and carry about its business.

The best way to study a process like this would undoubtedly be with a cell culture of cells from the animal itself, in which the process could be carefully observed under the most controlled conditionspossible. A cell culture would also help greatly towards mitigating one current bottle-neck effect on research in this area, which is the need to collect and prepare individual tardigrades in large quantities for any research project. However, despite having been the topic of some research for more than 300 years, there is no known way to create and maintain a growing cell culture of tardigrade cells as of yet (Wright, 2001).

Our intention with this project was to try and create a sustainable, growing cell culture of storage cells from tardigrades or at least investigate the behavior of these cells under different culturing conditions, thus getting us closer to creating a working culture in the future.

2. Problem

2.1 Problem Field

The majority of the project will be about the common biological method of cell culturing, both in general and specifically how we approach it for tardigrade storage cells. The theory of this method will mostly fall under physiology and the biochemistry of cryptobiosis and osmoregulation. It will also include cell biology.We will be using the tardigrade species *Richtersius coronifer* in all of our experiments. It will therefore also be the focus of most of the theory.
Since we are investigating the mostly unknown properties of tardigrade storage cells, we will also cover some theory about the physiology of tardigrades. This is necessary to give us an informed approach when changing the parameters of cell culturing, in trying to get the cells to survive and divide.

2.2 Problem Formulation

To investigate what factors are essential to observe, when working with storage cells from *R. coronifer* in a way conducible for the establishment of a cell culture with a focus on the viability of the cells.

2.3 Hypothesis

It is possible to maintain a high viability of *R. coronifer* storage cells, by suspending them in a medium that mimics the internal environment of the tardigrade.

2.4 Demographic

This report was written with the intention of being intelligible for students in the biological sciences entering their last year of the bachelor degree. Terms and concepts that are too specialized for it to be reasonable to expect such a reader to understand are elaborated in the report.

3. Theory

3.1 Tardigrades

3.1.1 Systematics

Tardigrades are a phylum under invertebrates, which are animals without a spine. One subgroup of invertebrates is the superphylum Ecdysozoa. All animals of this superphylum undergoes ecdysis, which is the moulting of the cuticle (Hickman et al.,2011). Figure 3.1.1 shows the relationship between the different clade and phyla under Ecdysozoa. The clade Panarthropoda is a collection of three taxa: Tardigrada, Onychophora (velvet worms) and Arthropoda, which is the group with e.g. insects, spiders and crustaceans (Hickman *et al*, 2011). Both tardigrades and arthropods have cuticles. All species belonging to the phylum Tardigrada is classified to have: Tardigrade leg claws, an exoskeleton called cuticle and buccal stylets (Hickman *et al*, 2011). There are two major classes in this phylum: Heterotardigrada and Eutardigrada. Eutardigrades are mostly terrestrial (Sands *et al*, 2008). The eutardigrades have three Malpighian tubules (Kinchin, 1994). The order Parachela,

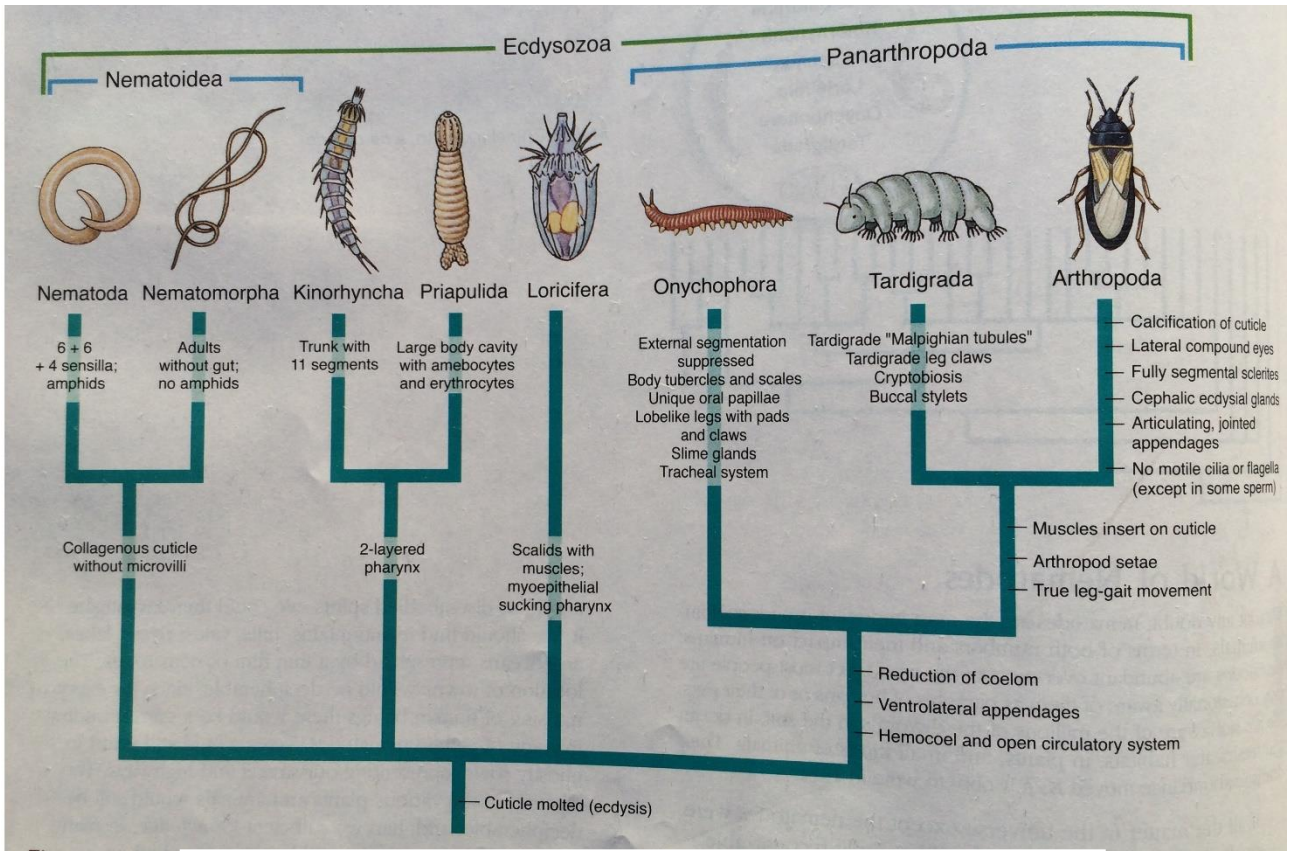


Figure 3.1.1: A cladogram of the different phyla under Ecdysozoa, it shows how close tardigrades and arthropods are (Hickman et al., 2008).

which used to have eight families, had recently been divided into three superfamilies: Isohypsibioidea, Hypsibioidea and Macrobiotoidea (Sands *et al*, 2008). *R. coronifer* is in the superfamily Macrobiotoidea and family Macrobiotidae, which is the family that contains most Eutardigrada species (Guidetti & Bertolani, 2001).

This study will concern genus *Richterius.* A taxanomic overview of the species *R. coronifer* is presented in table 3.1.1.

Table 3.1.1: Taxonomic overview of the species *R. coronifer* of the groups from kingdom to species. The taxonomic system has overall seven hieratic groups: Kingdom, phylum, class, order, family, genus and species (Rasmussen, 2005).

Kingdom:	Animalia	
Superphylum:	Ecdysozoa	Together with e.g. Nematoda
Clade:	Panarthropoda	Together with Arthropoda
Phylum:	Tardigrada	About 900-1000 species
Class:	Eutardigrada	
Order:	Parachela	
Superfamily:	Macrobiotoidea	
Family:	Macrobiotidae	
Subfamily:	Macrobiotinae	
Genus:	*Richtersius*	Before named *Adorybiotus*
Species:	*coronifer*	The name is *Richtersius coronifer*

Giribet et al. (1996) performed a molecular study that proofed an evolutionary relationship between arthropods and tardigrades. It indicates that Tardigrada should be a sister-group to the Arthropoda (Giribet et al., 1996).

Tardigrades have, like arthropods, true leg-gait movement, which means that they move by using their legs and not by moving their body (Hickman et al., 2008).

3.1.2 Morphology

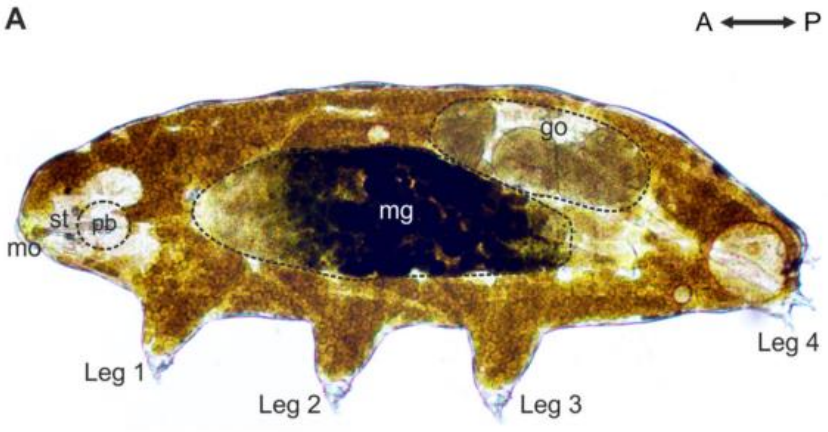


Figure 3.1.2: Light microscopy of a hydrated *R. coronifer* (lateral view) with the anterior (A) and posterior (P) end: mo is the mouthpart, st is the stylet, pb is the pharyngeal bulb, mg is the mid-gut, go is the gonads and with leg 1-4 (Halberg et al., 2013a).

Tardigrades have an average body length of 250-1200 μm (Nelson, 2002). In this context, *R. coronifer* is rather large, with a body length up to 1 mm. Because of its size and colour, *R. coronifer* is also known as the "giant yellow water bear" (Møbjerg et al., 2011). The elongated body consist of a head with a large brain, and four trunk parts each with a pair of legs terminating in claws (Halberg et al., 2013a). The body is unsegmented and the head is in the anterior part of the animal and is not a part of the first trunk segment (Hickman et al., 2008). The size, shape, numbers and structures of their claws are important to the systematics of tardigrades. Each pair of legs for the Eutardigrada have two double claws, an external and an internal one (Dewel et al., 1993).
The tardigrade has a small circular mouth opening with calcified stylets that are used for puncturing food particles, which are plant and animal cells (Nelson, 2002). The digestive system is divided into three parts: The foregut consists of the mouth, buccal tube, stylet mechanism and salivary glands, myoepithelial pharynx and oesophagus (see figure 3.1.2, 3.1.3 and 3.1.4). The midgut begins after the oesophagus and ends at the pylorus. The hindgut is, in eutardigrades, divided in two: rectum and a cloaca (Dewel *et al*, 1993). The primary urine is produced in the Malpighian tubule, which is located in the area between the midgut and the hindgut (see figure 3.1.4). The Malpighian tubule serves as the area of secretion for the tardigrades secretion-reabsorption system (similar to human kidneys). The reabsorption is in the proximal tubule, hindgut and rectum. (Halberg & Møbjerg, 2012). Malphigian tubules are known to be the excretory and osmoregulatory system in eutardigrades (Møbjerg & Dahl, 1996).

Tardigrades do not have a respiratory system or a circulatory system. Thus they “breathe” through their skin and the movement of the body acts as a pump to circulate the body-fluids (Miller, 1997).

The cuticle undergoes moulting periodically during the entire life span of the tardigrade. This cuticle consists of three different layers (from inside): endo-, meso- and exocuticle (Dewel *et al*, 1993). The moult requires 5-10 days to take place and during that time, the old cuticle is shed (Jönsson & Rebecchi, 2002; Nelson, 2002). The size of the tardigrade increase with each moult until it reaches maximum size (Biodiversity Institute of Ontario, 2010).

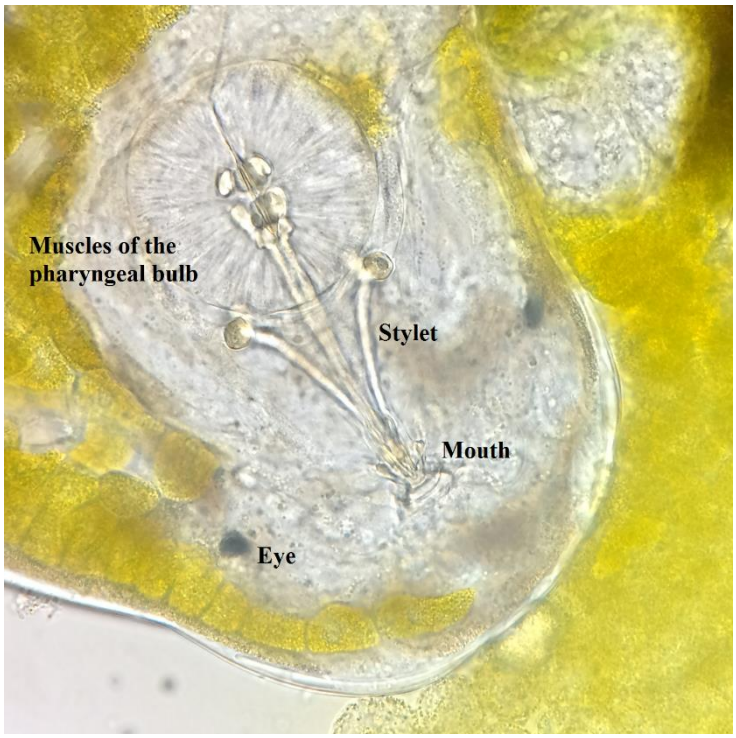


Figure 3.1.3: Ventral picture (Zeiss, Imager.M2m), which shows the head of a *R. coronifer* tardigrade that has been punctured.

The muscular system of tardigrades consists of long muscle bands, bound to the body wall, which have a hydrostatic pressure inside them. This can be regarded as analogous to our skeleton (Hickman *et al*, 2011). The muscles work against the hydrostatic skeleton. The tardigrade can also move by flexing their body (Dewel *et al*, 1993). This muscular structure consists of ventral, dorsal and lateral longitudinal fibres and transverse muscles, which are attached to the internal cuticle (Marchioro *et al*, 2013). The areas of attachment have an extra thick exocuticle–layer. Tardigrades also have small visceral muscles with the primary function of causing intestinal movement (Dewel

et al, 1993). There are also muscles attached to the pharyngeal and the stylet in the cephalic part of the animal (Marchioro *et al*, 2013). The nervous system of the tardigrade consists of a brain in the anterior-dorsal end of the animal, and four ganglia following the length of the animal on the ventral site. These four ganglia support the four pairs of legs the animal has (Kinchin, 1994). An illustration of the muscular system and nervous system can been seen in figure 3.1.4.

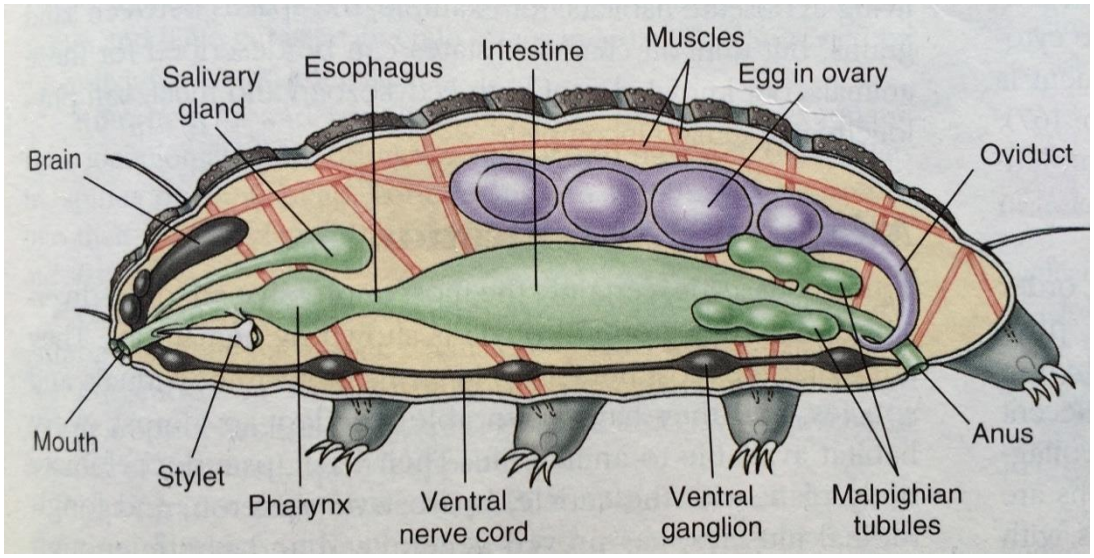


Figure 3.1.4: Illustration of the internal structure of a tardigrade with nervous system, digestive system, muscular system and ovary (Hickman et al., 2005).

Reproductive system

Depending on the species, tardigrades can be either hermaphroditic or have distinct sexual reproductive organs (Dewel *et al*, 1993). Female and male tardigrades both have one reproductive organ that lies over the intestine (Biodiversity Institute of Ontario, 2010). There is both the possibility of sexual and asexual reproduction (parthenogenesis).

Strains consisting exclusively of female tardigrades have been found. In these strains, the female lay eggs that without fertilization and mature into new female tardigrades (parthenogenesis) (Miller, 1997).

Sexual fertilization can occur in several ways. In some species it happens by deposit of sperm in the ovary. In others, it happens by deposit of the sperm under the cuticle, after which the egg is fertilized outside the female. When the female lay the eggs she also moults, and the eggs are fertilized in the old cuticle. In some special cases, the male penetrates the cuticle and deposit sperm

in the female's body cavity. The female tardigrade lays between 1-30 eggs at a time (Biodiversity Institute of Ontario, 2010).

Storage cells

Body cavity cells, also known as storage cells, Speicher cells or coelomocytes are found in the body cavity (Reuner et al., 2010).

Figure 3.1.5: Female *R. coronifer.* The black arrow points at the buccal tube, arrowhead are storage cells and the asterisk are two big oocytes. Scale bar at the bottom right is 100 µm (Rebecchi et al., 2007).

The storage cells are present in the the hemolymph, inside the body cavity also called the hemocoel (or haemocoel). The storage cells can be seen on figure 3.1.5 (arrowhead) inside the body and on figure 3.1.6 outside the body. The hemolymph contains lipid droplets and pigmented material.
The storage cells contain a large nucleus and the cytoplasm is packed with organelles, as well as storage vacuoles containing lipids, peptides and carbohydrates, they store food reserves for the tardigrade. Studies suggest that storage cells have a phagocytic function (Kinchin, 1994). The number of storage cells is approximately 200 per individual although they vary in size and number depending on the nutritional conditions of the animal (Kinchin, 1994). There are different opinions concerning if the tardigrade is eutelic (have a fixed number of cells) or not. According to Kinchin (1994) the tardigrade is not eutelic (Kinchin, 1994), and according to (Wright, 2014) it is.
When tardigrades are exposed to starvation, the number of storage cells decreases, indicating that the storage cells can sacrifice themselves for metabolism, instead of mobilizing the stored fat, as is

the case in human adipose tissue cells (Dewel *et al*, 1993).

The cells generally appear spherical, but they can be found in other shapes too. Tardigrades come in a variety of colours. This colouration results from pigment granules in the storage cells, the epidermis, or from the content of the gut. Storage cells from the *R. coronifer* species are characteristically yellow, giving the animal its yellow appearance (Dewel *et al*, 1993) (See figure 3.1.6).

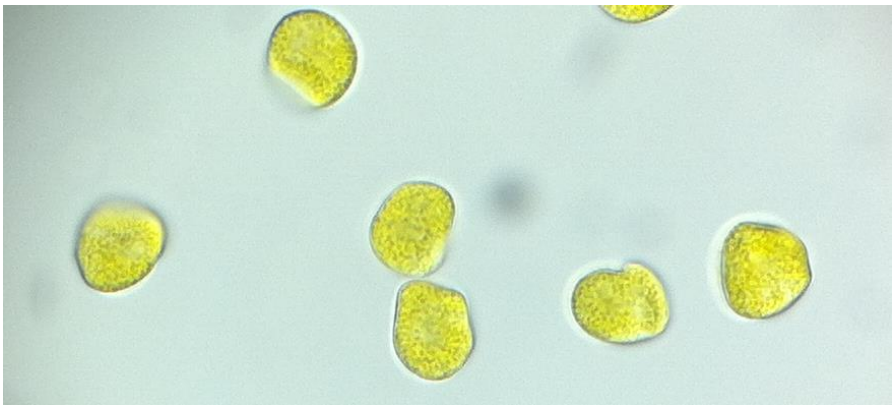


Figure 3.1.6: Microscopy picture of storage cells outside the body. The picture is taken with a mobil camera through the eyepiece of a Gundlach light microscope with a magnification of 400x.

In addition to the discussion about storage cell size related to the nutritional states, Jönsson & Rebecchi (2002) investigated mean area of storage cells. They found that an average storage cell in *R. coronifer* was 129.60 μm^2 before the cryptobiotic period and 109.93 μm^2 after. The storage cells decreased by 14 % in the studied time of cryptobiosis (Jönsson & Rebecchi, 2002). The diameter of *R. coronifer* storage cells differ between 11.83 μm and 12.85 μm (Reuner *et al*, 2010). Jönsson & Rebecchi, (2002) observed a negative correlation between cell size and animal size; however these findings were not statistically significant.

(Halberg & Møbjerg, 2012) investigated how pre-incubation in 2,4-dinitrophenol (DNP) affects anhydrobiotic survival in *R. coronifer*; DNP is a mitochondrial uncoupler. They induced anhydrobiosis by dehydrating the animals after pre-incubation in DNP. The study indicated that the animals failed to form the tun-state, suggesting that the ability to enter anhydrobiosis is dependent on mitochondrial energy production.

Barker Jensen (2010) performed an electrophysiological study on *R. coronifer* storage cells and observed that the intermediate size voltage-gated Cl^-channels of the storage cells were the main cause of the macroscopic current in the cells (Barker Jensen, 2010).

Ion composition

(Halberg et al., 2013b) studied the inorganic ion composition in *R. coronifer*. They used high-perfomance liquid chromatography (HPLC) to identify and quantify different ions present in tardigrade homogenates. The investigated ions were: Na^+, NH_4^+, K^+, Ca^{2+}, Mg^{2+}, F^-, Cl^-, PO_4^{3-} and SO_4^{2-}. HCO_3^- was not investigated. The exact findings are listed in appendix 1. The total osmotic concentration using nanoliter osmometry, which was found to be around 361 mOsm/kg in the *R. coronifer* homogenates, was also measured.

The inorganic ions contributed to the total osmolality with 171 mmol/L, however the study showed a summed osmotic concentration of 361 mOsm/kg. Consequently, there was an osmotic deficit of 190 mmol/L or about 50.8 %, which was ascribed to unidentified organic osmolytes. According to the authors, only the cryptobiotic tardigrades from the study showed such a large fraction of organic osmolytes (Halberg et al., 2013b).

Osmolality

It should be kept in mind that the total osmolality from the (Halberg et al., 2013b) study is measured on homogenates. In our project, we are interested in the osmolality as well as the ion composition of the fluid in which the storage cells are kept - the hemolymph. It is the environment of the hemolymph we would like to copy, in order to culture the cells.

The internal osmolality (hemolymph) in the *R. coronifer* when kept in demineralised water is 168 ±18 mOsm/kg (Bjørn-Mortensen, 2006; Møbjerg et al., 2011). The demineralised water is close to the osmotic concentration in moss water, in which the *R. coronifer* live, which has been measured to be 4.33 mOsm/kg (Halberg et al., 2013b). The animals are capable of adjusting their internal osmolality when exposed to higher salinities; thus for example in an external salinity around 200 mOsm/kg, the animal will adjust and have an internal osmolality of around 350 mOsm/kg. *R. coronifer* will however not survive an external osmolality above 500 mOsm/kg (Bjørn-Mortensen, 2006). This is illustrated in figure 3.1.7. This figure is copied from (Møbjerg et al., 2011)

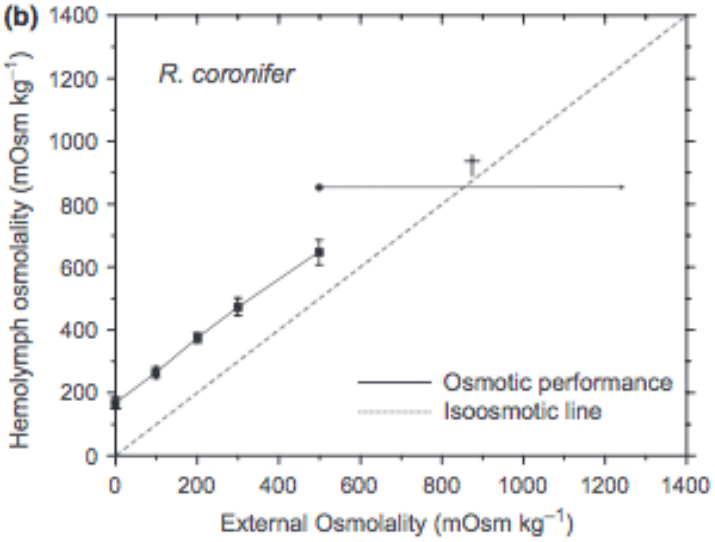


Figure 3.1.7: "*Hemolymph osmolality of Richtersius coronifer from Öland, Sweden as a function of external osmolality. External solutions with osmotic concentrations of 100, 200, 300 and 500 mOsm kg^{-1} were prepared from artificial seawater salt"* Figure text copied from: (Møbjerg et al., 2011).

Habitats

Tardigrades are found all over the world in aquatic as well as in terrestrial ecosystems. The environments in which the microscopic animals live are divided into marine, freshwater and limno-terrestrial, although all tardigrades depend on free water to be in their active state (Nelson, 2002). R. coronifer is a terrestrial species that is found in mosses on orthoceratit limestone rocks at Öland, Sweden (Ramløv & Westh, 1992). Tardigrades share their environments with other organisms such as rotifers and nematodes.

3.1.4 Cryptobiosis

The interest in tardigrades comes primarily from their ability to go into cryptobiosis. It is this state that allows them to survive extreme conditions such as desiccation and the vacuum of space (Jönsson et al., 2008; Persson et al., 2011) and it is this state that gives reasearchers hope of uncovering knew knowledge regarding how to preserve biological material, for example through dehydration or freezing (Møbjerg et al., 2011).

The word *cryptobiosis* was coined by David Keilin in 1959 and means *hidden life*. He defined the term as "*the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable, or comes reversibly to a standstill"* (Keilin, 1959).

There are four main types of cryptobiosis, which are defined by the environmental conditions they individually respond to. The most researched types are anhydrobiosis, which happens in response to dehydration, and cryobiosis, which happens in response to extreme sub-zero temperatures. Beside those, there are anoxybiosis, which is caused by a lack of oxygen supply, and osmobiosis, which happens in response to extreme osmotic conditions (Keilin, 1959; Møbjerg et al., 2011). These types are not equivalent and have been suspected of working by different mechanisms (Crowe et al., 1990). Anhydrobiosis is for example characterized by the active formation of a tun-state, involving withdrawn legs and contracted body as well as some internal organ packing (Bertolani *et al*, 2004).

Tardigrades can, as we have mentioned, survive many extreme conditions. Specifically, they have been shown to survive temperatures from 70 ^{o}C (Ramløv & Westh, 2001) to sub-zero temperatures as far down as -196 ^{o}C (Ramløv & Westh, 1992; Persson *et al*, 2011) with no known lower lethal temperature, osmolalities ranging from distilled water to saltwater with an osmolality of 2000 mOsm kg^{-1} (*Halobiotus crispae*) (Halberg et al., 2009), γ-radiation up to 5 kGy (Jönsson et al.,2005) as well as heavy iron radiation up to 8 kGy (Horikawa *et al*, 2006), and the cosmic radiation of space (Jönsson et al., 2008; Persson et al., 2011). In addition they have been shown to survive anoxia, dessication, high pressure, extreme pH, anatomical stress, toxic chemicals and the vacuum of space (Møbjerg et al., 2011).

Some of these feats are achieved not just by cryptobiosis, but by other features of some species of tardigrades through for example cyclomorphosis (Halberg et al., 2009; Kristensen, 2009). Cyclomorphosis is the phenomena where an animal like the tardigrade changes its physiology and morphology depending on the seasons. One feature of cyclomorphosis in some tardigrades is supercooling, where the tardigrades body temperature becomes as low as -20 ^{o}C in order to remain active at freezing temperatures (Halberg et al., 2009) and protect against extreme fluctuations in

salinity. Some tardigrades have been shown to exercise osmoregulation by adjusting their body volume, while in the active state. When put in extreme hypotonic solutions they swell with up to 60 % of their body volume before regulating back to normal volume and similarly in hypertonic solutions, they shrink and then regulate back to normal volume (Halberg et al., 2009). When exposed to temperatures around the -196 ^{o}C of liquid nitrogen, *R. coronifer* were able to survive in the active state as well as in the tun-state, although tolerating a much higher exposure time when in the tun-state (Ramløv & Westh, 1992).

In fact, some of the survival capabilities of tardigrades do not seem to be linked with cryptobiosis at all, such as their resistance to radiation. This is because active tardigrades have been shown to have as high, if not higher, tolerance for radiation as desiccated tardigrades, leading scientists to believe that this tolerance is caused, not by cryptobiosis, but by extremely efficient mechanisms of DNA repair (Horikawa et al., 2006; Jönsson et al., 2005). Keep in mind that the experiments comparing active tardigrades to desiccated ones are only indicative of the effects of anhydrobiosis on radiation tolerance, not all forms of cryptobiosis.

Interestingly enough, tardigrades are still somewhat vulnerable to extreme heat. As previously mentioned, they can survive up to 70^{o} C when in anhydrobiosis (Ramløv & Westh, 2001), which is still relatively extreme conditions in a broader sense, but not compared to the other extremes that they show little to no problem adapting to.

Cryptobiosis also prolongs the tardigrades life (if you count the time spent *in* cryptobiosis as life). The accumulation of DNA damage during cryptobiosis (Neumann, 2009) has been used to explain why the time it takes the tardigrade to wake up from anhydrobiosis correlates with the time the tardigrade has spent in anhydrobiosis, since the tardigrade would have to carry out considerable DNA-repair in the post-anhydrobiotic stage which would take progressively longer time with more accumulated DNA-damages (Møbjerg et al., 2011).

3.1.5 Biochemistry

The key interest at this point in the field, is to understand the underlying molecular mechanisms of cryptobiosis. It has been suggested that entry into and exit out of cryptobiosis involves the synthesis of certain bioprotectants that act as molecular stabilizers. These bioprotectants might be carbohydrates such as trehalose, proteins such as the heat shock proteins, or antioxidant enzymes that protect against the oxidizing reactions that, as we mentioned before, are suspected to be accomplice to the DNA damage that accumulate during cryptobiosis, as well as other free radical scavengers. Such bioprotectants could function either by interacting with macromolecular structures or simply by functioning as osmolytes. Other suggestions for possible bioprotectants include biological membranes containing certain phospholipid and powerful but unknown mechanisms of DNA repair (Møbjerg *et al*, 2011).

Trehalose

One molecule that has been studied as a potential molecular stabilizer is the carbohydrate trehalose. It gained interest due to being upregulated in some tardigrades during desiccation and was theorized to increase stability of cellular structures by replacing water (Hengherr *et al*, 2008). It has been suggested that trehalose forms hydrogen bonds with macromolecules and cellular structures in absence of water during desiccation and thus preserve native structures. This is known as the water replacement hypothesis (Hengherr *et al*, 2008).

Other organisms such as nematodes have shown to accumulate higher concentrations of trehalose in the dry state compared to tardigrades, which may indicate that *R. coronifer* does not need as high trehalose accumulation for optimal protection (Westh & Ramløv, 1991). Other evidence shows that trehalose does not have an effect on desiccation. A study investigated the importance of trehalose in a mutant strain of yeast *Sacharomyces cerevisiae* with a deleted trehalose-6-phosphate synthase gene (a gene that is involved in trehalose synthesis). The organisms were capable of dehydrating despite the absence of trehalose (Schill *et al*, 2009). Other organisms such as Bdelloid rotifers and *Milnesium tardigradum* do not synthesize trehalose during anhydrobiosis. This demonstrates that the upregulation is not consistent across species and is relatively low in *R. coronifer* compared to some other animals (Møbjerg *et al*, 2011). Furthermore, Pagnotta et al. (2010) observed that the concentration of trehalose only had an extremely minor effect on the amount of water molecules present and their intermolecular binding (Pagnotta et al., 2010).

It has therefore been suggested that trehalose alone is not essential for the capability to enter anhydrobiosis and that some organisms must have additional or alternative mechanisms making them able to enter anhydrobiosis (Hengherr *et al*, 2008; Schill *et al*, 2009).

Heat shock proteins

Heat shock proteins (HSP) are stress proteins that function as chaperones, which bind to other proteins, assist in protein folding and unfolding, prevent aggregation, intracellular localization and degradation of proteins (Schill *et al*, 2004; Jönsson & Schill, 2007). In addition, they provide microenvironments where proteins can fold (Nelson & Cox, 2013). Chaperones are thought to be important in organisms that are exposed to stress conditions, such as desiccation, heat, cold, UV radiation and extreme osmolarities (Ramløv & Westh, 2001; Nelson & Cox, 2013). Chaperones are found in all kinds of organisms, ranging from bacteria to mammals (Nelson & Cox, 2013). In vitro analysis indicates that they prevent desiccation-induced aggregation of proteins (Jönsson & Schill, 2007)

During entrance into anhydrobiosis, Ramløv & Westh (2001) demonstrated the upregulation of a protein with a molecular weight of 71 kDa. They proposed this to be part of the hsp70 protein family. The African chironomid *Polypedilum vanderplanki*, another cryptobiotic organism, showed a upregulation of six different Hsp upon dehydration (Gusev *et al*, 2011).

Schill (2004) then studied the expression of RNA for three isoforms of hsp70 and showed that one of them was significantly induced during transition to cryptobiosis and during the post-cryptobiotic stage, while the other isoforms were downregulated both during cryptobiosis and the transitional stages (Møbjerg et al., 2011). Jönsson & Schill, (2007) used immune-westernblot to show that hsp70 expression indeed increased during the transitional stages of cryptobiosis, but not during the cryptobiosis itself. Expression of hsp70 was lower for desiccated tardigrades during anhydrobiosis, than for the untreated control group. They suggested that hsp70 may have a role to play in the repair-mechanisms taking place after desiccation, but not as a molecular stabilizer during cryptobiosis (Jönsson & Schill, 2007). Other studies indicate that heat shock proteins only have a limited role to play in desiccation tolerance and their expression is species specific (Møbjerg *et al*, 2011) and thus unlikely to be fundamental to the molecular mechanisms of cryptobiosis.

Late embryogenesis abundant proteins

Another candidate for bioprotectant is the late-embryogenesis abundant proteins (LEA), which have been shown to give desiccation tolerance in other organisms, possibly analogously to the tolerance in tardigrades (Møbjerg *et al*, 2011). It has been speculated that they may protect the organism against the damaging effects during water loss, such as protein aggregation and prevent the inactivation of proteins (Goyal *et al*, 2005). Goyal et al. (2005) studied two types of LEA proteins from the nematode *Aphelenchus avenea* and from wheat and observed that these LEA proteins alone are not capable of preventing protein aggregation due to heat stress. Thus, it was observed that these LEA proteins reduced citrate synthase aggregation at high temperatures when trehalose were present, more so than when only trehalose was present. This may indicate that LEA proteins can work synergistically with trehalose (Goyal *et al*, 2005).

LEA proteins have won some interest due to one LEA protein being found present in the species *M. tardigradum* (Schokraie *et al*, 2010).

Anti-oxidant defences

One of the most deleterious effects of water depletion is the formation of Reactive Oxygen Species (ROS) and free radicals because of the increase in ionic concentration, which causes oxidative damage (Rizzo *et al*, 2010). ROS is an oxygen atom with an unpaired electron (Devasagayam *et al*, 2004). It attacks DNA in a way that may cause mutations, leading to inactivation of important enzymes and peroxidation of fatty acids in the cell membrane (Rizzo *et al*, 2010), which causes a decrease in membrane fluidity and lysis of the cell (Machlin & Bendich, 1987).

Rizzo et al. (2010) studied different antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione reductase and glutathione peroxidase in *Paramacrobiotus richtersi* in both desiccated and hydrated state. The results showed that SOD and glutathione peroxidase activity increased in desiccated tardigrades, while for the other enzymes no differences were observed (Rizzo *et al*, 2010). This suggests that the antioxidant enzyme system may not only play a role in desiccated tardigrades, but also in the rehydration phases, because of the de novo synthesis of ROS due to the exposure to oxygen (Rizzo *et al*, 2010). Antioxidant enzymes can neutralize free radicals and their actions at different stages. SOD can catalyze the transformation of superoxide to hydrogen peroxide, after which catalase can break it down to water (Devasagayam *et al*, 2004).

Other approaches

Several attempts have been made to gage the molecular mechanisms of cryptobiosis by genetically mapping cryptobiosis by first sequencing the genome of tardigrades and then correlating it to protein activity with transcriptomics and proteomics (Møbjerg *et al*, 2011).

Neuman (2006) tries to give an assessment of the challenge in understanding mechanisms of cryptobiosis from a theoretical physics perspective. It includes no data and is mainly concerned with discussing the relevant concepts, such as bootstrapping, thermodynamics and information processing, rather than describing or quantifying them in any way. It does however hypothesize that cryptobiosis may require a minimum of metabolic activity, making it not a completely ametabolic state, in order to maintain the organisation of the metabolic system in the cell, for when it is stimulated to awaken. The paper encourages future researchers to investigate this falsifiable prediction as an initial way to attack the problem of cryptobiosis from a more biophysical angle to supplement the molecular biology research already being carried out (Neuman, 2006).

3.2 Cell Culturing

The research of cells has a huge knowledge potential with regards to the study of various properties. Cryptobiosis, as seen in tardigrades, is no exception. This is the reason for the need to develop a cell culture of tardigrade storage cells.

The following sections will give an introduction to the terminology of cell culturing, as well as a brief overview of the key principles in establishing a cell culture. The chapter deals primarily with insect cell cultures, because of assumed similarities between insect- and tardigrade cells. Furthermore, this chapter contains a short overview of methods for keeping storage cells alive.

Requirements for cell division

The main principle in establishing a new cell culture is to get the cells to divide. Several aspects have to be taken into consideration, like gaining cell mass to a certain point, to have enough resources required to divide. Another important factor is the external signals, for example from adjacent cells, which may be responsible for starting the intracellular program that triggers cell division. It is therefore important that the in vivo conditions are imitated by the in vitro ones (Alberts *et al*, 2002).

The observation of the cell culture in the first few days after setting it up is essential to see if they are dividing or simply staying alive. In most cells, a natural apoptosis occurs after a few cell divisions, so primary cell cultures have a limited life-span of only a few cell divisions. It is difficult to create a cell line without any transformation of the genes that are responsible for limiting the cell division. This can be influenced to some degree by external factors, like adding growth hormones (Alberts *et al*, 2002)

Key principles in establishing a cell line

The establishment of a new cell line usually follows similar protocols. The start is making a primary culture. This means that the cells are not available as a cell line yet, but instead come directly out of the target organism.

Cell cultures generally fall into one of two categories: adherent monolayer cultures or suspension cultures. Adherent cells should be grown on a solid substrate in order to survive, while suspension cultures have the cells freely suspended in a fluid medium (Freshney, 2000).

The procedure is to extract the desired cells and transfer them to an appropriate growth medium in a flask, tube or well, where they can grow. The next step is to place the vessel in an incubator at a specific temperature and sometimes also exposed to gas (like CO_2) for a few days, depending on the growth rate of the cells. The gas is simply to maintain the buffer-system that regulates the pH of the medium.
How cells grow varies dramatically and it depends mainly on how they have been grown before, in the in vivo environment, whether they will attach to a surface or not. In the case of tardigrade storage cells, they are found in the hemolymphic fluid, where they are freely suspended, but sometimes attach to the surface of the body cavity (Dewel *et al*, 1993).

The requirements of using the medium and the cells correctly can be found from the ATCC description, which is an independent organization for bio-laboratory equipment usage.

Medium composition

The medium contains amino acids and vitamins, an energy source of carbohydrates (most commonly glucose or sucrose in the case of insect cells), inorganic salts and antibiotics. Furthermore, the temperature and pH are both important factors in simulating the in vivo conditions of the original organism (Harrison & Rae, 1997).

Basically there are two different types of medium, which should be distinguished between:

- Medium with serum (also called *undefined medium*): Contains amino acids, vitamins, inorganic salts, an energy source and serum. The right amount of nutrients is not exactly known, due to the serum, which delivers components of unknown concentration.
- Serum free media: More standardization possible through exact knowledge about concentrations of the ingredients (lifetechnologies).

Adjustable medium parameters

pH: Most mammalian cell lines grow with a neutral pH around 7, but insect cells prefer a more acidic environment with a pH at 6.2. As the cells grow the environment will become more acidic because of their waste products, so a buffer should be used to maintain a stable pH value. A buffer could be for example bicarbonate, commonly used for mammalian cell lines (Davis, 2011). Whereas the insect cell lines prefer a phosphate buffer (Schlaeger, 1996).

The description from ATCC says that cells in Grace's Insect Medium should be incubated without CO_2, but does not give a reason for this at the beginning. Therefore we asked directly at the company why that is the case.

Here is the response they gave:

> *"CRL-1711 is cultured in Grace's Insect Medium Supplemented (GIBCO/Invitrogen Cat. No. 11605-094. This medium is formulated for use in a free gas exchange with atmospheric air. The standard sodium bicarbonate/CO_2 buffering system is replaced by a combination of phosphate buffers, free-base amino acids, higher levels of sodium pyruvate and galactose. A CO_2 and air mixture is detrimental to cells when using this medium for cultivation. If cells in this medium are incubated with CO_2, the medium may quickly turn acidic and likely kill the culture."*
>
> - Catherine, ATCC

Amino acids and vitamins: Which amino acids and vitamins are needed depends on the cell type.
Carbohydrates: The carbohydrates function as energy source for the cells. It is mostly glucose in media, but it can also be fructose, maltose, sucrose or galactose. For insect cells mostly used is glucose and sucrose (Schlaeger, 1996).
Inorganic salts: The concentration of inorganic salts should be known because the media should have the same osmolality as the cells (Freshney, 2000).
Hormones and growth factors: Can be added to serum-free media. They should support the growth of the cells. If serum is contained then there are already certain growth hormones included.
Antibiotics: antibiotics in the media are used to avoid bacterial growth. In a primary cell line, this is necessary because the cells that are used are extracted directly from the animal along with many

different microorganisms. However, the use of antibiotics in medium also comes with disadvantages, e.g. the risk of making an antibiotic resistant cell line. The focus should be kept on good antiseptic technique, then using antibiotics if that is not sufficient (Vlak *et al*, 2006).
Temperature: The incubator should have a temperature around 37 ºC for mammalian cells and between 25 ºC and 28 ºC for insect cells (Harrison & Rae, 1997).

Subculturing

When the cells have been dividing and are approaching confluency, then the cells can be sub-cultured. Sub-culturing is when a fraction of the cells are passed to a new vessel in order to support growth. If the cells are adherent, then generally trypsin is used to detach the cells before transfer (Davis, 2011)

Methods for keeping storage cells alive

Due to the fact that culturing storage cells from tardigrdes is a completely unresearched field, the first challenge in establishing a primary culture of storage cells, is keeping the cells alive.

Christel Barker studied storage cells form *R. coronifer* in her master thesis from 2010 and managed to keep them alive for at least 4 hours. The cells were kept in a media with a calculated osmolality around 192 mOsm/kg. Barker Jensen (2010) used a medium with an ion composition that corresponds to amphibians, since the intracellular ion composition of storage cells is unknown (Barker Jensen, 2010). Different solutions were used for her experiments, but the base solvent in most of the solutions was a common Frog Ringer solution, with and without additional ions. The pH values of the used Ringer solutions were 8.5 and 7.5. Barker Jensen (2010) also used Hoyles medium with additional glucose, which has a pH of 6.5.

A Ringer solution is an aqueous salt solution. There are different recipes for Ringer solutions, which should be chosen between, based on the relevant cells' ion composition (Helmenstine, 2015). Ringer solutions are not intended for cell culture use, but for short-term maintenance of examination material.

Viability test

It is necessary to be able to determine whether the cells are alive or not, before starting a new primary culture.

A common way of doing a viability test is the Trypan Blue staining method. (Lynn, 2002)

The blue dye only diffuses into the cells that have unstable membranes, but not the ones with stable membranes. Therefore only dead cells will be dyed blue, while the viable cells will remain in their original color (Barker Jensen, 2010).

It is also possible to test the viability of cells by other methods, such as studying ATP production or fluorescence (Barker Jensen, 2010).

4. Methods

This section include descriptions of both procedures and experiments. The techniques for basic handling of tardigrades had to be learned and practiced before the initiation of experiments. In total we describe eight individual experiments.

4.1 Apparatus

VAPRO Osmometer 5520, Wescor.
The osmolalities of all mediums used were measured on a VAPRO Osmometer 5520, Wescor, as seen on Figure 4.1.1.
The measurements were triplicates The unit of the osmolality was mOsm/kg.

Figure 4.1.1: This is a picture of a VAPRO Osmometer 5520, Wescor.

Microscopes
All the microscopes had an Occular magnification of 10x.

Zeiss Imager. M2m.

Software: AxioVision 4.8

Objectives: 20-63x, leading to a total magnification of 200-630x.

Gundlach Stereo Microscope

Total magnification: 10-40x magnification.

Inverted Leica Microscope

Software: LAS V4.2.

Objectives: 20-40x, leading to a total magnification of 200-400x.

Gundlach light microscope.

Objectives: 10-100x, leading to a total magnification of 100-1000x.

Medias

Antibiotics

The antibiotics used in the following experiments were made from a penicillin-streptomycin stock with a concentration of 10 mg/mL (Pen/Strep; 10 mg/mL; CSE/OV; 16.07.2015). A concentration of 0.1 mg/mL was used. If contamination occurred, the dose could be increased.

Trypan Blue

We made a 0.4 % Trypan Blue dye solution by mixing 59.3 mg of Trypan Blue (10x) with 14.8 mL of 0.9 % NaCl. This solution was kept at 5 °C and used in all viability tests.

Grace's Insect Medium

Grace's Insect Medium (1x), supplemented from Thermo Fisher Scientific.
LOT number: 1663821.

Frog Ringer Solution

Frog Ringer Solutions were made using the recipe found in appendix 2. The ingredients were 0.578 g of HEPES buffer, 3.60 g of NaCl, 0.12 g of KCl, 0.11 g of $CaCl_2$, 0.91 g of glucose and 0.10 g of $MgCl_2$. We made a final volume of 500 mL. The pH value was adjusted with 0.5 M NaOH until approximately 6.49, 7.47 and 8.60, respectively. After each adjustment, 150 mL was transferred to a smaller flask and 16.6 mL of water was added. In total, 3 different solutions were prepared and then autoclaved.

4.2 Procedures

Extracting tardigrades from moss

The *R. coronifer* used in all of our experiments were extracted from moss, collected at Öland in Sweden. The moss was collected on a rainy day on April 1, 2015 and was dried out and kept in coffee filters in the laboratory for about 6 months before the experiments began.

To extract tardigrades from the moss a shaft-shredder, a sieve column, a watch glass, a Gundlach Stereo Microscope and an Irwing loop was used (an Irwing loop is a prepared chopstick with a wire and a loop at the end). We began the extracting-procedure by adding two or three dried moss balls to a shaft-shredder. They were chopped into a sieve column of progressively smaller size. The small pieces of moss in the sieve column were flushed with tap water for about five minutes and the contents of the 250 µM and 125 µM columns were put into a Petri dish with water. The Petri dish contained the chopped moss, tardigrades, some free-floating eggs and nematodes. The tardigrades were extracted from the Petri dish with an Irwing loop, and transferred to a watch glass containing tap water.

R. coronifer is easy to recognize because of their color size and habit. Also there are only few other species in these samples, which are only found in low numbers and neither of them resemble *R. coronifer*. Figure 4.2.1 illustrates the content of a Petri dish under the Gundlach Stereo Microscope.

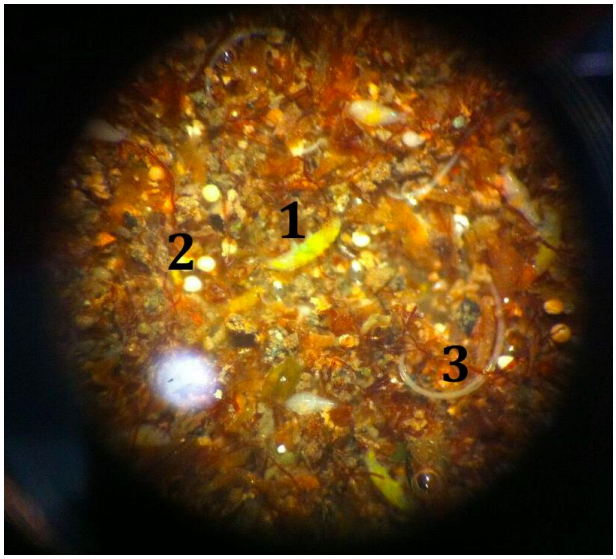


Figure 4.2.1: This picture shows a Petri dish with chopped moss soaked in water under the Gundlach Stereo Microscope with a magnification between 10-40x. The numbers in the picture shows 1: a *R. coronifer, 2:* eggs and 3: a nematode.

Extracting storage cells from living tardigrades

As described in the theory sections, the storage cells are suspended in the hemolymph, which is found inside the body cavity of all tardigrade species. To get the cells out of the hemocoel, the bodies were punctured using two fine insect needles; this procedure killed the animals.

To carry out this procedure we used an Irwin loop, two thin insect needles with chopstick handles, a 20-200 µL pipette, a watch glass, a Gundlach Stereo Microscope, an Eppendorf tube, paraffin oil, and a collection of extracted active *R. coronifer* kept in tap water.

We began the procedure by filling the watch glass with 2 mL of paraffin oil and then we placed about 200 µL of a chosen medium containing antibiotics, below the paraffin oil. The bubble should be completely covered by the oil, to avoid evaporation of any liquid leading to a change in the osmolality.

Active tardigrades were transferred from the watch glass with water, directly into the droplet of medium under the paraffin oil. They were stabbed with the two insect needles. The procedure was carried out under a Gundlach Stereo microscope. The stabbing of the tardigrades allowed the yellow storage cells to float out into the medium, along with free-floating eggs and perhaps some waste from the mid gut. After having punctured 5-10 tardigrades in a droplet we transferred the extracted storage cells into an Eppendorf tube with a pipette. The pipette tip was wetted before the transfer, to avoid that the cells got stuck in the pipette tip. The Eppendorf tube with the cells suspended in media, was then referred to as the *primary cell suspension*.

4.3 Experiments

Since we are continuously developing the methods for working with storage cells, we will give brief objectives for each experiment in this section to give a better understanding of how we proceed from one experiment to the next.

1. Survival of *R. coronifer* in water

Objective: To investigate the time span that *R. coronifer* could be kept in tap water without any additional nutrients.

Experiment: 27 tardigrades were placed in a watch glass filled with tap water. This watch glass was kept in the refrigerator at 5 °C for four days.

2. Cell Culture A

Objective: To make a cell culture, while getting information about the practical obstacles in the process. Triplicates were made in a 24 well plate, and tested for two different parameters: Cell concentration and additional glucose. This let us probe two different factors, the osmolality of the medium and the amount of nutrients available to the cells.

Experiment: For this experiment, double amount of glucose was used in half of the wells. The concentration of glucose in Grace's Insect Medium is 700 mg/L (see appendix 3). The wells had a total volume of approximately 1000 µL. We transferred 20 µL of a glucose stock. Following calculations were made (1-3):

$$700\ \frac{mg}{L} \times 0.001\ L = 0.7\ mg \qquad (1)$$

There were 0.7 mg glucose in each well. To double that amount, we added 0.7 mg in 20 µL of Milli-Q water.

$$c = \frac{0{,}7\ mg}{0.00002\ L} = 35000\ \frac{mg}{L} = 35\ \frac{g}{L}. \qquad (2)$$

We achieved this concentration by measuring out 3498 mg ≈ 3500 mg and dissolved it in 100 mL Milli-Q water:

$$\frac{3500\ mg}{0.1\ L} = 35000\ \frac{mg}{L} \qquad (3)$$

When we prepared the glucose stock we made a calculation mistake, we thought that the glucose stock had a 10 times higher concentration than it actually had. Therefore we diluted it by another factor 10 by adding 100 µL of this stock to 900 µL Milli-Q water and added 20 µL of this dilution to the wells instead. Therefore, instead the glucose concentration was change to 110 % rather than the desired 200 %.
In total 57 *R. coronifer* were punctured and the storage cells were transferred to a primary cell suspension, before adjusting the cell concentrations for the different wells.
We worked with 4 different cell concentrations by adding 40 µL, 30 µL, 20 µL and 10 µL of our primary cell suspension to the different wells.

A schematic drawing of the content of the different wells is shown in Figure 4.3.1.

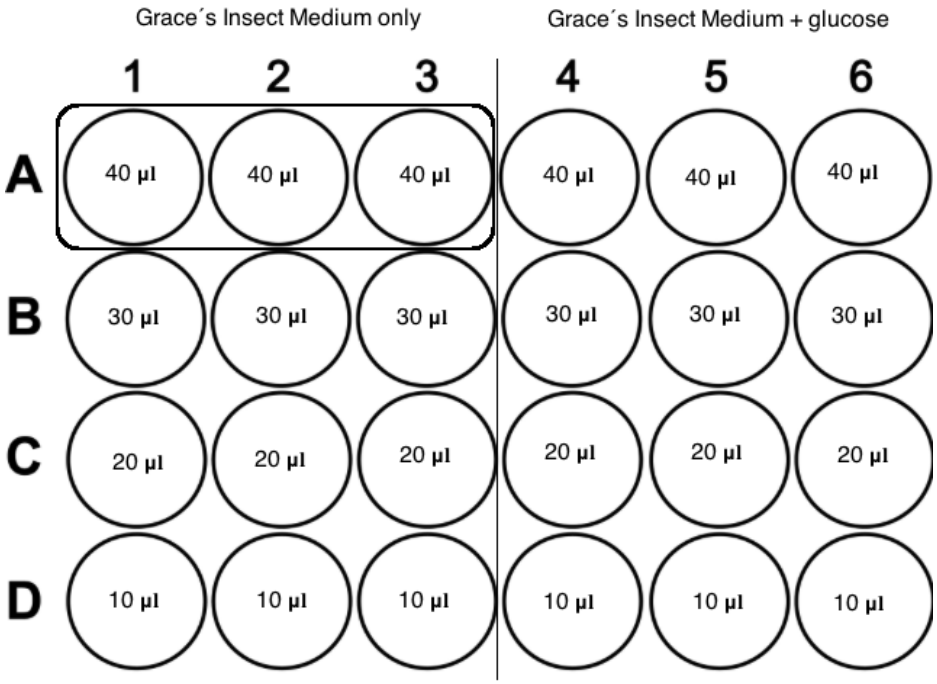


Figure 4.3.1: Schematic drawing of the content of the wells in the 24 well plate. Column 1-3 contain Grace´s Insect Medium +20 µL of Mill- Q water whereas column 4-6 contain Grace´s Insect Medium and 20 µL of the glucose stock. Going from row A to D the cell concentration decreases. The circle around A1, A2 and A3 illustrates that we have made the different wells in triplicates.

The 24 well plate was taken to the cell laboratory along with the media, primary cell suspension, glucose solution and Milli-Q water and filled in the following way (Using aseptic techniques as described in appendix 4):

First 960 µL of pure medium were added without antibiotics to row A, then 970 µL to row B, 980 µL to row C and 990 µL to row D. After that we added 40 µL cell suspension to row A, 30 µL to B, 20 µL to C and 10 µL to D. Finally, we added 20 µL Milli-Q water to column 1, 2 & 3 and 20 µL glucose solution to column 4, 5 & 6. This adds up to a total volume of 1020 µL in all wells (Figure 4.3.2).

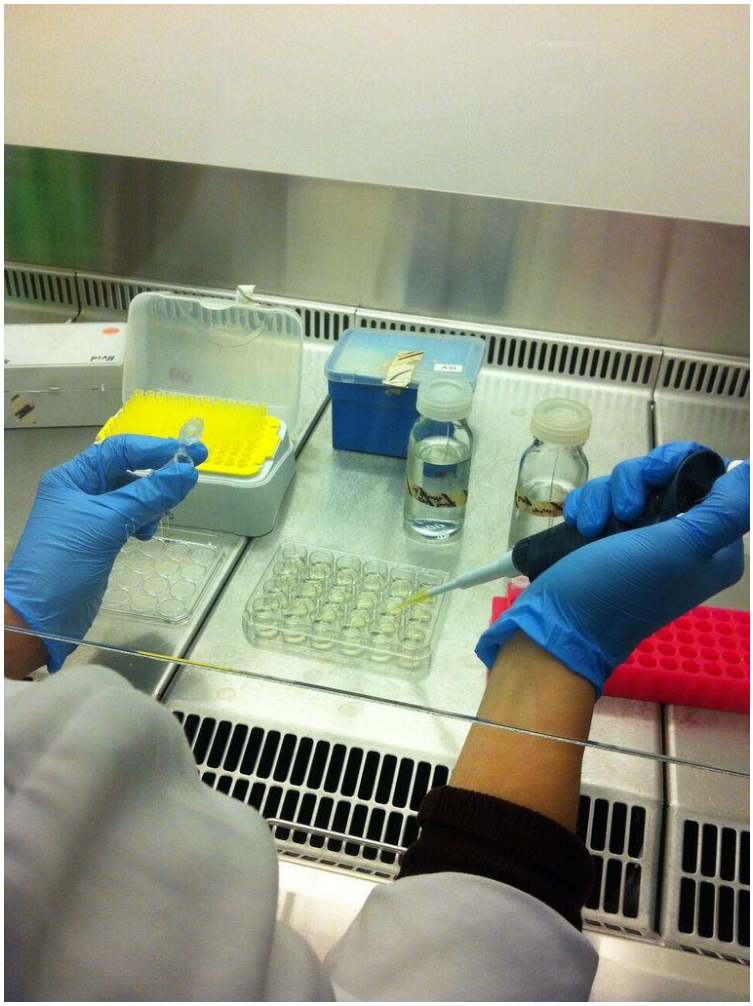


Figure 4.3.2: Setting up our first cell culture in a sterile laboratory. The flow-hood was cleaned with 70 % ethanol before use and all the used equipment was sterilized as well to avoid the risk of contamination.

Once the cell cultures were done we examined the wells using the Gundlach Stereo microscope. The cell concentrations were decreasing from A to D in the expected proportions, variations between wells with the same contents were insignificant and some amount of waste from the moss and tardigrades had been transferred to all wells.

The cell cultures were now placed in the incubator at 15 °C without CO_2. After two days of incubation, the cultures were observed again. Because of contamination at this time the experiment was terminated.

3. Tardigrade response to Grace´s Insect Medium

Objective: To investigate *R. coronifer* response to the Grace´s Insect Medium.

Experiment: On day 1, 50 *R. coronifer* were transferred to a watch glass with Grace´s Insect Medium and 50 tardigrades to one with water as control. Both watch glasses were kept in the incubator at 15 °C, without CO_2, and the lid 90 % closed to avoid evaporation without suffocating the animals. The tardigrades were observed after two hours and again three days later.

At day 3, 30 of the 50 tardigrades kept in water were transferred to a new watch glass with Grace´s Insect Medium containing antibiotics. The watch glass was left in the incubator for 15 minutes, and the medium was removed and replaced with fresh tap water.

4. Failed viability test

Objective: To investigate if the storage cells die when extracted in Grace´s Insect Medium, water or salt water (0.9 % NaCl).

Experiment: The storage cells were extracted as described in the previous method section. Afterwards the cells were transferred to wells containing Trypan Blue. Droplets from these wells were transferred to glass slides and covered with coverslips. The slides were studied at a Gundlach light microscope to determine if the blue color had dyed the cells, which would suggest that the cells were dead.

5. Tardigrade response to osmolality

Objective: To investigate the effect of osmolality on living *R. coronifer*.

Experiment: We made six glucose-water solutions and four dilutions of Grace's Insect Medium of varying osmolality. Two living tardigrades were placed in each dilution to see if they would keep their active state or go into the tun-state (or die, but we did not check that).

All of the dilutions were made with Milli-Q water, which was kept in a plastic bottle in an uncertain time period and therefore have a much higher osmolality than expected. We measured the osmolality of the different dilutions of Grace´s Insect Medium and water with glucose with the osmometer. The osmolality was only measured ones. In Table 4.3.1 we have listed the 12 different medias used in this experiment. The experiment was made in 12 Eppendorf tubes with the respective medias. The tubes were covered with parafilm and incubated at 15 °C until the next day.

The description of how we made the glucose dilution can be found in appendix 5.

Table 4.3.1: A table of the medias used in this experiment, the measured osmolalities and the placements of the tardigrades + medias in a 96 well plate. In this table, Grace's refer to Grace's Insect medium.

Media	mOsm/kg	Placement
Milli-Q water	77	a1
Glucose 84	84	a2
Glucose 163	163	a3
Glucose 196	196	a4
Glucose 246	246	a5
Glucose 311	311	a6
Glucose 404	404	a7
Grace´s (1:1)	363	c1
Grace´s (1:2)	226	c2
Grace´s (1:2.5)	151	c3
Grace´s (1:3)	129	c4
Grace´s (1:3)	115	c5

The next day, we realized that it was not possible to determine whether the tardigrades were moving or not moving in the Eppendorf tubes. The tardigrades were therefore transferred with a pipette to specific wells in a 96 well plate and observed under a Gundlach Stereo microscope. During the transfer, three tardigrades were lost (a4, a7 and c1). The new placement of the individual medias + tardigrades is written in column three in the table a bow.

6. Cell Culture B

Objective: We want to test the storage cells response to different osmolalities of Grace's Insect Medium and different cell concentrations on cell culturing.

Experiment: This time, a plate with 96 wells was used. The total volume of each well was 200 µL.

Four different dilutions of Grace's Insect Medium containing 0.1 mg/mL of antibiotics were used in this experiment: Undiluted 1:1, diluted 1:2, diluted 1:2.5 and diluted 1:3. We diluted the medium in autoclaved Milli-Q water, and the dilutions were made under sterile conditions in the flow hood (see appendix 4).

Tardigrades were extracted as described in the standard procedure. They were punctured in the respective media dilutions and put into Eppendorf tubes with that same dilution (primary cells suspensions). For each primary cells suspension, 15 tardigrades were punctured and their cells suspended in a total volume of approximately 500 µL. To increase the concentration of cells in the Eppendorf tubes, the cells were briefly spun down, using a table centrifuge. 50 µL of the medium was discarded and the cells were mixed carefully. All of these procedures were carried out under non-sterile conditions.

The four different primary cell suspensions were brought to the sterile laboratory and then we used the well setup shown on figure 4.3.3.

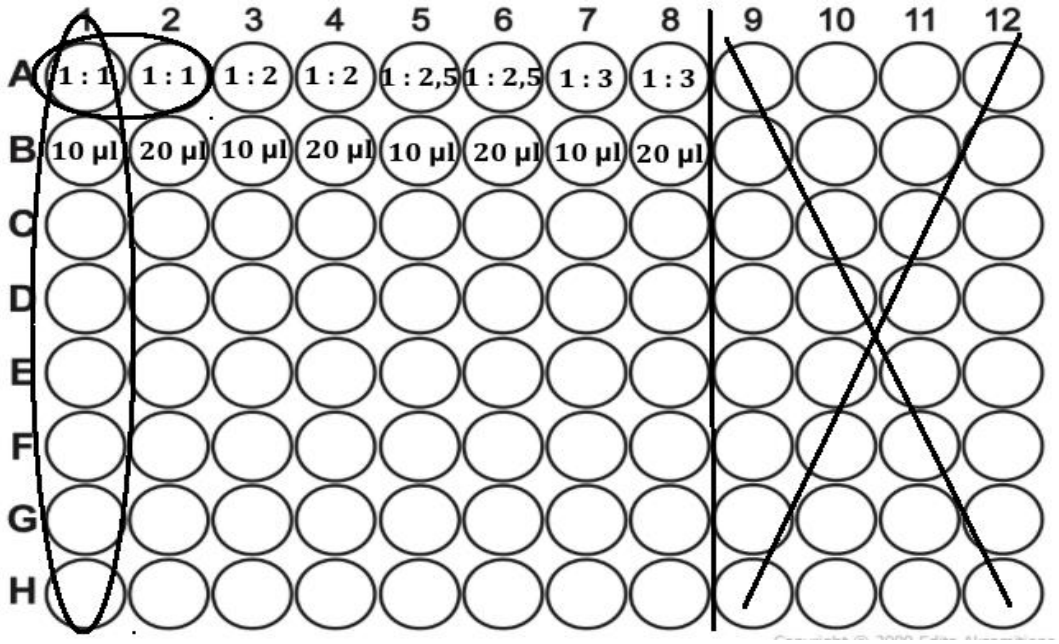


Figure 4.3.3: Well setup. We used column 1-8 in a 96 well plate, column 9-12 were not used. All off the wells were made with a total volume of 200 µL with varying cell concentrations. See the text for a specific description.

We used column 1-8 in a 96 well plate, column 9-12 were not used. All off the wells were made with a total volume of 200 µL with varying cell concentrations. For example in column 1 we added 190 µL of Grace's Insect Medium undiluted (1:1) + 10 µL primary cell suspension, and in column 2 we added 180 µL of Grace's Insect Medium undiluted (1:1) and 20 µL of primary cell suspension. The same goes for column 3 + 4 and so forth, only with different medias. The columns were made as octaplicates meaning that row A-H contents are identical in the same column. This was done to make sure we would have enough samples for future viability tests. Column 1 + 2 was loaded with undiluted Grace's Insect Medium, column 3 + 4 with diluted media 1:2, column 5 + 6 with diluted media 1:2.5 and column 7 + 8 with diluted media 1:3.

The wells were checked under a light microscope. We could see that the wells with the smallest cell concentration of 10 µL were thin, but in the 20 µL the cells seemed to be dispersed evenly across the bottom of the wells. The cell culture was then incubated at 15 ºC.

Lastly, we made viability tests using the leftovers from the four primary cell suspensions. At this time the storage cells were approximately 3 hours old. The Trypan Blue dye solution was added directly into the Eppendorf tubes with the cell suspensions. About 20 µL of the colored cell suspension was then pipetted to a slide, a cover slip was added and the slides were observed under the Gundlach light microscope.

At day 3 we made new viability tests again.

7. Comparing microscopes for viability test

Objective: We wanted to test how to differentiate between dead and alive storage cells on 3 different microscopes, when using the Trypan Blue viability test.

Experiment: Slides with the following setup was prepared: A 10-20 µL droplet of glucose solution (129 mOsm/kg) was placed on a slide. One *R. coronifer* was transferred to the droplet and punctured in it. Afterwards, 5-10 µL of Trypan Blue dye solution was added together with a coverslip. The slides were then examined under three different microscopes: Zeiss Imager, Inverted Leica and Gundlach light microscope. Pictures were taken to document our results.

8. Storage Cell Viability Assay

Objective: To test the survival rate of storage cells over time while suspended in different types of media.

Experiment: We used three different dilutions of Grace's Insect Medium, three different glucose-water solutions of varying osmolality and three Frig Ringer solutions with different pH values. The measured osmolalities of the different medias can be found in the table 5.2.1.

All the tardigrades used in this experiment were extracted at the day of the experiment. The slides for the "*t* = 0 measurements" were made by adding 2 x 15 µL of media to a slide, using a pipette, and then transferring 2-3 living tardigrades with an Irving Loop to each droplet. The tardigrades were punctured with two insect needles directly on the slides to avoid any loss of cells during the transfer procedure. Afterwards, we added 5 µL of Trypan Blue solution to the droplets and a cover slip was placed on top of them.

The slides that needed to be measured after 1 or 2.5 hours were made in almost the same way. The only difference was that after the tardigrades had been punctured, the slides were incubated at 15 °C for the relevant time in a "moist chamber" made from a Petri dish with a wet napkin. Once the slides had been incubated for 1 or 2.5 hours, the Trypan Blue was added and then the coverslip.

The slides were imaged with the microscope Zeiss Imager. M2m just after the coverslips had been placed. Then, after 10-25 minutes, the slides were counted under Gundlach Stereo Microscope at 20x magnification. The survival of approximately 100 cells was registered for each droplet. Cells were considered dead if they were blue and alive if they were yellow with yellow granulas. Cells that appeared blue due to having blue coloured granulas were also categorized as being dead. Some of the cells seemed to have partly destroyed membranes, because the intracellular substance was leaking out of them. These cells were also categorized as being dead.

It took 5-10 minutes to count each slide. The time consuming part of the process was to find the cells, because, as we found out, 200-600 storage cells on a 32 x 24 mm cover slip are not easy to find. Therefore the slide scheduled to be measured after 0 hours, 1 hour and 2.5 hours were measured in a slightly later time.

5. Results

5.1 Results of Experiments

1. Survival of *R. coronifer* in water

The tardigrades were alive and moving in the water on the second and the third day after the experiment. On the fourth day however, the water had evaporated from the watch glass. Water was poured on the tardigrades and observed under the microscope. Their cuticles had been slightly displaced from the rest of their bodies, indicating that they were dead and not in anhydrobiosis. All of the 27 tardigrades in the experiment were dead.

2. Cell Culture A

The 24 wells of cell cultures were observed after 1 and 2 days using a Gundlach Stereo microscope, which can magnify images 10-40 times. In this magnification, the wells contained lots of yellow spots, that were interpreted as being viable storage cells.
On day 5 the wells were also observed under an Inverted Leica microscope. We saw that a contamination had spread in the wells and the cells had ruptured, leaving cell fragments in place of the whole cells that were observed on day 1.
Macroscopically, the contamination gave the wells a milky appearance and seemed to infect 20 of the 24 wells. The remaining 4 wells were still clear, however, the cells in these wells had also ruptured in the same way as in all the other wells (see Figure 5.1.1).

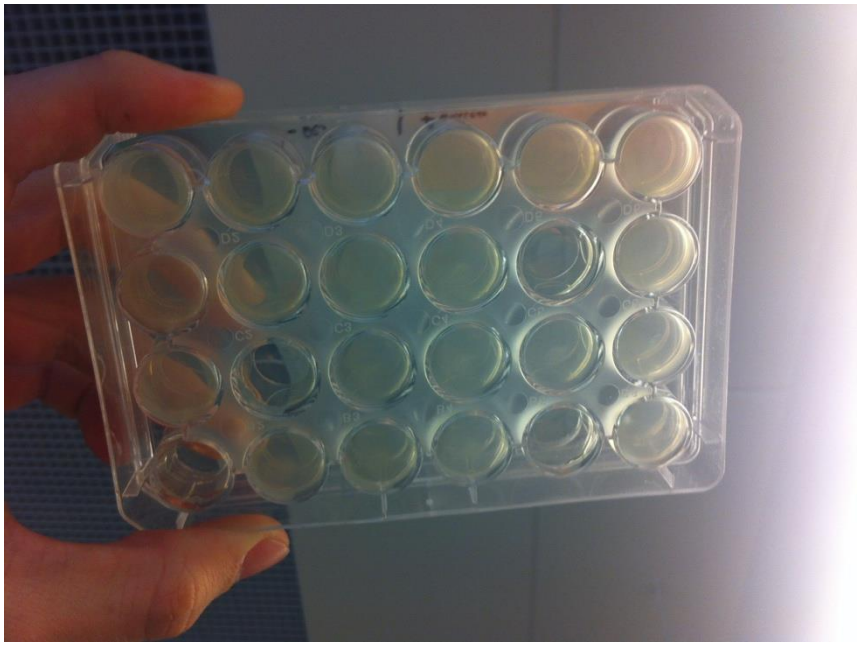


Figure 5.1.1: Four of the wells are uncontaminated: A1, A5, B2 and C 5 as opposed to the rest of the wells, which are all contaminated. There is a clearly visible difference in the transparency of the medium in the uncontaminated wells, compared to the contaminated ones.

3. Tardigrade response to Grace's Insect Medium

The tardigrades placed in Grace's Insect Medium quickly entered the tun-state, while the ones in water stayed active, except for one. They were checked up on several hours later, but changes were observed.

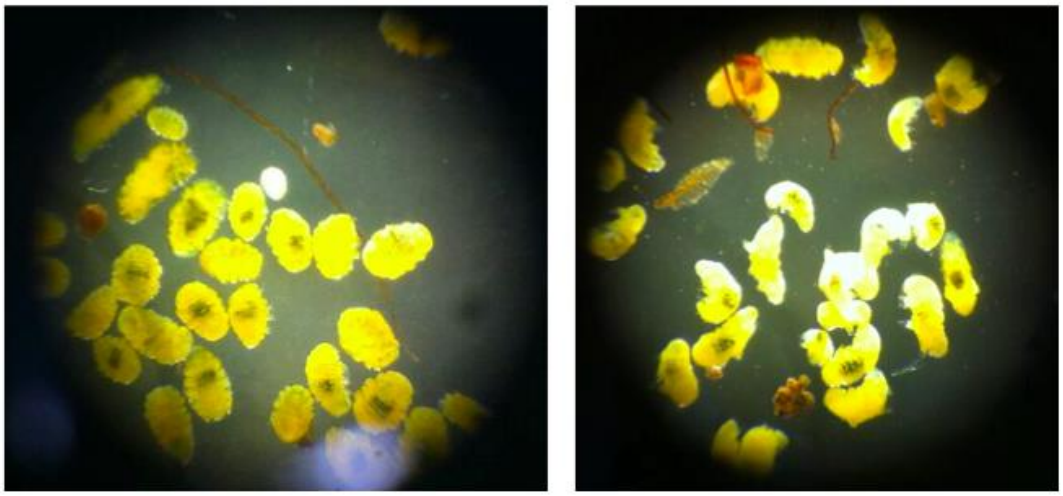


Figure 5.1.2: The picture to the left shows *R. coronifer* in Grace's Insect Medium, we can see that they are in tun-state. The picture to the right there can be found many tardigrades of *R. coronifer* in tap water, these tardigrades are in their active state. Both of the pictures are from a Gundlach Stereo microscope with a magnification between 10-40x**.**

On day 3, 30 of the tardigrades kept in tap water were transferred to a watch glass with Grace's Insect Medium. After 15 minutes, all of the tardigrades had entered the tun-state. When the medium was replaced with tap water, the tardigrades gradually started moving and after 15 minutes, all 30 were active again (see Figure 5.1.2).

4. Failed viability test

This experiment yielded no reliable results, because it suffered from several uncertainties. We had no experience analyzing the storage cells with the Gundlach light microscope and were not sure what to expect seeing. Some of the slides had been made with overly large coverslips, possibly crushing the cells and definitely spreading them much more thinly on the slide than desired.

Initially, we took our results to suggest that all the cells were dead, but this is not a reliable result, since we later confirmed that, what was studied were air bubbles under the coverslip. This can be recognized by the shiny look of the barrier surrounding a drop of Trypan Blue dye (see Figure 5.1.3).

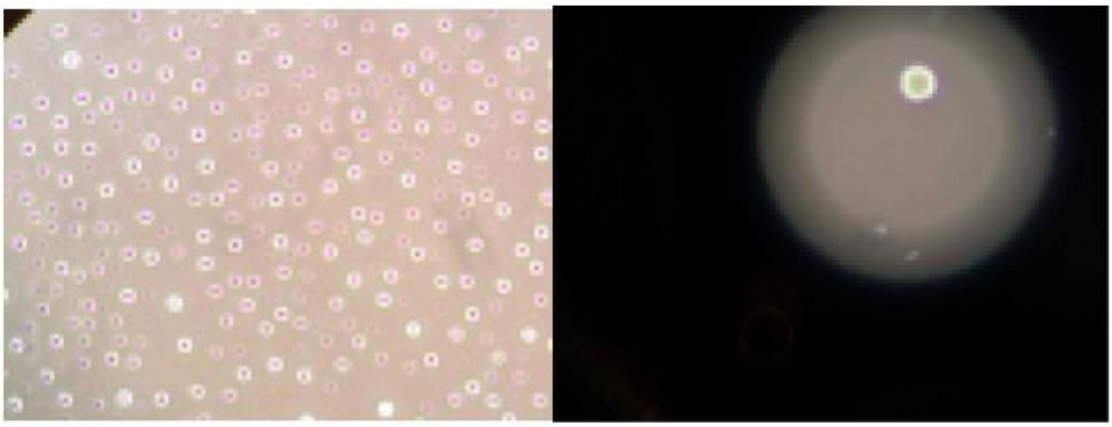


Figure 5.1.3: Images of Gundlach light microscope: In the picture to the left we have many air bubbles and in the picture to the right we only see one air bubble. The used microscope is Gundlach light microscope. The magnification is unknown.

Although the experiment failed to produce any useful results, we did gain important experience working with both Trypan Blue tests and the Gundlach light microscope.

5. Tardigrade response to osmolality

We have listed the results from the experiment in Table 5.1.1 below.

Table 5.1.1: In this table we summarize the findings of the ”Tardigrade response to osmolality” experiment. The experiment included 24 *R. coronifer,* but during the transfer, three tardigrades were lost, one from each of these wells: a4, a7 and c1.

Media	mOsm/kg	Placement	Tardigrade
Milli-Q water	77	a1	two moving
Glucose 84	84	a2	two moving
Glucose 163	163	a3	one moving, one moving slowly
Glucose 196	196	a4	one moving slowly
Glucose 246	246	a5	not moving
Glucose 311	311	a6	not moving
Glucose 404	404	a7	one not moving
Grace’s (1:1)	363	c1	one not moving
Grace’s (1:2)	226	c2	two moving

Grace's (1:2.5)	151	c3	one moving, one moving slowly
Grace's (1:3)	129	c4	one moving, one moving slowly
Grace's (1:3)	115	c5	two moving

The Table 5.1.1 shows that *R. coronifer* seem to be most comfortable in environments with an osmolality ranging around 77-196 mOsm/kg for the glucose solutions and 115-225 mOsm/kg for the Grace's medium.

6. Cell Culture B

The viability test made from the leftovers of the four primary cell suspensions did not give us any result because the cell concentrations were too low. Therefore we were not sure if the cells were viable after the transfer to the wells in the different kind of dilutions at day 1.

At day 3 we made new viability tests again. This time we transferred 10 μL from the bottom of one of the wells to a slide and added 5 μL and a cover slip. The slide was observed using a Zeiss Imager.M2m microscope. We could not find any storage cells to count at the first slide. The test was repeated with other wells but unfortunately we still could not find any cells on the slides. We managed to find a few characteristically yellow fragments; however, it was not sure if they have been cell fragments or body parts of the animal.

We checked the wells under the Gundlach Stereo light microscope, and we could see yellow dots on the bottom of the wells. Using this small magnification (40x) though, we really could not say if it was storage cells or cells fragments that we saw. We did however, surprisingly found one tardigrade in well F5. It was alive.

We decided to remove the whole media from the wells and stain cells directly in the wells instead. The wells were observed the Inverted Leica microscope. We only saw cells fragments. In Figure 5.1.4 we have captured our observations from well A8, which contained diluted Grace's Insect Medium (1:3).

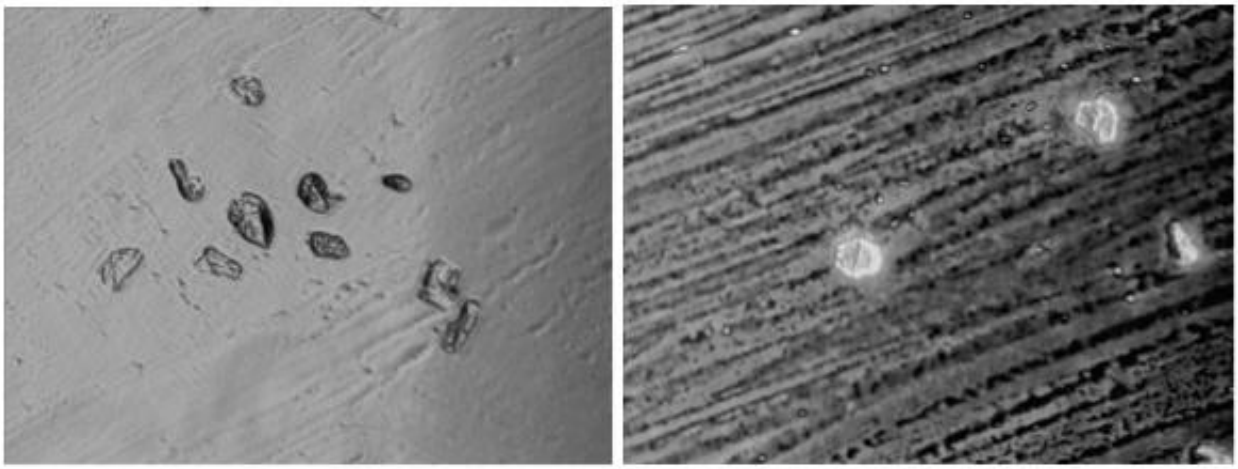


Figure 5.1.4: Images from the Inverted Leica microscope. The picture to the left is from well (A8), which contains 1:3 Grace's Insect Medium and storage cells (as they look like on day 3). The picture to the right is from the same well, but Trypan Blue dye Solution has been added directly in the well after removing most of the media. The magnification is unfortunately unknown. The different shaped dots on the two pictures appear to be cell fragments**.**

7. Comparing microscopes for viability test

We compare the Trypan Blue coloured storage cells under different microscope. The characteristically yellow colour of the storage cells cannot be recalled using the Inverted Leica microscope. Below we present two microscope pictures using two different filters on the Inverted Leica microscope. Figure 5.1.5 shows the same two storage cells with a magnification of 200x - the cell at the top is assumed to be dead and the one at the bottom is assumed to be alive.

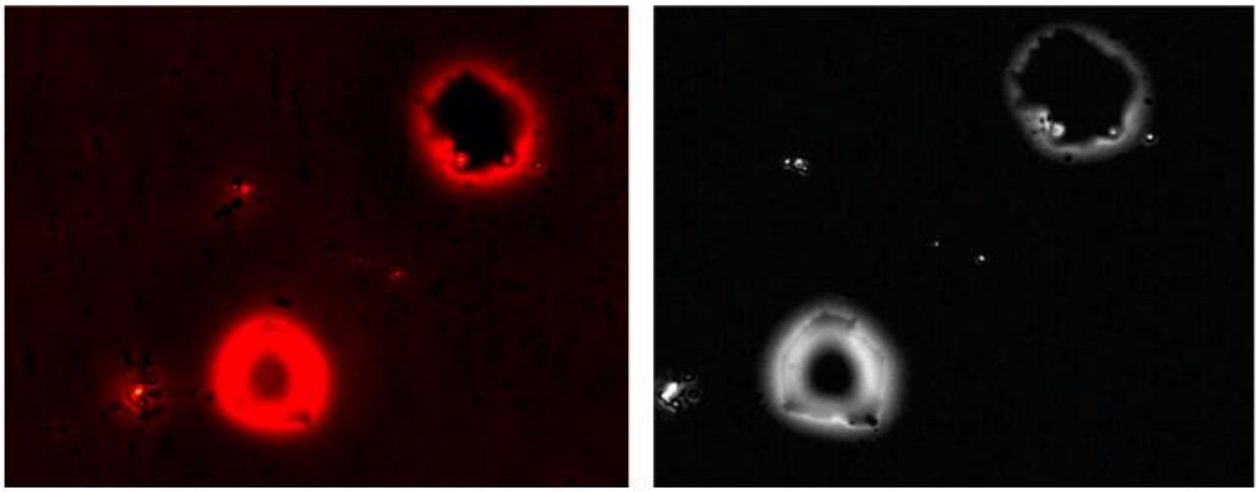


Figure 5.1.5 Microscopi pictures from an Inverted Leica microscope using a 200x magnification, filter 4 (red/black) and filter 1 (black/white). The two images show two Trypan Blue coloured storage cells from a *R. coronifer* in a water-

glucose solution (196 mOsm/kg). The cell at the bottom is interpreted as being alive whereas the cell at the top is assumed to dead due to its dark color.

Using a Zeiss Images.M2m microscope we can see the true colors of the storage cells. The software, however, due to a software problem, we were only able to get the pictures in black and white color. The on the left side of Figure 5.1.6 is taken with an iphone camera and the image to the right is taken with the camera that is integrated in the Zeiss Imager.M2m microscope. We can distinguish the yellow and blue color easily in the picture to the right. In the picture to the left the dark cells are “truly” blue/dead and the light-grey cells are “truly” yellow/alive.

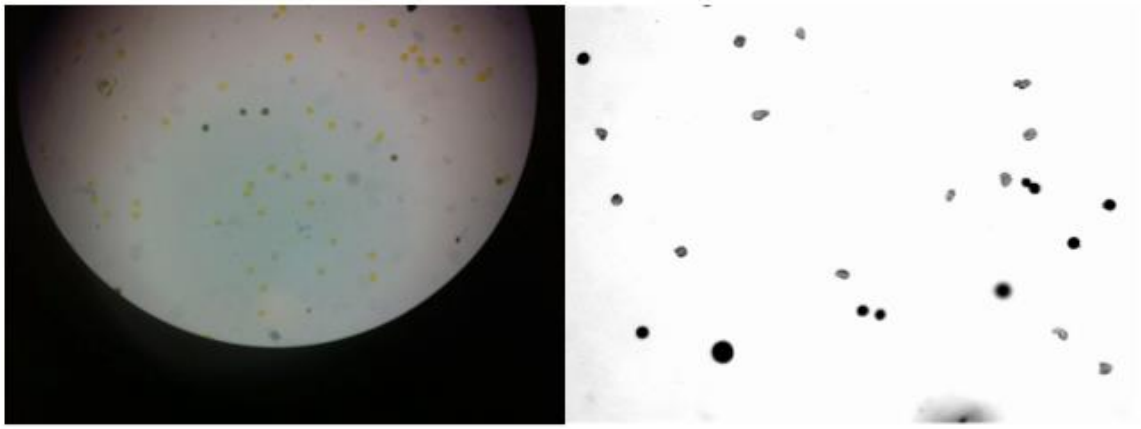


Figure 5.1.6: Microscopy pictures taken from a Zeiss Imager. M2m microscope with a 100x magnification. The two photos are of storage cells in glucose water (196 mOsm/kg) coloured with Trypan Blue dye solution. The left image is taken with a mobile camera and the image to the right is taken with the integrated microscope camera - the software is not updated and therefore we can only get the pictures in grayscale.

Using a Gundlach light microscope we can only take mobile pictures through the eyepieces. In the Figure 5.1.7 we see two storage cells that have been coloured with Trypan Blue dye solution, the blue cell is dead and the yellow cells is alive.

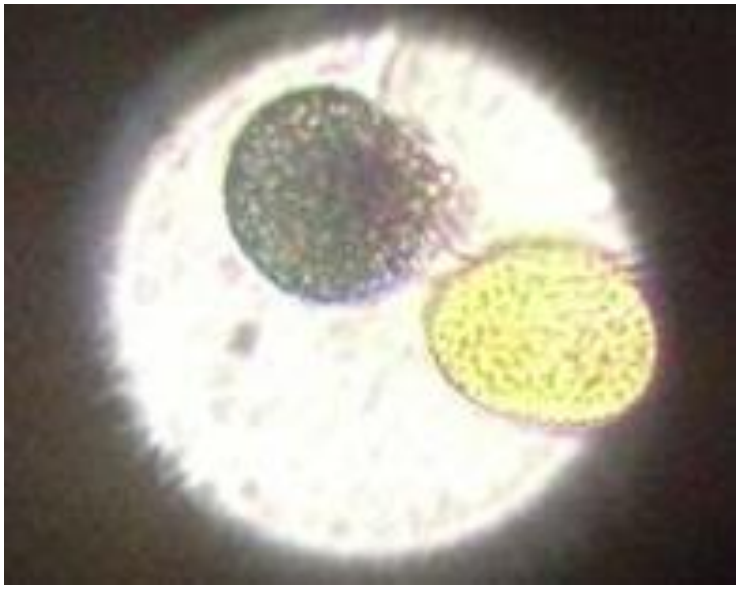


Figure 5.1.7: Picture of two storage cells coloured with Trypan Blue. The microscope used is a Gundlach light microscope and the magnification is 1000x (so we used oil). The blue cell to the right is dead and the yellow cell to the right is alive.

The best and most reliable method of differentiating between living and dead cells after a Trypan Blue staining was with the Zeiss Imager microscope and the Gundlach light microscope, where the true color of the cells were clearly visible.

8. Storage Cell Viability Assay

In the following table we present the results from experiment 8. Storage cell viability assay.

Table 5.1.2: Mean osmolality of Grace's Insect Medium, glucose solutions and Frog Ringer Solutions with calculated variance and standard deviation.

Medias	Number of Measurements	Mean	Variance	Standard deviation
(1) Grace's medium Undiluted	3	349	4	2
(2) Grace's medium 1:2	3	186.67	14.33	3.79
(3) Grace's medium 1:3	3	136.67	8.33	2.89
(4) Glucose (196 mOsm/kg)	1	196		
(5) Glucose (246 mOsm/kg)	1	246		
(6) Glucose (404 mOsm/kg)	1	404		

(7) Ringer pH = 6.5	3	302.67	2.33	1.53
(8) Ringer pH = 7.5	3	304.33	16.33	4.04
(9) Ringer pH = 8.5	3	278	13	3.61

Table 5.1.3, 5.1.4., 5.1.5 below show the results from the storage cell viability test after 0, 1 and 2.5 hours. In the first column, the 9 different medias that were used in the analysis are listed. The second to the fifth column all contain the results from the first droplet of the duplicate, whereas the sixth to the ninth column contain the results from the second droplet.

In these 2 x 4 columns, we have listed the number of dead cells, number of living cells, the total sum counted and the survival in percent. The single-star (*) marked areas indicate that less than 100 cells have been counted. The two-star (**) marked areas in table 1 indicate that slides have been remade because the first slides were undetectable. These new slides were prepared and counted immediately after being stained, as opposed to the rest of the slides, which were counted after they had been imaged. This meant a reduction in waiting time of 15–25 minutes. The areas marked "N/A" indicate that the cell counting could not be performed, for various reasons. Most of the time it was due to low cell numbers.

Table 5.1.3: Results at t = 0 hours

***t* = 0 hours**	**1. Droplet**				**2. Droplet**			
	Dead	**Alive**	**Sum**	**% Alive**	**Dead**	**Alive**	**Sum**	**% Alive**
(1) Grace's medium Undiluted	68	39	107	36.45	48	71	119	59.66
(2) Grace's medium 1:2	6	102	108	** 94.44	7	98	105	** 93.33
(3) Grace's medium 1:3	18	130	148	87.84	10	90	100	90
(4) Glucose (196 mOsm/kg)	48	52	100	52	42	114	156	73.08
(5) Glucose (246 mOsm/kg)	16	119	135	** 88.15	18	89	107	** 83.18
(6) Glucose (404 mOsm/kg)	60	80	140	** 57.14	25	74	99	** 74.75
(7) Ringer, pH = 6.5	46	75	121	** 61.98	39	75	114	** 65.79
(8) Ringer, pH = 7.5	8	107	115	** 93.04	N/A	N/A	N/A	** N/A
(9) Ringer, pH = 8.5	5	107	112	95.54	10	90	100	90

Table 5.1.4: Results at *t* = 1 hour

***t* = 1 hour**	**1. Droplet**				**2. Droplet**			
	Dead	**Alive**	**Sum**	**% Alive**	**Dead**	**Alive**	**Sum**	**% Alive**
(1) Grace's medium Undiluted	27	118	145	81.38	37	68	105	64.76
(2) Grace's medium 1:2	74	144	218	66.06	46	74	120	61.67
(3) Grace's medium 1:3	35	95	130	73.08	82	81	163	49.69
(4) Glucose (196 mOsm/kg)	66	108	174	62.07	110	54	164	32.93
(5) Glucose (246 mOsm/kg)	N/A	N/A	N/A	N/A	11	89	100	89
(6) Glucose (404 mOsm/kg)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
(7) Ringer, pH = 6.5	41	79	120	65.83	11	26	37	* 70.27
(8) Ringer, pH = 7.5	69	37	106	34.91	71	35	106	33.02
(9) Ringer, pH = 8.5	62	38	100	38	100	15	115	13.04

Table 5.1.5: Results at *t* = 2.5 hours

***t* = 2.5 hours**	**1. Droplet**				**2. Droplet**			
	Dead	**Alive**	**Sum**	**% Alive**	**Dead**	**Alive**	**Sum**	**% Alive**
(1) Grace's medium Undiluted	36	77	113	68.14	35	23	58	* 39.66
(2) Grace's medium 1:2	98	2	100	2	34	85	119	71.43
(3) Grace's medium 1:3	45	79	124	63.71	69	41	110	37.27
(4) Glucose (196 mOsm/kg)	96	4	100	4	95	31	126	24.6
(5) Glucose (246 mOsm/kg)	N/A	N/A	N/A	N/A	53	0	53	* 0,00
(6) Glucose (404 mOsm/kg)	21	9	30	* 30.00	34	66	100	66
(7) Ringer, pH = 6.5	82	18	100	18	12	91	103	88.35
(8) Ringer, pH = 7.5	38	64	102	62.75	33	0	33	* 0,00
(9) Ringer, pH = 8.5	36	63	99	63.64	42	81	123	65.85

5.2 Statistics

The following calculations and graphs are made from the results presented in the chapter above from experiment 8. Storage Cell Viability Assay.

Table 5.2.1 : Mean, variance and standard deviation (SD) on survival percentage of *R. coronifer* storage cells after 0 hours.

***t* = 0 hours**	**Droplet 1 % Alive**	**Droplet 2 % Alive**	**Mean**	**Variance**	**SD**
(1) Grace's Undiluted	36.45	59.66	48.06	269.35	16.41
(2) Grace's medium 1:2	94.44	93.33	93.89	0.62	0.78
(3) Grace's medium 1:3	87.84	90	88.92	2.33	1.53
(4) Glucose (196 mOsm/kg)	52	73.08	62.54	222.18	14.91
(5) Glucose (246 mOsm/kg)	88.15	83.18	85.66	12.35	3.51
(6) Glucose (404 mOsm/kg)	57.14	74.75	65.95	155.06	12.45
(7) Ringer, pH = 6.5	61.98	65.79	63.89	7.26	2.69
(8) Ringer, pH = 7.5	93.04	N/A	93.04	N/A	N/A
(9) Ringer, pH = 8.5	95.54	90	92.77	15.35	3.92

Table 5.2.2: Mean, variance and standard deviation (SD) on survival percentage of *R. coronifer* storage cells after 1 hours.

***t* = 1 hour**	**Droplet 1 % Alive**	**Droplet 2 % Alive**	**Mean**	**Variance**	**SD**
(1) Grace's medium Undiluted	81.38	64.76	73.07	138.11	11,.75
(2) Grace's medium 1:2	66.06	61.67	63.87	9.64	3.1
(3) Grace's medium 1:3	73.08	49.69	61.39	273.55	16.54
(4) Glucose (196 mOsm/kg)	62.07	32.93	47.5	424.57	20.61
(5) Glucose (246 mOsm/kg)	N/A	89	89	N/A	N/A
(6) Glucose (404 mOsm/kg)	N/A	N/A	N/A	N/A	N/A
(7) Ringer, pH = 6.5	65.83	70.27	68.05	9.86	3.14
(8) Ringer, pH = 7.5	34.91	33.02	33.97	1.79	1.34
(9) Ringer, pH = 8.5	38	13.04	25.52	311.5	17.65

Table 5.2.3: Mean, variance and standard deviation (SD) on survival percentage of *R. coronifer* storage cells after 2.5 hours.

***t* = 2.5 hours**	**Droplet 1 % Alive**	**Droplet 2 % Alive**	**Mean**	**Variance**	**SD**
(1) Grace's Undiluted	68.14	39.66	53.9	405.56	20.14
(2) Grace's medium 1:2	2	71.43	36.72	2410.26	49.09
(3) Grace's medium 1:3	63.71	37.27	50.49	349.54	18.7
(4) Glucose (196 mOsm/kg)	4	24.6	14.3	212.18	14.57
(5) Glucose (246 mOsm/kg)	N/A	0	0	N/A	N/A
(6) Glucose (404 mOsm/kg)	30	66	48	648	25.46
(7) Ringer, pH = 6.5	18	88.35	53.18	2474.56	49.74
(8) Ringer, pH = 7.5	62.75	0	31.38	1968.78	44.37
(9) Ringer, pH = 8.5	63.64	65.85	64.75	2.44	1.56

Following figures present the survival percentage of the storage cells over time. Each figure presents a type of solution. (Figure 5.2.1, Figure 5.2.2, Figure 5.2.3).

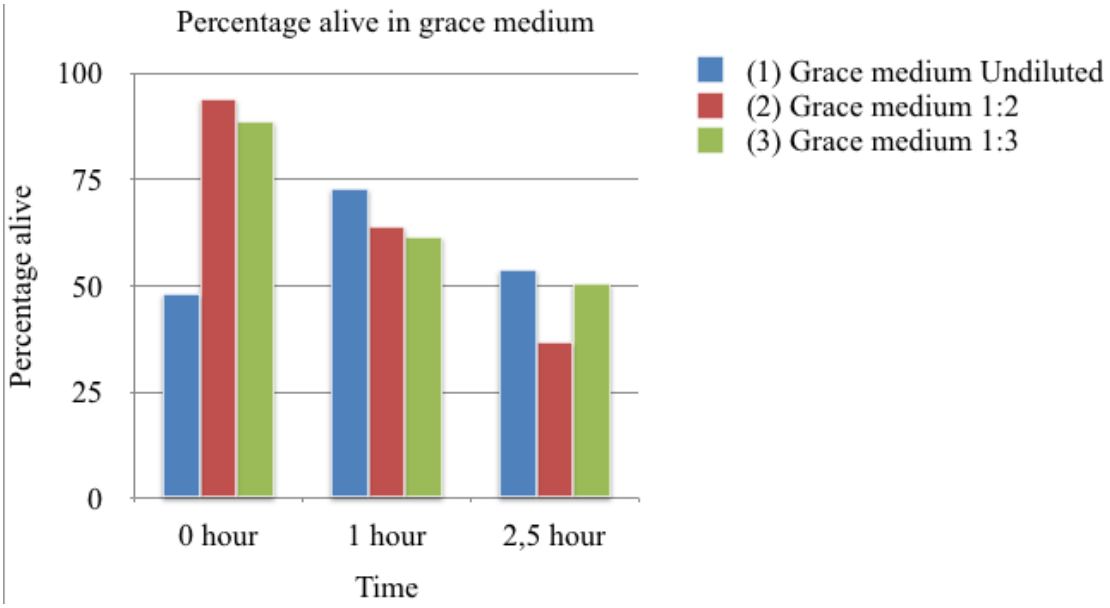


Figure 5.2.1. The survival in percentage for storage cells from *R. coronifer* in 3 different dilutions of Grace's Insects Medium over time.

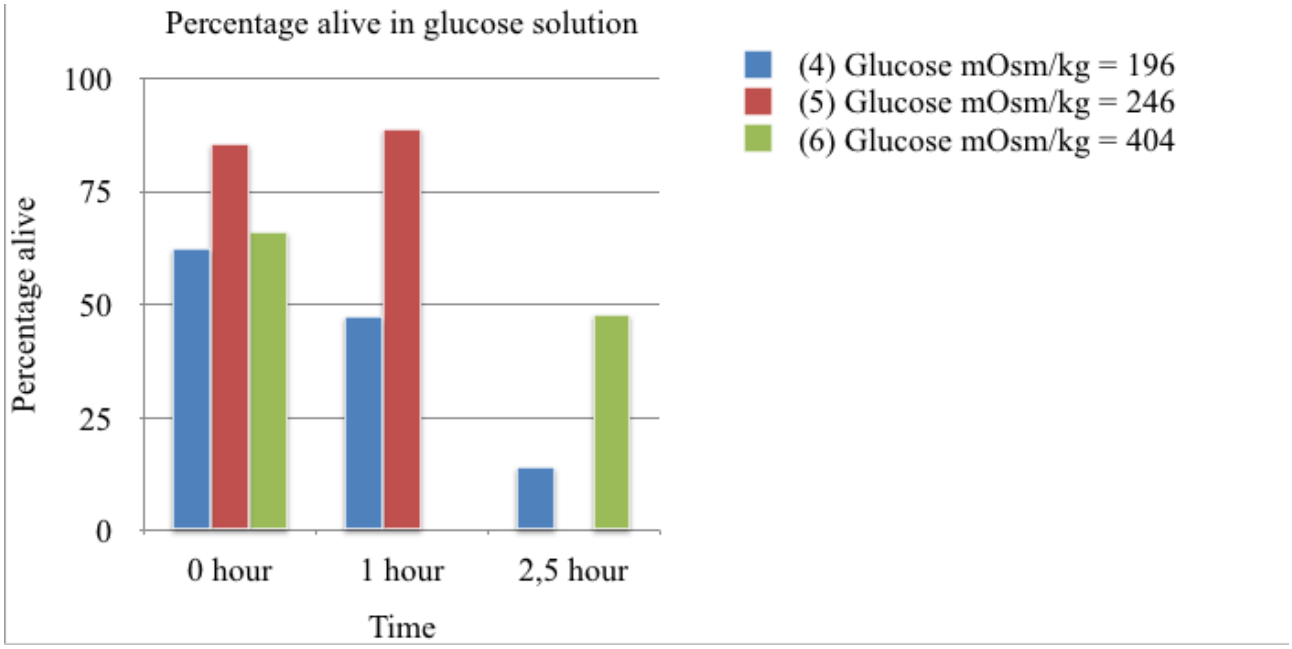
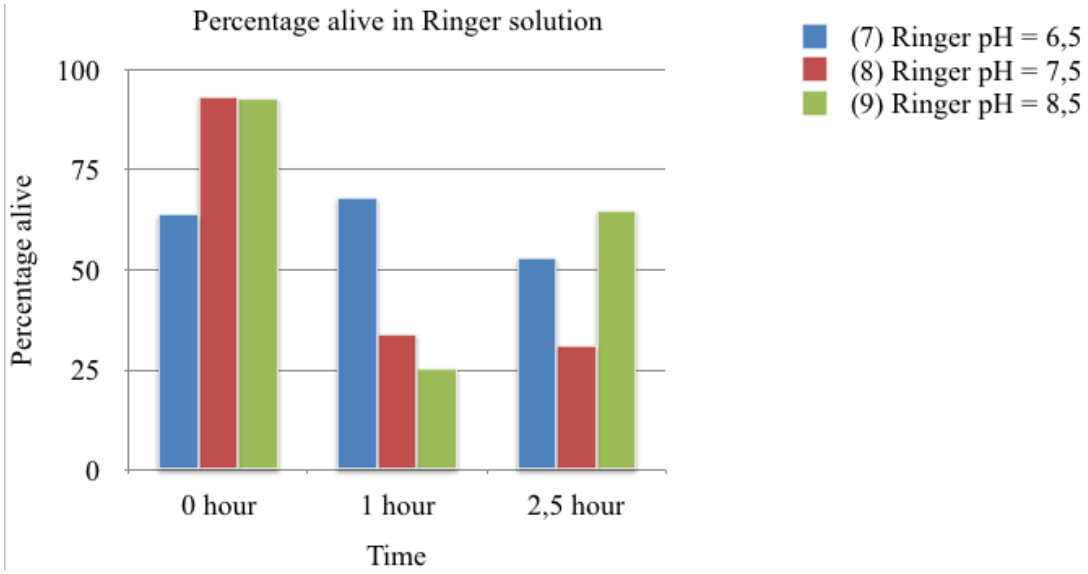


Figure 5.2.2 The survival in percentage for storage cells from *R. coronnifer* in 3 different glucose dilutions over time.



Figur 5.2.3. The survival in percentage for storage cells from *R. coronnifer* in 3 different ringer solutions over time.

Table 5.2.4: Students T tests between different kinds of solutions

***t* = 0 hours**	
Solution	**p-value**
Grace's Undiluted and 1:2	0.157177008
Grace's Undiluted and 1:3	0.173500966
Grace's 1:2 and 1:3	0.087645279
Glucose 4 and glucose 5	0.258665035
Glucose 4 and glucose 6	0.827968298
Glucose 5 and glucose 6	0.248624371
Ringer 6.5 and Ringer 7.5	N/A
Ringer 6.5 and ringer 8.5	0.018900209
Ringer 7.5 and Ringer 8.5	N/A

Table 5.2.5: Students T tests between different kinds of solutions

***t* = 1 hour**	
Solution	**p-value**
Grace's Undiluted and 1:2	0.46049005
Grace's Undiluted and 1:3	0.508805474
Grace's 1:2 and 1:3	0.867439941
Glucose 4 and glucose 5	N/A
Glucose 4 and glucose 6	N/A
Glucose 5 and glucose 6	N/A
Ringer 6.5 and Ringer 7.5	0.019705386
Ringer 6.5 and ringer 8.5	0.172460626
Ringer 7.5 and Ringer 8.5	0.621077664

Table 5.2.6: Students T tests between different kinds of solutions

***t* = 2.5 hours**	
Solution	**p-value**
Grace's Undiluted and 1:2	0,710654725
Grace's Undiluted and 1:3	0,876928273
Grace's 1:2 and 1:3	0,762642798
Glucose 4 and glucose 5	N/A
Glucose 4 and glucose 6	0,275937366
Glucose 5 and glucose 6	N/A
Ringer 6.5 and Ringer 7.5	0,689678236
Ringer 6.5 and ringer 8.5	0,797705159
Ringer 7.5 and Ringer 8.5	0,480242297

Table 5.2.7: Overview over the Student T-Values

Students t-test	***t* = 0 hours**	***t* = 1 hour**	***t* = 2.5 hours**
	p-value	p-value	p-value
Grace's 1:2 & Glucose 5	0.172857443	N/A	N/A
Grace's 1:2 & Ringer, pH = 6,5	0.02771726	0.312070397	0.770768398

6. Discussion

Experiments 1-7

Following chapter is a summation of what we discovered and learned during our experiments, and a discussion of the results.

Many of the early experiments did not contribute with results, we can use for our final conclusion. Instead they served to discover the challenges we faced, working with storage cells, and thus informed and shaped the later experiments, so we could design those to yield more reliable results. In addition, the early experiments also served as practice for us to work experimentally with the tardigrade storage cells.

The first experiment, *1. Survival of* R. coronifer *in water,* confirmed that tardigrades can be extracted one day and stored in water for a later experiment for at least two days. We learned that it was necessary to reduce the surface area with a lid, in order to avoid evaporation of water and thus damage to the tardigrades.

Experiment *2. Cell Culture A,* resulted in contamination and cell fragmentation. This illustrated the necessity of using antibiotics in the wells of the cell cultures. There was no reliable way to determine the cause or time of cell fragmentation. This indicated the necessity to determine whether or not the cells had been viable at initial conditions, which greatly influenced the direction of the project.

The time it take for cells to break into cell fragments could be studied by extracting cells in a watch glass, and then observe the cells in a microscope over time. Another way is to use a microscope that takes pictures automatically through the experiment.

Because *R. coronifer* had been observed to go into tun-state immediately after being placed in a drop of medium, it seemed interesting to investigate why the tardigrades reacted to the medium as a hostile environment. In experiment *3. Tardigrade response to Grace's Insect Medium,* 50 tardigrades all entered the tun-state after 15 minutes of exposure to the medium, while the control remained active in water. When the medium was replaced with water, all the tardigrades returned to their active state within another 15 minutes, showing that the medium, while hostile, was not deadly to the animals, during the time of exposure.

In experiment: *5. Tardigrade response to osmolality, R. coronifer* was exposed to various dilutions of Grace's Insect Medium and various dilutions of glucose solution. The tardigrades were active at osmolalities ranging between 77-196 mOsm/kg in the glucose dilutions, and 115-225 mOsm/kg in

the Grace Insect's Medium. The tardigrades entered the tun-state above these ranges. This suggested that it was the osmolality that induced the tun-state and not specific harmful components in the Grace's Insect Medium. Bjørn-Mortensen (2006) tested *R. coronifer* response to osmolality, and found that the tardigrades could survive within osmolality up to 500 mOsm/kg. The difference between experiment 5 and Bjørn-Mortensens study was the contents of the solutions. Bjørn-Mortensen used saline solutions in his experiment and we used glucose and Grace's Insect Medium. The variations in results could be due to differences in the molecule size in the solutions.

To find out whether or not the cells were alive at initial conditions when extracted in the seemingly unfriendly media, we performed a viability test in experiment: *7. Comparing microscopes for viability test.* We used three different microscopes, to test how to differentiate between dead and alive storage cells, when using the Trypan Blue viability test. Although we did not reliably determine the viability of storage cells after extraction, we did learn to identify the storage cells and distinguish stained from unstained ones, using the Zeiss Imager. M2m and the Gundlach light microscope.
The second cell culture: *6. Cell Culture B.* was a test of the parameters of medium osmolality and cell concentration. We used octaplicates, since the plan was to do continuous viability tests at the different conditions over time to map the survival rate of cells in relation to osmolality.
A viability test was performed two days after setting up the experiment, it was impossible to find any intact cells to study, but there appeared to be cell fragments in the wells.
After this, we decided to abandon trying to study the cells over a span of days and instead chose to map the survival rate of the cells from 0 to 2.5 hours.

8. Storage Cell Viability Assay

Our final experiment suffers from a number of problems. These will be addressed in the following section, and should be taken into consideration when analyzing the results.
As it can be seen on table 5.1.3, 5.1.4 and 5.1.5 in the result section, some of the result are missing since it was not possible to count any cells. A lot of time was spend finding the cells on some samples, since the cells were often located in one or two areas. Furthermore, the counter had not tried to count tardigrade storage cells in this extent before, so there is the possibility of some cells being misjudged.

There was a change in cover slips halfway through the experiment. The new cover slips were larger than the first ones. The larger coverslips made it more difficult to locate the cells and therefore it was more time consuming to count the cells. It has also been suggested that the larger cover slips might have increased the risk of crushing the cells, which may have increased the percentage of dead cells.
There were two tardigrades in each drop of medium. The size of the tardigrades varied, which may leave a slight uncertainty that could have been avoided, by only using adult tardigrades. The samples were imaged immediately after they were made. The amount of time used on taking pictures varied, which resulted in the countings not being done at the exact same time.

Statistics on experiment 8. Storage Cell Viability Assay

The following section is a discussion of the statistics performed on *8. Storage Cell Viability Assay*. The statistics can be found in the result section.
Table 5.2.1, 5.2.2 and 5.2.3 in the result section show the calculated mean, variance and standard deviation. The means are mainly based on two observations, one from each droplet. For some of the samples, we were only able to count the cells in one of the drops. For those samples, we simply use that one data point, instead of a mean. Of course this adds an uncertainty to the data, but it was done to compare as many results as possible. The standard deviation is calculated to examine how uneven the results are.
The standard deviations from the results at $t = 0$ hours are small, except the result from: Grace's Insect Medium undiluted which is 16.41, glucose with an osmolality of 196 mOsm/kg (From now on called Glucose 4) which is 14.91 and glucose with an osmolality of 404 mOsm/kg (From now on called Glucose 6) which is 12.45. The result will be used, regardless the standard deviations. There are high standard deviations on the result after 1 hour on the following solutions: Grace's Insect Medium undiluted (11.75), Grace's Insect Medium 1:3 (16.54), Glucose 4 (20.61) and Ringer pH 8.5 (17.65). Again it is not so critically high that the results will not be used, but it should be kept in mind when analyzing the results. All standard deviations of the results after 2.5 hours are high except for Ringer pH 8.5. This is probably due to difficulties when counting the cells. The cells where more difficult to find on the plate, so the results after 2.5 hours include more irregularities.

Tables with Student's t-test performed on the results from experiment *8. Storage Cell Viability Assay*, are listen in the result section. The test is a two-tailed Student's t-test for results with unequal

variance. It can be seen from table 5.2.1, 5.2.2 and 5.2.3 that the variance of the different means are not the same. The tests are performed to examine if there is an actual difference in the means, which can be ascribed to differences in the extraction medias. The tests are performed with the following hypothesis:

H_0: $\mu = \mu_0$, H_1: $\mu \neq \mu_0$. Significance: $\alpha \leq 0.05$.

It was not possible to perform t-test on some results, due to lack of data. These are marked *N/A*. The p-values on tables 5.2.4, 5.2.5, 5.2.6 and 5.2.7 shows that it is possible to reject H_0, and thereby deduce that there is a statistically significant difference between the results, in the test from*:* $t = 0$ between Ringer pH 6.5 and Ringer pH 7.5, Grace 1:2 and Ringer pH 6.5 and from $t = 1$ between Ringer pH 6.5 and pH 7.5. The rest of the p-values are above the level of significance and H_0 can therefore not be rejected. We interpret this as a need to increase the amount of data used. Since this is not a possibility for the project, our data will still be analyzed, but with this knowledge in mind.

Survival percentage in different dilutions of Grace's Insect Medium

Figure 5.2.1 presents a graphical overview of the average survival percentage of the storage cells in different dilutions of Grace's Insect Medium over time. The figure is based on means of the survival percentages presented in the results section. The graph shows that the survival percentage decrease in all dilutions of media over a time span of 2.5 hours. The results from the undiluted Grace's Insect Medium show some irregularities. The survival percentage after $t = 0$ hours is 48.1 %, after $t = 1$ hour is 73.1 % and after $t = 2.5$ hours is 53.9 %. It seems strange that the survival percentage from undiluted Grace's Insect Medium *t = 0* hours is a lot smaller than the rest of the survival percentages measured at the same time, since the survival percentage in undiluted Grace's Insect Medium almost follows the survival percentage of the other dilutions in $t = 1$ hour and $t =$ 2.5 hour. Our earlier experiment: *3. Tardigrades response to Grace's Insect Medium* concerning how living tardigrades survive in undiluted Grace's Insect Medium, shows that the tardigrades quickly enter the tun-state. It is difficult to extract the cells, when tardigrades are in the tun-state, and it might increase the risk of uneven samples. Note that according to Bjørn-Mortensen (2006), there is no evidence for *R. coronifer* being able to perform osmobiosis, yet it enters a tun-state when exposed to osmotic stress.

This potential source of error could have been avoided by a different experiment design. For example we could have transferred the cells of 50 tardigrade to the same suspension and then made the viability tests from these cells over time.

We chose not to use this approach because of the risk of losing or damaging the cells in the process of transferring them e.g. by having the cells get stuck in the pipette.

The survival percentage over time is more consistently declining in the 1:2 Grace's Insect Medium than with the undiluted Grace's Insect Medium. The survival percentage at t = 0 hours is 93.9 %, at t = 1 hour 63.9 % and at t = 2.5 hour 36.7 %. 1:2 Grace's Insect Medium has the highest survival percentage of all 9 medias after 0 hours. This result fits with the theory, since the osmolality of the 1:2 Grace's Insect Medium is measured to be 226 mOsm/kg, which is the close to the internal osmolality of the tardigrades (168 ±18 mOsm/kg), measured on tardigrades in demineralized water (Bjørn-Mortensen, 2006).

The results from Grace's Insect Medium 1:3 are also consistent over time. The survival percentage at t = 0 hours is 88.9 %, after t = 1 hour 61.4 % and after t = 2.5 hours 50.5 %. The 1:3 dilution of Grace's Insect Medium shows a relatively successful survival rate, which could also be due to the similarities in the osmolality of the hemolymph. The osmolality of Grace's Insect Medium 1:3 is 137 mOsm/kg. It could be interesting in further experiments to try a dilution that has an osmolality between those of dilutions 1:2 and 1:3, so it would be even closer to the osmolality of the hemolymph.

We expected that the survival percentage would decrease over time. The goal of the experiment was to gain information useful for reducing cell mortality upon extraction. Therefore, it is interesting to examine which medium causes the cells to die the slowest. The result of the undiluted Grace's Insect Medium after t = 0 hour will not be used in the calculations because of the uncertainty of the measurement. We are aware that the following calculations suffer from the limited amount of data.

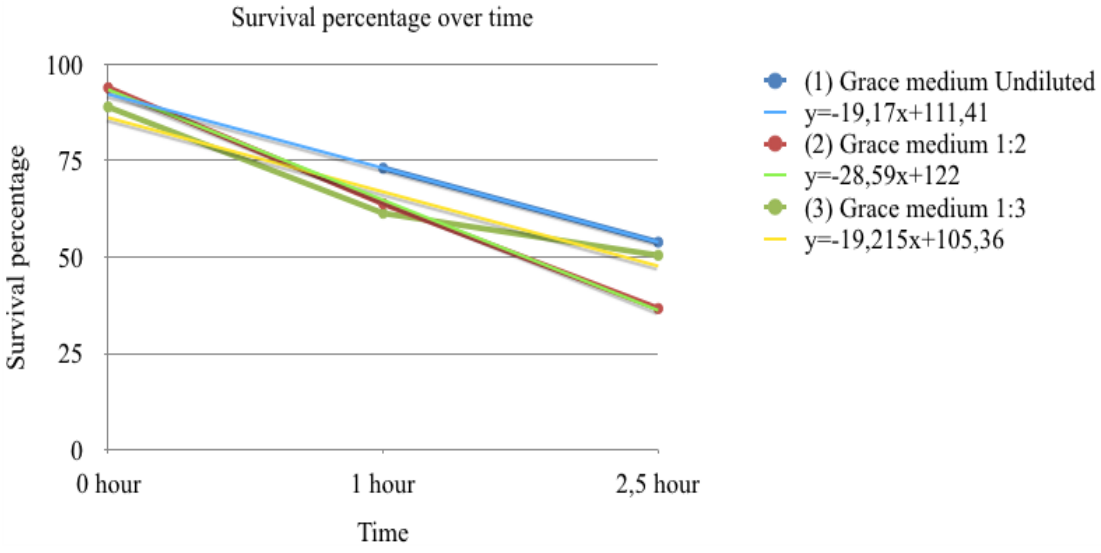


Figure 6.1.1 Survival percentages over time in Grace's Insect Medium with trend lines.

Figure 6.1.1 is based on the same results as presented on figure 5.2.1, but with linear trend lines. The R^2-values are between 0.94 and 1, so it is acceptable to use a linear trend line. According to the slopes of the trend lines, the Grace's Insect Medium 1:3 shows the most promising result, i.e. the slowest decrease in survival, with a slope of -19.215. The 1:2 dilution of Grace's Insect Medium shows higher survival percentages if only the first two results of dilution 1:2 is compared with the results of dilution 1:3 (Figure 5.2.1). Again, it should be taken into consideration that this is based on a small amount of data.

Survival Percentage in Frog Ringer solutions with different pH

The survival rate of storage cells in the Frog Ringer solutions are shown in figure 5.2.3. The 3 different Ringer solutions showed different effects on the survival of storage cells. For the Ringer with a pH of 6.5 (from now on called Ringer 7), the survival percentage was 63.89 % at t = 0 hours, 68.1 % at t = 1 hour, and 53.2 % at t = 2.5 hour, showing relatively low changes in survival percentage.

The Ringer with a pH of 7.5 (from now on called Ringer 8) showed a relatively high survival percentage of 93.04 % at t = 0 hours, but decreased to 34 % at t = 1 hour and finally 31.4 % at t = 2.5 hours, showing a drastic decline in the first hour after extraction.

The Ringer solution with a pH of 8.5 (from now on called Ringer 9), has a high initial survival percentage of 92.77 % at t = 0 hours, then it decreases to 25.52 % at t = 1 hour and increases to 64.75 % at t = 2.5 hours.

For Ringer 8 (and to a much lesser extend Ringer 7), the survival percentage does not decline continuously, as it should be expected. This is probably due to same problems as discussed earlier. The work with tardigrades in a Frog Ringer, tailored to the needs of frog hearts, is not necessarily ideal for tardigrades. However, Barker Jensen (2010) was able to keep tardigrade storage cells alive in a Frog Ringer solution for 4 hours.

Of the three Ringer solutions, the survival percentage is most continuously for Ringer 7, which corresponds with the expectation that tardigrade cells would survive in a slightly acidic environment, similar to insect cells. Ringer 8 showed the clearest decrease in survival percentage, while Ringer 9 showed very inconsistent results that likely were caused by a mistake with those measurements during the experiment.

Survival percentage in different glucose dilutions

Figur 5.2.2 shows an overview of the average survival percentage of the storage cells kept in different glucose dilutions over time. The survival percentage of glucose solution with an osmolality of 196 mOsm/kg (called glucose 4 from now on) after t = 0 hours is 62.5 %, after t = 1 hour 47.5 and 14.3 % after t = 2.5 hours. The results from glucose 4 are the only ones that seem to be consistently declining over time. The glucose dilution with an osmolality of 246 mOsm/kg has a survival rate of 0 % after 2.5 hours and we were not able to get any information from glucose with an osmolality of 404 mOsm/kg (called glucose 6 from now on) at t = 1 hour.

The survival percentage of cells in the solution of glucose 6 is after t = 0 hours 65.95 % and after t = 2.5 hours 48 %. There are no results from t = 1 hour. This is not a very successful survival percentage compared to glucose 4, which could well be due to the high osmolality of the solution.

The survival percentage of glucose 5 is 85.56 % after t = 0 hour, 89 % after t = 1 hour and 0% after t = 2.5 hours. So the survival percentage of glucose 5 rises in the first hour, and then suddenly drops to 0 %. It does not make sense that the survival percentage should increase over time, which further indicates a problem with the experiment, as discussed earlier. It also seems unlikely that the survival percentage should go from 89 % after t = 1 hour and then suddenly drop to 0 % after another 1.5 hours. Other than this drop (which is likely due to an error in the experiment), the results for glucose 5 are acceptable and are therefore still used.

Like Grace's Insect Medium 1:2, Glucose 5 shows promising results in the first two measurements, which may be because their osmolalities are similar. The experiment was planned with both media and glucose test, so it was possible to isolate the factor of osmolality, because the glucose solutions do not contain all the extra ingredients that the media does. The similarities in results from media 1:2 and glucose 5 may indicate that the osmolality of the media is of importance. As mentioned earlier: Bjørn-Mortensen (2006) observed that the osmolality of *R. coronifer* hemolymph is 168 ±18 mOsm/kg, when measured on tardigrades in demineralized water. But it should be taken into consideration that the same study shows that *R. coronifer* regulate their internal osmolality to be approximately 170 mOsm/kg higher then the surrounding osmolality, since they are osmoregulators. In our experiment, the cells were extracted from tardigrades that were punctured while in the target medium. This means that there is a possibility that the tardigrade starts to regulate its internal osmolality in response to this medium. So it is a possibility that the storage cells are susceptible to a media with a higher osmolality then 168 ±18 mOsm/kg. Bjørn-Mortensen (2006) measured animals that had been in the solution for 30 minutes. This is not the case in our

experiment, where the animals are in the solution 5-10 minutes before they are punctured. It could be interesting to make an experiment similar to Bjørn-Mortensens, but where the animal are in the solution for 10 minutes instead of 30 minutes, because the knowledge of the speed of the internal regulation could be useful for further planning of experiments.

It seems reasonable that the internal environment of the tardigrade should be imitated as closely as possible, when trying to make a cell culture of storage cells. However, this is made difficult by the constant change in osmolality likely taking place in the organism, in response to the external environment.

This regulation is an important issue in the consideration of the possibility to make a cell culture from storage cells from adult tardigrades. It also identifies the possibility that the cell culture should be developed from embryonic cells instead of adult cells (Christensen *et al*, 2002; Lynn, 2001). This approach also addresses the problem that it is a risk that the storage cells are not able to divide, since the adult tardigrades might be eutelic (Wright, 2014). As mentioned earlier, there is a disagreement whether the tardigrades are eutelic or not.

Attempts at working with tardigrade and nematode cells have implied some of the same complications: The animal is small, and getting a reasonable amount of cells for experiments is demanding. It makes sense to study the experiments made for establishing nematode cell cultures, since there are many shared morphological characteristics between the tardigrade and nematode (Nelson, 2002). Nematode cell cultures have successfully been established with the usage of embryonic cells. The in vitro differentiated muscle and neuron cells of nematodes show to be similar to the in vivo, so they can be used for further study (Christensen *et al*, 2002). The protocol for establishing a cell culture from nematode embryonic cells can be found in the study of Christensen et al. (2002). This protocol would be interesting to use as a template for a similar experiment with tardigrade embryonic cells.

7. Conclusion

Although many of our results had p-values above the level of significance and therefore will not be used to draw conclusions, they clearly show a trend for viability of the storage cells to decline substantially over a time span of 2.5 hours, regardless of the medium.

The 1:2 and 1:3 dilutions of Grace's Insect Medium show some of the highest survival percentages, which may be because they best match the *in vivo* osmolality of the tardigrades. Glucose 5 also has a very high survival percentage for the first two measurements.

The similarities between Glucose 5 and the 1:2 dilution of Grace's Insect Medium in both osmolality and survival percentage somewhat substantiates the conclusion from *5. Tardigrade response to osmolality* that osmolality is an important factor determining if the medium is perceived as a hostile environment. However, this would require a much more detailed iteration of this experiment to verify.

It was always our intention to tailor a medium that would mimic the *in vivo* conditions as closely as possible. However, we conclude that this is difficult, because of the constant osmoregulation in the tardigrade, in response to that same tailored medium.

Most insect cell cultures use embryonic cells. This was also done with nematodes, which share many morphological characteristics with tardigrades. This leads us to believe that it would be interesting to use embryonic tardigrade cells for establishing a cell culture, since osmoregulation is unlikely to be an issue in such a scenario.

8. Perspective

This section covers possible further studies for establishing a storage cell culture from tardigrades. As mentioned before, possible parameters to change are the pH, osmolality, temperature, ion composition, amino acids, carbohydrates and buffer systems.
Grace's Insect Medium is supplemented with the hemolymph of certain organisms (Schlaeger, 1996). A possible approach to finding the right medium for tardigrade cell cultures could be to extract the hemolymph of the tardigrade and use it as a supplement to the medium. It may therefore be interesting to do further research in the hemolymphs composition.
Another potential improvement is the use of animal serum. Using 10 % of fetal bovine serum, Schlaeger (1996) showed an improvement together with yeastolate on insect cell cultures.
The medium used for the experiments in this project contained no serum. It could be interesting to test a medium containing serum in further experiments, and observe if there is a higher survival percentage. Although the variable composition of serums deliver inconsistent quality in product (Schlaeger, 1996).
Another approach to establish a cell culture would be adjusting factors that stimulate cell division, for example hormones, vitamins and ions. Examples of vitamins could be choline, biotin and inositol, and a potentially useful ion could be Cl^-, which has been shown to stimulate the growth of insect cells (Baines, 1996).
There are still some uncertainties in the study of insect cell growth factors (Lynn, 1999), but experiments indicate that the three growth factors: Juvenile hormone, 20-hydroxyecdysone and insulin-like-hormone play a significant role in development and growth of insect cells.
Because of the limited knowledge of tardigrade storage cells, we do not know how susceptible the cells may be to mechanical stress. They may be damaged by pipetting or centrifugation. This risk could be reduced by adding pluronic F-68, which is a surfactant used to stabilize the membrane and thus protect the cells (Vlak *et al*, 2006). However, this method may reduce the reliability of the Trypan Blue viability test, since the membranes of dead cells could be stabilized, preventing the dye from entering them (Flickinger, 2013).

A common way to manipulate cells in the field of genetic engineering is to insert foreign DNA into a cell through transformation, for example by viral infection (Griffiths *et al*, 2000). This way, foreign DNA can be integrated into the cell and inhibit apoptosis, which would give the cells a

cancer-like behavior. An example of such a virus for transformation of insect cells is baculovirus (Griffiths *et al*, 2000).
For this method to work, the foreign DNA needs to be designed to share homologous sequences with the tardigrade genome, which requires knowledge about the tardigrade genome (University of Utha).

Of course, many of the approaches suggested in this section could be combined.

One reason why it is so difficult to do it is because the animals are very small and the research on the animal itself has been relatively limited.

There is still a general lack of information that is necessary, in order to maintain the cells and decide between the many possible approaches for cell culturing on an informed basis. Many attempts will likely have to be made, before a cell culture can successfully be established.

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10. Appendix

Appendix 1

Table 10.1: Investigated ions from the study by Halberg et al. 2012.

	Concentration (mM)	Osmotic contributions (%)
Na^+	29±5	8.0
NH_4^+	11±5	3.0
K^+	22±4	6.1
Ca^{2+}	23±3	6.4
Mg^{2+}	7±1	1.9
F^-	7±3	1.9
Cl^-	48±15	13.3
PO_4^{3-}	16±5	4.4
SO_4^{2-}	15±4	4.2
Total ionic conc.	171	49.2
Osmotic deficit	190	50.8
Unidentified cation	Peak found	-
HCO_3^-.	Not investigated	-

Appendix 2

Table 10.2: Frog Ringer Solution (copied from: webs.wofford.edu/davisgr/bio342/Ringers.htm).

Chemical	Molec. Wt.	Concentration	to make 500 ml	to make 1 liter	to make 2 liters
HEPES (a buffer)	238	5 mM	0.595 g	1.19 g	2.38 g
NaCl	58	125 mM	3.63 g	7.25 g	14.5 g
KCl	75	3 mM	0.113 g	0.225 g	0.450 g
CaCl2	147	1.8 mM	0.113 g	0.265 g	0.53 g
Glucose (Dextrose)	180	10 mM	0.90 g	1.80 g	3.60 g
MgCl2	203	1.6 mM	0.162 g	0.324 g	0.648 g

Procedure:

Use only glassware that is labeled "Ringer's Only."

Rinse weighing spatula with distilled water and dry with a Chem-Wipe before and after scooping each chemical.

Set up a beaker with stir bar on a stir plate. Add about 90% of the final volume of distilled water to the beaker and have the water stirring while adding the ingredients.

Once all chemicals have been added, bring to the final volume in a graduated cylinder.

Return the Ringer's solution the beaker on the stir plate and adjust the pH while agitating.

Rinse of the pH probe with distilled water, blot dry with a Chem-wipe and suspend in the Ringer's Solution.

pH should be adjusted to 7.3 to 7.45 for amphibians with 1M NaOH.

Rinse pH probe with distilled water before returning to storage solution. Transfer Ringer's solution to a bottle with cap, label, and store in refrigerator.

Appendix 3

Table 10.3: Composition of Grace's Insect Medium

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75.0	650.0	8.666667
L-Alanine	89.0	225.0	2.52809
L-Arginine hydrochloride	211.0	700.0	3.3175356
L-Asparagine	132.0	350.0	2.6515152
L-Aspartic acid	133.0	350.0	2.631579
L-Cystine 2HCl	313.0	28.68	0.09162939
L-Glutamic Acid	147.0	600.0	4.0816326
L-Glutamine	146.0	600.0	4.109589
L-Histidine	155.0	2500.0	16.129032
L-Isoleucine	131.0	50.0	0.3816794
L-Leucine	131.0	75.0	0.57251906
L-Lysine hydrochloride	183.0	625.0	3.4153006
L-Methionine	149.0	50.0	0.33557048
L-Phenylalanine	165.0	150.0	0.90909094
L-Proline	115.0	350.0	3.0434783
L-Serine	105.0	550.0	5.2380953
L-Threonine	119.0	175.0	1.4705882
L-Tryptophan	204.0	100.0	0.49019608
L-Tyrosine disodium salt	225.0	62.14	0.27617776
L-Valine	117.0	100.0	0.85470086

beta-Alanine	89.0	200.0	2.247191
Vitamins			
Biotin	244.0	0.01	4.0983607E-5
Choline chloride	140.0	0.2	0.0014285714
D-Calcium pantothenate	477.0	0.02	4.192872E-5
Folic Acid	441.0	0.02	4.5351473E-5
Nicotinic acid (Niacin)	123.0	0.02	1.6260162E-4
Para-Aminobenzoic Acid	137.0	0.02	1.459854E-4
Pyridoxine hydrochloride	206.0	0.02	9.708737E-5
Riboflavin	376.0	0.02	5.319149E-5
Thiamine hydrochloride	337.0	0.02	5.934718E-5
i-Inositol	180.0	0.02	1.11111105E-4
Inorganic Salts			
Calcium Chloride ($CaCl_2$) (anhyd.)	111.0	500.0	4.5045047
Magnesium Chloride (anhydrous)	95.0	1070.0	11.263158
Magnesium Sulfate ($MgSO_4$) (anhyd.)	120.0	1358.0	11.316667
Potassium Chloride (KCl)	75.0	2800.0	37.333332
Sodium Bicarbonate ($NaHCO_3$)	84.0	350.0	4.1666665
Sodium Phosphate monobasic (NaH_2PO_4-H_2O)	138.0	1013.0	7.3405795
Other Components			

Alpha-Ketoglutaric acid	146.0	370.0	2.5342467
D-Fructose	180.0	400.0	2.2222223
D-Glucose (Dextrose)	180.0	700.0	3.8888888
Fumaric acid	116.0	55.0	0.47413793
Lactalbumin hydrolysate		3303.0	Infinity
Malic acid	134.0	670.0	5.0
Succinic acid	118.0	60.0	0.5084746
Sucrose	342.0	26680.0	78.011696
Yeastolate		3330.0	Infinity

OBS: When the table says that the concentrations of Lactalbumin hydrolysate and Yeastolate are "Infinity", we understand it to mean that it is an unknown, but finite concentration.

Appendix 4

Aseptic Techniques

A major risk in cell culturing is the risk of contamination, so there should be put great consideration into using the correct aseptic technique. Here are some of the ground rules for aseptic technique:

- Good personal hygiene. Hands should be washed and gloves used. Clean the gloves with 70% ethanol before starting. Lab coats should be worn, and long hair put up. A person with a cold should also wear a mouth cover.
- The workspace (in this project that is a flow-hood) should be cleaned with 70% ethanol before and after use. The same goes with all the equipment that goes into the hood.
- Keep the working area tidy and try to have a lot of space so that contamination between things are avoided. Do not work on the edge of the flow hood. Any spillages should be cleaned immediately with 70% ethanol.
- If working with different cell lines, you should clean the hood between each to reduce the risk of cross-contamination.
- Use a new pipette for each manipulation to avoid e.g. getting cells into the media bottle. Make sure that the tip of the pipette does not touch anything before use.
- When opening a bottle, open the bottle with one hand and keep the cap and the bottle in the same hand, so it can be put back on right after use. Place the cap downwards on the table if this is not possible, so dust doesn't fall into it. The table should be sterile.
- Never reach over an open bottle or dish. There is a risk of getting a sleeve in the product, knocking things over or having dirt or bacteria fall in to the container.

(Harrison & Rae, 1997, 70f).

Usually, a contamination can be identified from the milky look it gives to the medium, as opposed to the clear look of uncontaminated cultures.

If for some reason this is not sufficient to determine the contamination, it can also be seen through a regular light microscope. If you hold the well or flask still under the microscope, you will see the bacteria moving around, while the cells will be still.

Appendix 5

Preparation of the used glucose dilutions

To prepare the glucose solutions the right amount of glucose is weighed exactly and added to 100 mL Milli-Q water with an osmolality of 77 mmol/kg. The right amount can be calculated through the assumption that mOsm/kg and mmol/L is equal.

For 100 mL sample we want to have a Glucose solution of 50 mOsm/kg:

$M_{glucose}$ = 180 g/mol

n= 50 mmol = 0,05 mol

$$m = n \cdot M$$

$$m = \ 0{,}05 \ \cdot 180 = 9g$$

9 g are for 1L, therefore it has to be divided by 10 to get the right amount of glucose for 100mL, which is 0,9g glucose.

g glucose/100mL

Table 10.4: Osmolality of glucose solutions.

Preferred osmolality	50 mOsm/kg	150 mOsm/kg	200 mOsm/kg	250 mOsm/kg	300 mOsm/kg	400 mOsm/kg
Required mass of glucose	0,9 g	2,7 g	3,6 g	4,5 g	5,4 g	7,2 g
Measured osmolality	84 mOsm/kg	163 mOsm/kg	196 mOsm/kg	246 mOsm/kg	311 mOsm/kg	404 mOsm/kg