The University Of Sydney

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Behavioural Variation of Acellular Slime Moulds

By

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Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Arisa Hosokawa

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Abstract

Protists are represented in every biome and have a diverse range of ecosystem roles. However, protists are severely under-represented in the scientific literature with few studies on how their diversity or behaviour affects ecosystem functioning. One group of protists, the acellular slime moulds, have been extensively studied for the behavioural abilities of the model species *Physarum polycephalum*. Although the decision-making and problem-solving abilities of *P. polycephalum* are well known, it is unclear whether the behaviour of *P. polycephalum* is representative of acellular slime mould species and whether their behaviour varies within individuals or strains. I investigated variations in acellular slime moulds at the species, strain and individual level and found a wide range of variability. Age affected two strains of *P. polycephalum* in a non-linear pattern and I observed age-related fluctuations in behaviour, physiology and cellular measures (Chapter 1). I found a non-linear relationship between age and decision-making, as well as distinct differences in decision-making between strains (Chapter 2). I found variation in foraging behaviour between three species of acellular slime moulds and each species also showed behavioural variation depending on the foraging environment as well as variations in interactions between species (Chapter 3). I was able to observe facilitation between species where foraging success improved in the presence of other acellular slime mould species (Chapter 3). The diversity of behaviour and physiology found within individuals and strains of *P. polycephalum* demonstrates the importance of including information on strains and age in future behavioural investigations. In addition, variation in behaviour between species demonstrates the diversity of behaviour within this group of protists and highlights the need for further research to understand how the behaviour of these protists affect species diversity and ecosystem functioning.

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Introduction

Protists account for the majority of taxa of eukaryotic life on Earth (Geisen et al., 2018). They are present in every biome and have a diverse range of morphologies and ecological functions. Protists range in size including single-celled, bacterium sized organisms, acellular organisms such as slime moulds, and even multicellular organisms such as kelp (Geisen et al., 2018). The protist kingdom includes phototrophs, heterotrophs and mixotrophs and they play key roles in the ecosystem as part of the soil microbiome community (Geisen et al., 2018; Wu et al., 2022) and within the plant microbiome (Bamforth, 1973; Flues et al., 2017). Protists can be involved in primary production (Jassey et al., 2015; Ward and Follows, 2016), element cycling (Aoki et al., 2007; Puppe et al., 2014; Sommer et al., 2013) and predation as bacteriovores (Glücksman et al., 2010; Rosenberg et al., 2009; Sherr et al., 1983), fungivores (Foissner, 1999), and by consuming soil eukaryotes (Berney et al., 2013; Geisen et al., 2016; Hess et al., 2012; Yeates and Foissner, 1995).

An abundance of protist organisms are found in the soil microbiome (Wu et al., 2022). For example, over 100 active protist organisms can be found per gram of soil or litter in forest soils (Adl and Gupta, 2006). In fact, protists are estimated to make up 31% of biomass and 69% of respiration in soil ecosystems (Wu et al., 2022). Despite their abundance and importance for ecosystem stability and as providers of ecosystem services, protists are critically understudied (Geisen et al., 2016; Hünninghaus et al., 2017; Wu et al., 2022).

A recent review found that the majority of publications on the soil microbiome focused on bacteria and fungi, with only 4.37% of publications including protists (Wu et al., 2022). Exclusion of protists from studies of the soil microbiome obscures their importance as predators for bacterial and fungal turnover as well as on the structure of soil biodiversity. Publications focused on protists have highlighted the significant effect that protists have on decomposition in a range of ecosystems (Clarholm, 1985; Geisen et al., 2021; Hünninghaus et al., 2017; Ribblett et al., 2005). Through predation of decomposers, many protists act as an important link between decomposers and the rest of the ecosystem through the mobilisation of important nutrients and

minerals such as carbon and nitrogen (Hünninghaus et al., 2017). Many studies of protists assess the impact of their presence on decomposition communities but few delve deeper into their behavioural complexities.

Every protist organism is brainless and as such suffers from the misconception that their behaviour is of less importance compared to their neural counterparts, despite empirical evidence that shows no qualitative distinction between the behavioural complexities of neural and aneural organisms (Van Duijn, 2017). A common behaviour seen in brainless organisms is decision making. Decisions in brainless organisms are made by monitoring the current state of their environment, often through chemotaxis or signal transduction (Lyon, 2015). Using chemotaxis, brainless organisms can decide to move towards nutrient sources or away from toxic compounds. Examples of this behaviour include bacteria that use motility mechanisms (Westerhoff et al., 2014), plant roots that move through the soil (Casper and Jackson, 1997; Fransen et al., 2001; Mommer et al., 2012; Rubio et al., 2003) and protists that move towards high calorie food sources (Latty and Beekman, 2010). Single-celled organisms, such as the bacterium *Escherichia coli*, use signal transduction to control cytoplasmic gene expression (Hsieh and Wanner, 2010) and make decisions on whether to transport or assimilate ammonia (van Heeswijk et al., 2013). Bacteria can also work collectively, mimicking a multicellular system, through quorom sensing. Quroum sensing is a type of cell-cell communication where bacteria produce small diffusible molecules that trigger a transcriptional response when detected (Westerhoff et al., 2014). Bacteria from the same or different species react to these molecules, creating a positive feedback loop that allows the bacterial community to respond to environmental change (Westerhoff et al., 2014). Habituation, a basic component of learning where an organisms' response to stimuli diminishes with repeated stimulus, has also been observed in brainless organisms, including ciliates (Hamilton et al., 1974; Osborn et al., 1973; Wood, 1972), slime moulds (Boisseau et al., 2016; Vogel and Dussutour, 2016) and plants (reviewed in Abramson and Chicas-Mosier, 2016).

Research on brainless organisms is also of interest due to the parallels that can often be found in behaviour between anueral and neural organisms. Behaviour and intelligence in neural or-

ganisms stem from the chemical interactions between neurons in the brain. Aneural organisms similarly use information processing units to make decisions or exhibit behaviour which helps them adapt to their environment. For example, the single-celled bacterium *E. coli* has large clusters of interacting sensory receptors that are linked to motor activity, allowing it to move toward stimuli or away from toxicity (van Heeswijk et al., 2013). Bacterial communities communicate and interact between many individual cells, allowing them to adjust their behaviour based on changes in their environment (Westerhoff et al., 2014). When members of the protist group, dictyostelids, are ready to form their reproductive life stage, individual amoebae will release signal molecules to create a swarm and travel together towards a suitable environment (Strassmann et al., 2000). Swarming displays in colonies of myxococcal bacteria also show similarities to groups of neural organisms such as flocking in birds, shoaling in fish and swarming in insects (Wu et al., 2009). In fact, the voltage-dependent sodium channels used in the nervous system of neural organisms are believed to have evolved from calcium channels, one of the major systems utilised in the behaviour of brainless organisms (Liebeskind et al., 2011). Studying the behaviour of brainless organisms provides further insight into the fundamental mechanisms and evolution of behaviour across all organisms.

Classification and life cycle of acellular slime moulds

One group of brainless organisms that have gained recent interest due to their "intelligent" behaviour are acellular slime moulds. Although their name may point their classification towards the fungi kingdom, acellular slime moulds are protists. They are part of a larger group of slime moulds which comprise of myxomycetes (acellular slime moulds), dictyostelids (cellular slime moulds) and protosteloids (amoeboid slime moulds). Acellular slime moulds are distinctive as they have an acellular vegetative stage called a plasmodium, which is a large single cell with multiple nuclei. A plasmodium is formed when two compatible haploid myxamoebae (which are free-living amoebae) or swarm cells (flagellated cells) fuse to create a diploid cell (Fig. 1). This cell continues to replicate without undergoing cytokinesis, which leads to the build-up of multiple nuclei in a large macroscopic single cell.

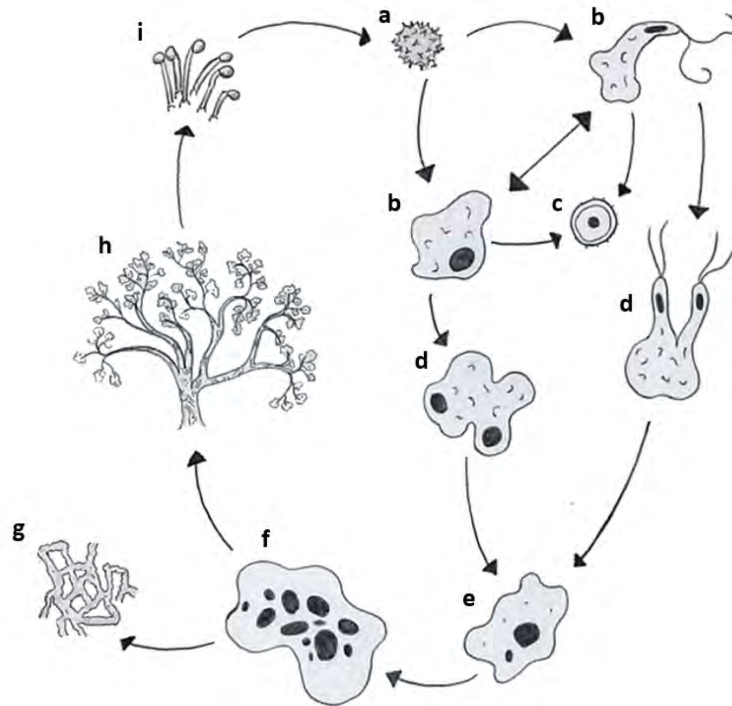


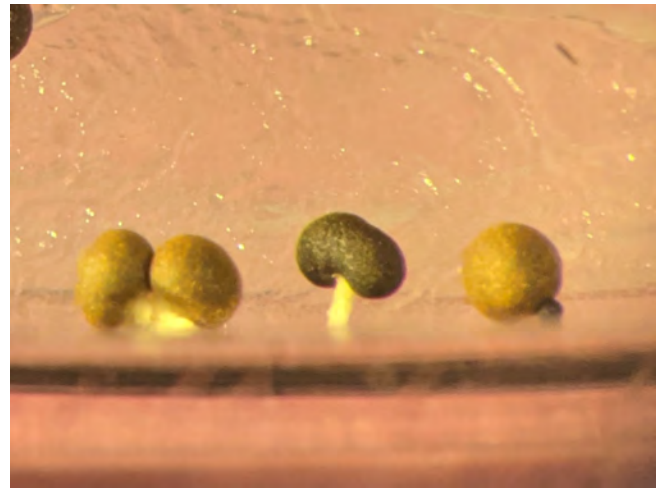
Figure 1: Lifecycle of myxomycetes: (a) Spore. (b) Unicellular myxamoeba (left) or swarm cell (right). These two forms interchange readily (c) Microcyst (d) Compatible pairs of myxamoebae or swarm cells fuse to form a diploid zygote (e) Zygote (f) Plasmodium. The nucleus of the zygote divides by mitosis continuously without cytokinesis to form a single large cell with many nuclei. (g) Under adverse conditions the plasmodium can form a resistant sclerotium. (h) Portion of a mature plasmodium. (i) Fruiting bodies. (Adapted from Stephenson (2011))

Plasmodia are often found on rotting logs or in leaf litter and continue to feed until environmental cues prompt them to form their reproductive life stage, called a fruiting body. Depending on the species of acellular slime mould, a single plasmodium can become hundreds of small fruiting structures or a singular large structure. All fruiting bodies contain haploid spores, which will re-enter the environment and become myxamoebae. Fruiting bodies of acellular slime moulds have a vast diversity of shapes and forms and microscopic observations of these structures are used to identify species groups (Fig. 2).

(a) *Arcryria cinerea*

(b) *Lamproderma scintillans.* Image reproduced with permission from Theotime Colin

(c) *Stemonitis virginiensis*



(d) *Physarum melleum*

Figure 2: Acellular slime mould fruiting bodies

89 Both myxamoebae and plasmodia have important functional roles in decomposition systems.
90 Myxamoebae and swarm cells are interconvertible, with the latter dominating habitats with
91 free water while the former are soil-dwelling. Acellular slime moulds at this life stage affect the
92 species composition of decomposition systems by preying on microbes such as bacteria, spores of
93 fungi and small protists (Stephenson and Stempen, 1994). Plasmodia also feed on bacteria and
94 fungi and directly break down organic matter (Stephenson and Stempen, 1994). Acellular slime
95 moulds are especially pervasive in the environment as they can retreat into resistant life stages
96 in adverse environmental conditions. This ability is present in both myxamoebal and plasmodial

life stages and is shared by many protists (Adl and Gupta, 2006). Myxamoebae transform into dormant structures called microcysts whereas plasmodia reduce their biomass in dry conditions and become structures called sclerotia (Stephenson and Stempen, 1994). These resistant stages are reversible and can remain viable for long periods, with sclerotia able to persist for at least two years (Anderson, 1992). The resistant stages of acellular slime moulds allow them to persist in harsh conditions and may be the reason they are ubiquitous in terrestrial environments.

The plasmodial cell

Studies of acellular slime mould behaviour mainly focus on the plasmodial life stage. Plasmodia are a fascinating life form that consist of a network of tubes containing cytoplasm (Durham and Ridgway, 1976; Kobayashi et al., 2006). These tubes are made of two specialised types of cytoplasm: the outer gel (ectoplasm) and the inner sol (endoplasm) (Kobayashi et al., 2006). Between these two layers of cytoplasm, contractile actomyosin filaments provide a mechanical force to help push cytoplasm rhythmically back and forth throughout the network (Fig. 3). The exterior of the network is lined with pores that increase the surface area of the plasmodia and act as channels in and out of the cell (Oettmeier et al., 2018). The entire network contracts asynchronously and can be thought of as consisting of units of coupled non-linear oscillators (Kobayashi et al., 2006). These coupled non-linear oscillators increase their oscillations when they are near attractants and decrease oscillations when near repellents, creating a pressure gradient which allows cytoplasm to flow towards areas of high oscillations (Durham and Ridgway, 1976; Kobayashi et al., 2006). The complex biochemical network throughout the plasmodia utilises both chemical receptors and photoreceptors to make decisions about which direction the cell will move (Oettmeier et al., 2018).

Plasmodia also excrete an extensive slime layer that facilitates movement through their environment, prevents dehydration (Oettmeier et al., 2018) and acts as an external memory system (Reid et al., 2012). This layer, also known as extracellular secretions, has a complex chemical makeup which differs between species. Extracellular secretions can consist of mucopolysaccharides, acidic polysaccharides, glycoproteins and enzymes (Oettmeier et al., 2018). Finally, due

124 to the multinucleate nature of the cell, plasmodia can be severed into multiple fragments, with
125 each fragment becoming an individual plasmodial cell as soon as 20 minutes after being severed
126 (Yoshimoto and Kamiya, 1978). This potential for rapid replication of cells makes acellular
127 slime mould plasmodia an excellent group of organisms for laboratory investigations of their
128 behaviour.

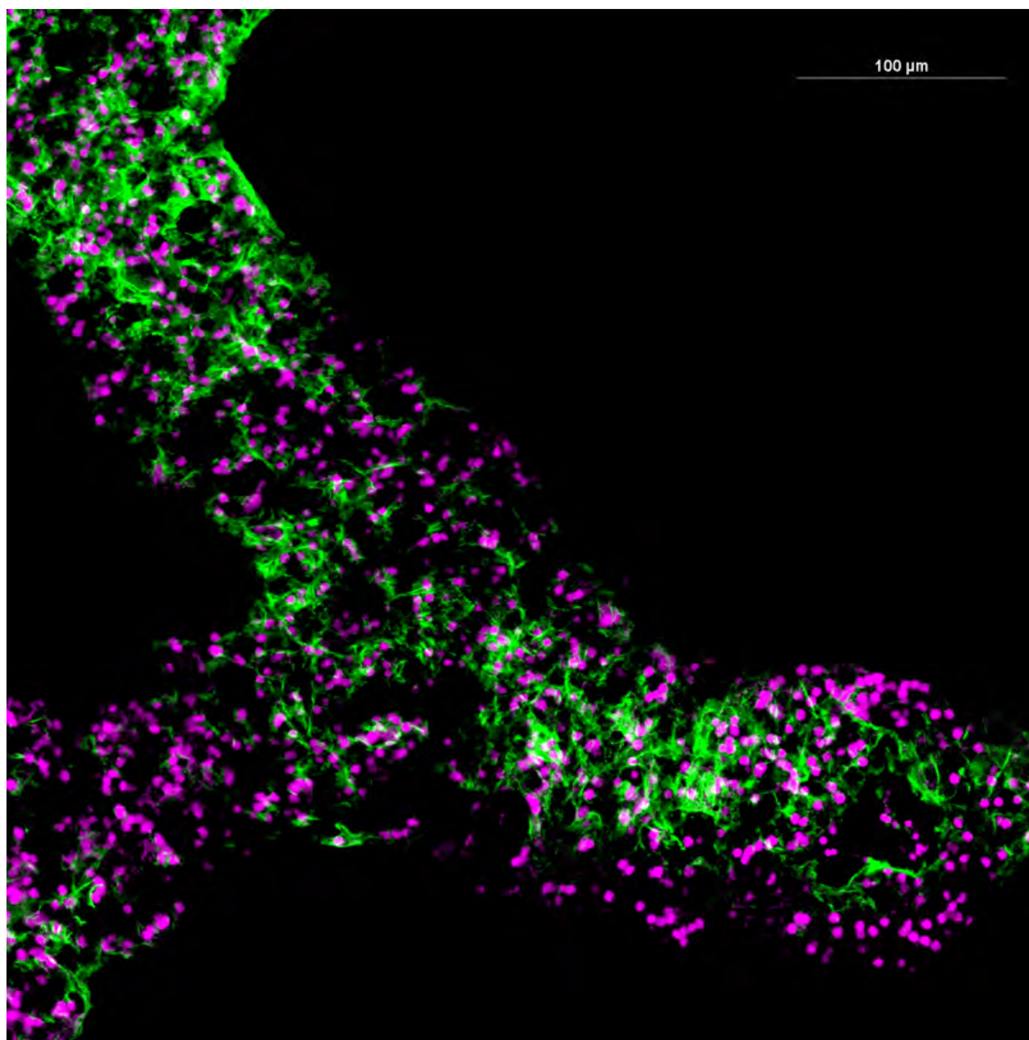


Figure 3: Fluorescence microscopy image of a plasmodial tube. Actomyosin filaments are green and nuclei are magenta.

129 Behaviour of acellular slime moulds

130 Investigations of the behaviour of acellular slime moulds have focused on the model species
131 *Physarum polycephalum*. *Physarum polycephalum* plasmodia can make decisions, as they can
132 discriminate between high concentration and low concentration food (Latty and Beekman,

133 2011a, 2009; Reid et al., 2016). Plasmodia also make trade-offs between the nutrient concen-
134 tration of food and environmental conditions. When given the choice between two options, *P.*
135 *polycephalum* plasmodia choose food in safe environments even if it is lower in quality, and only
136 choose food in stressful environments if the nutrient concentration is sufficiently higher (Latty
137 and Beekman, 2009). Speed-accuracy trade-offs have been demonstrated in *P. polycephalum*
138 where stressed individuals made faster but less accurate decisions in difficult discrimination
139 tasks (Latty and Beekman, 2011b). *Physarum polycephalum* can also balance their nutritional
140 intake by regulating their growth over separate carbohydrate and protein food sources to con-
141 sume an optimal ratio of nutrients (Dussutour et al., 2010). Another notable behaviour was
142 the demonstration of irrational decision-making, where *P. polycephalum* food preference was
143 affected by the presence of a third, low quality food option (Latty and Beekman, 2011a).

144 Due to the decentralised nature of plasmodia, acellular slime moulds can simultaneously feed
145 from multiple sources. Plasmodia of *P. polycephalum* have the ability to organise their network
146 of tubules to connect food using the shortest path (Pastorino et al., 2019; Reid and Beekman,
147 2013; Shirakawa and Gunji, 2006; Tero et al., 2010). Habituation has also been observed in *P.*
148 *polycephalum*, where plasmodia can learn to cross a bridge made of negative stimuli to reach a
149 food reward (Boisseau et al., 2016). Habituation can be transferred to naive clones through cell
150 fusion (Vogel and Dussutour, 2016) and is also maintained after habituated plasmodia enter their
151 resistant stage, sclerotia, and then return to plasmodia again (Boussard et al., 2019). *Physarum*
152 *polycephalum* can also anticipate periodic changes to environmental conditions, where trained
153 plasmodia will slow down growth rate in anticipation of dry and cold conditions (Saigusa et al.,
154 2008).

155 **Species level variation**

156 The limited studies conducted on the behavioural variations between acellular slime mould
157 species have yielded a diverse range of behavioural responses. Westendorf and colleagues (2018)
158 compared plasmodial growth and network development of three species of acellular slime moulds,
159 *P. polycephalum*, *Badhamia utricularis* and *Fuligo septica*. Variations in plasmodial growth

appeared to relate to phylogenetic differences. The plasmodia of *F. septica*, the most distantly related species, did not display self-avoidance as is typical in *P. polycephalum* and *B. utricularis*, and instead grew and retracted over the same area multiple times. The more closely related species, *P. polycephalum* and *B. utricularis*, responded similarly to chemo-attractants and only differed in growth rate. Differences in foraging behaviour have been found even in closely related species. For example, plasmodia of *Didymium iridis* moved slower but with higher directionality after contact with food compared to plasmodia of *Didymium bahiense*, a species from the same genus, that showed no change in foraging behaviour after contact with food (Yip et al., 2014). *Didymium bahiense* also showed no reaction to light exposure while foraging (Latty and Beekman, 2015). This reaction to light is particularly interesting, as light exposure was commonly understood to be detrimental to acellular slime moulds based on observations of *P. polycephalum* (Latty and Beekman, 2015). It is possible that *D. bahiense* is affected by a different wavelength of light not tested in the study, or perhaps the darker brown colour of the species allows them to be more resistant to light stress.

Strain level variation

Initial studies of *P. polycephalum* and *D. iridis* that began in the 1960s carefully noted the strains of each plasmodium. Until the late 1990s, strains could be traced back to isolates from the 1970s and careful genetic analysis by Kawano and colleagues (1997) showed that all existing strains of *P. polycephalum* at that time could be traced back to laboratory groups from Pennsylvania, North Carolina, Indiana, Iowa, Wisconsin, Osaka, Nagoya and Hitotsubashi. Around this time, sclerotia of *P. polycephalum* became available through biological supply houses; many current researchers cite Carolina Biological or Southern Biological supply houses as the source of their plasmodia. This has implications on current findings from acellular slime mould research as we are coming to understand that there are intraspecific differences in behaviour of *P. polycephalum* and it is difficult to know how strains produced by biological supply houses are cultivated and whether this could impact our understanding of *P. polycephalum* behaviour.

Behavioural differences have been found between strains of *P. polycephalum*. All current research

187 has focused on comparisons between the following three strains: a strain supplied by Carolina
188 Biological supply house, a strain supplied by Southern Biological supply house and another
189 strain known as HU192xHU200 that was supplied by Hakodate University in Japan. Zabzina and
190 colleagues (2014) found that smaller plasmodia were more likely to exploit only one food source
191 whereas larger plasmodia were more likely to exploit both food sources. They found significant
192 differences between strains where larger plasmodia of the strain from Southern Biological were
193 more likely to exploit both food sources than both the strains from Hakodate University and
194 Carolina Biological (Zabzina et al., 2014). The strains studied were also found to have distinct
195 and recognisable behaviour. The strain from Southern Biological moved slowly by creating
196 multiple thin tubes that spread radially (Dussutour et al., 2019; Zabzina et al., 2014). The
197 behaviour of the strain from Hakodate University were significantly different, where plasmodia
198 grew fewer tubes that were thicker, more directional and grew quickly (Dussutour et al., 2019).
199 The behaviour of the strain from Carolina Biological was a mixture of the previous two strains,
200 where plasmodia moved quickly but spread radially rather than directionally (Dussutour et al.,
201 2019). There were differences in decision accuracy between the three strains, where the slowest
202 strain, Southern Biological, were the most accurate and the fastest strain (from Hakodate
203 University) were the least accurate (Dussutour et al., 2019). When the strains were placed in
204 competition with each other, the behaviour of the strain from Hakodate University was the
205 most advantageous in environments with one food source, as it could move quickly and exploit
206 the food before the other strains (Dussutour et al., 2019). However, plasmodia of the strain
207 from Carolina Biological were the most successful in environments with multiple food sources,
208 as their higher accuracy meant they were able to exploit the higher concentration food before
209 the other strains (Dussutour et al., 2019).

210 Vogel and colleagues (2015) used chemical analysis to determine the drivers of behavioural dif-
211 ferences between the three strains. They found that plasmodia excrete calcium onto food sources
212 while feeding which acts as an attractant to other cells. Plasmodia of strains from Southern
213 Biological excreted the highest concentration of calcium and were also the most accurate. They
214 suggest that slow and accurate movement may be caused by the excretion of large amounts

215 of calcium, as this process may be more energetically costly and produce stronger signals for
216 plasmodia to follow.

217 Individual level variation

218 Behavioural variation can even be found within clones of the same strain of acellular slime
219 mould. Clones of the same strain can be produced by severing a single plasmodium into mul-
220 tiple fragments, as each severed fragment becomes a functioning individual plasmodium after
221 20 minutes (Yoshimoto and Kamiya, 1978). Due to the multinucleate nature of plasmodia,
222 individuals formed by fragmentation are not strictly clones as they have different compositions
223 of nuclei. Therefore, I will be referring to plasmodia of the same strain as individuals. Variation
224 within individuals is common in many experiments, for example in a foraging task up to 10%
225 of plasmodia moved away from food when they were within 1 cm of food stimulus (Pastorino et
226 al., 2019). When plasmodia approach a zone of toxic quinine, plasmodia demonstrate a range
227 of responses such as continuing across the zone, avoiding the zone or exhibiting both behaviours
228 simultaneously (Kunita et al., 2017; Shirakawa et al., 2020). Landscape variations can change
229 the behaviour of *P. polycephalum,* where plasmodia in correlated landscapes (defined patches
230 of high or low concentration food) gained more weight than plasmodia foraging in uncorrelated
231 landscapes (high and low concentration food distributed randomly) (Latty and Beekman, 2009).
232 Stress caused by hunger or light exposure also changes behaviour of *P. polycephalum.* In binary
233 discrimination tests, stressed plasmodia made faster decisions in difficult tests where the quality
234 of food options were similar and made slower decisions in easy tests where food options differed
235 largely in quality (Latty and Beekman, 2011b).

236 The cause of individual variation in the plasmodia of acellular slime moulds are largely un-
237 known. A likely cause of variation may be ageing of plasmodia in the duration of behavioural
238 experiments. As many researches source sclerotia from biological supply houses the true age
239 of plasmodia studied in experiments is often unclear. Previous research on ageing in acellular
240 slime moulds have shown that they exhibit signs of ageing and senescence (Abe et al., 2000;
241 Clark, 1984; Clark and Lott, 1981; Clark and Mulleavy, 1982; Hu et al., 1985; Kerr and Waxlax,

1968; Lott and Clark, 1980; McCullough et al., 1973; Nakagawa et al., 1998). All of these studies were undertaken before the 2000s, prior to the advent of behavioural experimentation on acellular slime moulds. This means that there is a major gap in our understanding of whether the behaviour of acellular slime moulds changes with age. In addition, as plasmodia appear "ageless" in current research, there are few observations made on the health of plasmodia before behavioural experimentation. It is likely that the health of plasmodia affects physiological and behavioural performance in plasmodia, and may be a major factor in the individual variation that is observed in many experiments.

As the study of *P. polycephalum* behaviour deepens, there appears to be a disconnect in how their behaviour affects their ecological function. The focus on *P. polycephalum* may be detrimental to our understanding of acellular slime mould behaviour as a whole, as strains of *P. polycephalum* have been cultivated for over 60 years with little interaction with wild type species. Studies of other acellular slime mould species have uncovered a diverse range of different behaviour, including different foraging strategies (Yip et al., 2014), decision-making (Latty and Beekman, 2015), extracellular secretion use (Masui et al., 2018; Reid et al., 2013), plasmodial growth and network development (Westendorf et al., 2018). This diversity in behaviour raises significant questions about how these behaviours can affect ecological function of acellular slime moulds. How do these different behaviours influence the success of acellular slime mould species in the environment? What environmental factors could drive differences in behaviour between species of acellular slime moulds? Additionally, *P. polycephalum* decision-making mechanisms have been extensively modelled and used for biological computing (Evangelidis et al., 2017; Ishiguro and Umedachi, 2018; Jones, 2016; Kalogeiton et al., 2015; Schumann and Pancerz, 2016; Tsompanas et al., 2015). Assuming that all acellular slime mould species move by utilising similar biochemical mechanisms, modelling the behaviour of different species may provide interesting alternatives to current biological computing approaches.

Aims

The aim of my thesis was to explore variations of acellular slime mould behaviour at the level of species, strains and individuals. My first two chapters explore variations in individuals over time and between strains. My third chapter observes behavioural variation between species. My fourth chapter outlines further experiments which were unable to be completed due to restrictions caused by the Covid-19 pandemic but were highly relevant to the questions raised in my thesis. This includes experiments that would have observed behavioural variation in individuals after different life stages and behavioural variation between species from different climates and habitats.

Plasmodia of *P. polycephalum* have been shown to age and senesce (Abe et al., 2000; Hu et al., 1985; McCullough et al., 1973; Nakagawa et al., 1998), but modern studies of *P. polycephalum* behaviour lack observations of plasmodial age and health. In Chapter 1, I observed how the behaviour of two strains of the acellular slime mould species, *P. polycephalum,* changed with age. In addition to behaviour, I assessed physiological and cellular changes to develop a well-rounded assessment of ageing in *P. polycephalum* and to help identify possible drivers of behavioural change. In Chapter 1, I specifically assessed network-building behaviour and search behaviour. For physiological changes I assessed movement speed and growth, and for cellular changes I assessed nuclei shape, circularity and density as well as analysing mtDNA genetic variation. Repeated testing of *P. polycephalum* over the plasmodia's lifespan allowed me to understand whether the behaviour of acellular slime moulds changed with age and whether changes in behaviour were correlated to physiological or cellular changes.

In Chapter 2, I specifically investigated changes in decision-making behaviour in ageing *P. polycephalum*. Previous research has found that speed-accuracy trade-offs are exhibited by hunger stressed plasmodia of *P. polycephalum* but not plasmodia that have been stressed by light exposure (Latty and Beekman, 2011b). I used binary discrimination tasks to observe whether speed-accuracy trade-off behaviour differed with plasmodial age. I also compared the behaviour of two strains of *P. polycephalum* and investigated differences in any age related

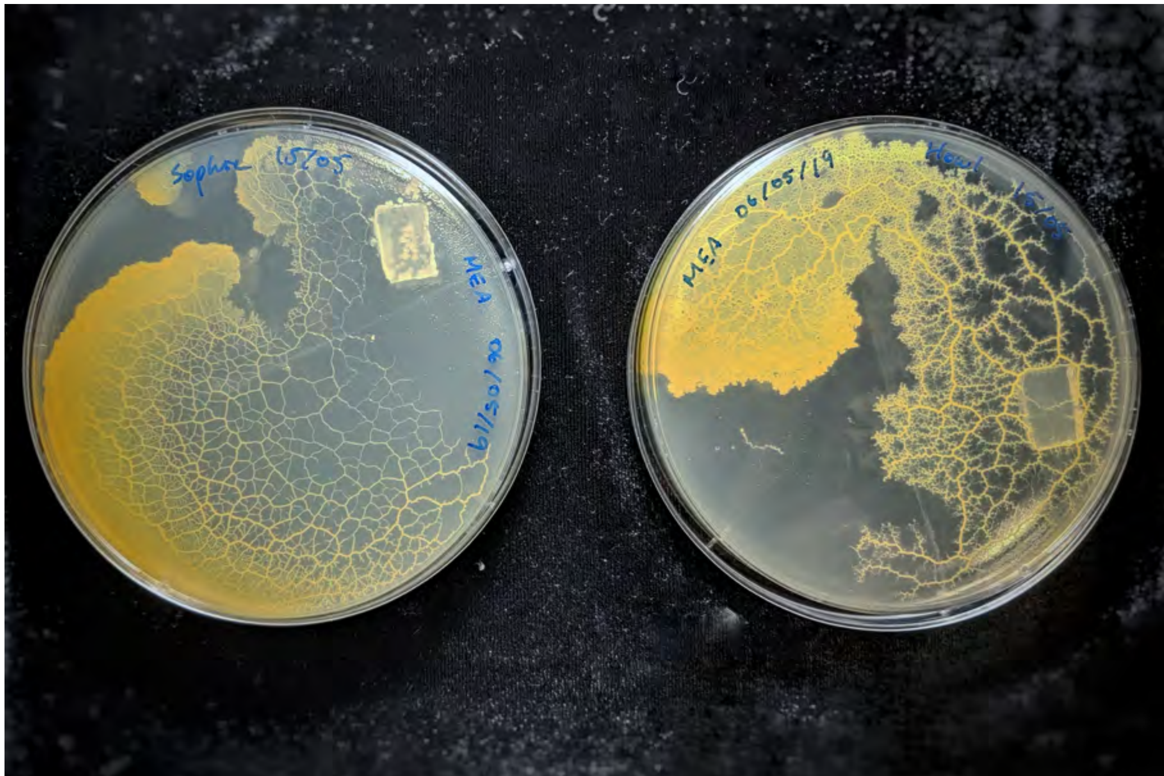
294 changes to decision-making ability.

295 Although variation in behaviour has been observed in different species of acellular slime mould,
296 interactions between species with different behavioural types have not been investigated. In
297 Chapter 3, I observed interactions between pairs of acellular slime moulds in a shared environ-
298 ment. I used three species of acellular slime moulds, *P. polycephalum, D. iridis* and *Physarum*
299 *melleum* and compared their foraging behaviour. I also assessed individual variation of forag-
300 ing behaviour by testing their ability in two different environments. In one environment food
301 was placed in a homogeneous distribution and in the second environment food was placed in
302 a heterogeneous distribution. My experimental design allowed me to observe both individual
303 variation of behaviour in different environments as well as behavioural variation at the species
304 level. By observing interactions between different species of acellular slime moulds I was also
305 able to observe whether foraging behaviour changed in the presence of other species.

306 The Covid-19 global pandemic occurred during the second and third year of my PhD. Due to
307 disruption to access to laboratory facilities, some planned components of my thesis were not
308 completed. In Chapter 4, I will discuss additional experiments I had planned to complete. The
309 first experiment was a continuation of my experiment in Chapter 1. I had created sclerotia,
310 the resistant life stage of acellular slime mould, every 30 days during my experiment. My aim
311 was to investigate whether the differences in behaviour of aged plasmodia would be consistent
312 after entering the sclerotia life stage. The second planned experiment was a continuation of my
313 experiment in Chapter 3. I planned to repeat this experiment by isolating wild slime mould
314 species from different geographical locations to observe whether interactions between species of
315 acellular slime moulds were influenced by environmental variables of their habitat.

Chapter 1

A Tale as Old as Slime: Ageing in plasmodia of *Physarum polycephalum*



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Author contributions: AH, MB, CR and TY contributed to the study design. AH conducted the experiment with assistance from VK for mtDNA isolation and analysis. AH collected and analysed the data. VK, CR and TY provided valuable feedback and editing for the article prepared by AH.

1.1 Abstract

Ageing in an organism typically leads to loss of function and death. Research on ageing has typically centered around animals, with less focus on ageing in unicellular organisms. Observing ageing in unicellular organisms provides valuable insight on ageing processes at the cellular level. In addition, parallels can be found between simple bio-electrical processes within unicellular organisms and complex processes in multicellular organisms. The acellular slime mould, *Physarum polycephalum*, utilises bio-electrical mechanisms similar to neural networks found in animals with brains to move and forage for food and presents an interesting model for studying ageing in a unicellular organism. *Physarum polycephalum* has been studied extensively for their behavioural abilities such as decision-making, creating short paths and habituation, but the effect of ageing on their behaviour is largely unknown. We observed physiological, behavioural and cellular changes monthly in two strains of *P. polycephalum* as they aged over 300 days. To observe changes in physiology we measured growth area and movement speed and to measure behavioural change we observed foraging strategy and network building ability. We also assessed cellular change by measuring nucleus shape and density as well as analysing changes in mtDNA at three age-points. We found non-linear relationships with age as the health of *P. polycephalum* cells fluctuated with age. Growth area, nucleus area and mtDNA sequences also had a non-linear relationship with age, although only growth area showed similar fluctuation patterns to cell health. Factors such as movement speed and foraging strategy had a linear relationship with age, where ageing plasmodia became faster and foraged in a more directional pattern. Additionally, density of nuclei in plasmodia decreased with age. Network building ability, a behavioural measure, was not affected by age. Lastly we found significant physiological, behavioural and cellular variation between the two strains of *P. polycephalum.* Our research demonstrates non-linear patterns in physiology, behaviour and within the cell over the first half of the lifespan of *P. polycephalum.* Acellular slime moulds may be a promising model species for studying ageing in brainless and unicellular organisms as fluctuations in cell health, nucleus shape and mtDNA sequences suggest that they may have cellular repair mechanisms to mitigate ageing stress.

1.2 Introduction

Ageing leads to the gradual loss of function in cells. Typically, as age increases in an organism, the ability of the organism to withstand extrinsic stresses decreases, leading to an increased probability of loss of function and death (Arking, 2006). Ageing has been extensively studied in a range of species, including invertebrates such as nematodes and flies, as well as vertebrates such as mice and primates (Lees et al., 2016). Research on ageing has also extended to unicellular organisms such as yeasts and bacteria (Ackermann et al., 2007; Lees et al., 2016; Ogrodnik et al., 2019). Previously, many unicellular organisms were thought to be unaffected by ageing as populations of these organisms could survive indefinitely in laboratory conditions, but it is now known that individual cells within the microbial populations do in fact age (reviewed in Moger-Reischer and Lennon, 2019).

Chronological age of an organism is typically split into sequential phases; starting from birth until the organism reaches reproductive age and then eventually dies (Gatsuk et al., 1980). Once an organism reaches reproductive age, physiological abilities such as growth rate and speed can begin to decline. Damage can accumulate at the cellular level as DNA, protein molecules and lipids can be damaged by mutations, UV radiation, free radicals or oxidative stress (Ogrodnik et al., 2019). This damage can accumulate more rapidly when cellular repair mechanisms degrade and become overburdened (Kaushik and Cuervo, 2015). The inability of an ageing organism to efficiently produce new cells leads to a decrease in growth rate (Nyström, 2007; Ogrodnik et al., 2019). Unicellular organisms, such as the bacterium *Escherichia coli*, are not immune to these cellular breakdowns, as their cell asymmetrically replicates and experiences slower growth rate in the one cell that inherits a higher proportion of damaged cell components during binary fission (Ackermann et al., 2007; Stewart et al., 2005).

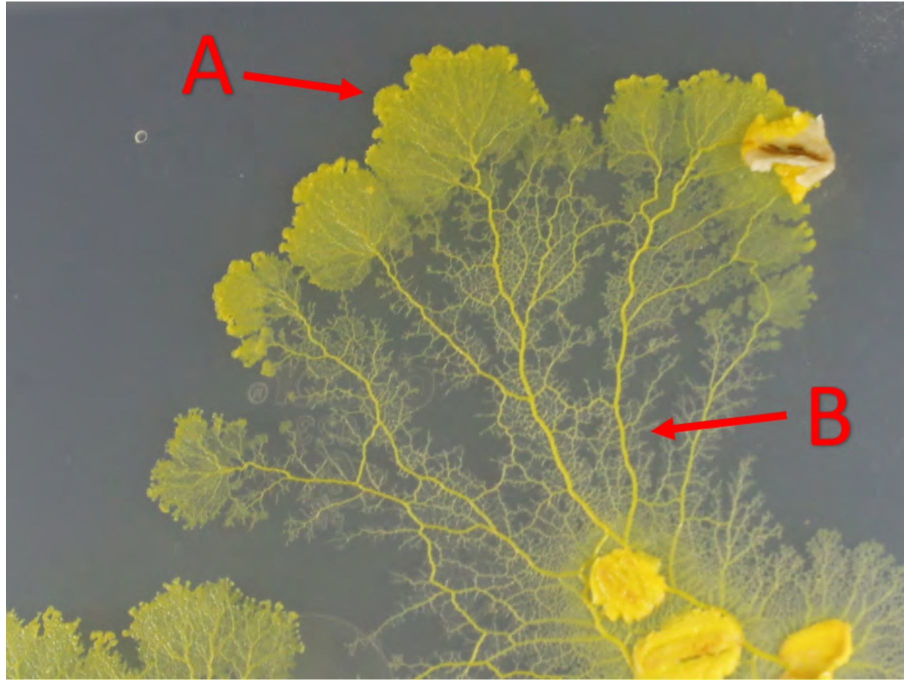

Figure 1.1: Section of plasmodium of *P. polycephalum.* A) Search front (or pseudopod) of plasmodia. B) Mid section of plasmodia

378 Historically, ageing research has focused on animal study species (Jones et al., 2014). Animal
379 models have restrictions, as it can be difficult to differentiate ageing at the cellular and molecular
380 level (Lees et al., 2016). Unicellular organisms present a powerful solution for observing ageing
381 at the cellular level, which can help researchers further dissect ageing. Many processes found
382 in unicellular organisms mimic those found in more complex multicellular organisms (Baluška
383 and Levin, 2016). For example, some unicellular organisms utilise bio-electrical mechanisms,
384 similar to the neural networks found in the brains of animals (Baluška and Levin, 2016). Other
385 unicellular organisms have been found to track past behaviour or events through a rudimentary
386 memory system that can be either internal or external to the cell (Caudron and Barral, 2013;
387 Cooper et al., 2012; Reid et al., 2012). An example of a unicellular organism that uses bio-
388 electrical mechanisms and has an external memory system is the acellular slime mould.

389 Acellular slime moulds are multinucleate organisms that have a lifestage called a plasmod-
390 ium, which consists of a complex network of cytoplasm. These unicellular organisms use bio-
391 electrical mechanisms to move cytoplasm through its network of branching tubes towards food

392 and favourable environments (Nakagaki et al., 2004). In recent years, acellular slime moulds
393 have been studied extensively for their behavioural abilities such as decision-making (Dussu-
394 tour et al., 2010; Latty and Beekman, 2010; Nakagaki et al., 2004; Reid et al., 2016; Reid et
395 al., 2012), habituation (Boisseau et al., 2016; Vogel and Dussutour, 2016) and speed-accuracy
396 trade-offs (Dussutour et al., 2019; Latty and Beekman, 2011b). Although many laboratories
397 culture acellular slime mould plasmodia, the age of the specimens are often disregarded or un-
398 known. Plasmodia can enter a dormant state called a sclerotium when they gradually dry out,
399 which can stay viable for long periods of time (Rojas and Stephenson, 2021). Researchers can
400 re-activate sclerotia a few days before running experiments by introducing sclerotia into a moist
401 environment. Due to this practice, changes to acellular slime mould plasmodia due to age are
402 rarely noted.

403 Our aim was to investigate how acellular slime moulds changed with age. We used the species,
404 *Physarum polycephalum*, to investigate this question as it is the model species used in the ma-
405 jority of behavioural experiments. In addition, physiological changes in ageing *P. polycephalum*
406 plasmodia have been observed (Poulter, 1969) and the maximum lifespan of the species has been
407 recorded at approximately 200 days (Hu et al., 1985). To develop a well rounded understand-
408 ing of ageing in *P. polycephalum* we assessed physiological, behavioural and cellular changes in
409 plasmodia with chronological age.

410 We investigated three aspects of physiology in ageing plasmodia, specifically cell health, move-
411 ment speed and growth rate. Observational research on ageing acellular slime moulds started
412 from the late 1960s on the plasmodia of *P. polycephalum*. The growing edge of plasmodia,
413 known as the search front (Figure 1.1A), became slightly irregular and then developed a lace-
414 like appearance as it aged, after which the plasmodia lysed (Poulter, 1969). Slower growth rate,
415 reduced cytoplasmic streaming and loss of yellow pigment were also observed in unhealthy and
416 aged plasmodia (Hu et al., 1985; Poulter, 1969). We used these symptoms to describe the cell
417 health of plasmodia for the duration of our experiment. For the remaining physiological as-
418 pects, we used assays to quantify movement speed and growth rate in plasmodia as they aged.

We hypothesised that movement speed and growth rate would decrease with increasing age. Slower growth has been observed in aged plasmodia in previous investigations (Hu et al., 1985; Poulter, 1969), suggesting that the acellular slime mould's ability to create new cell components and cytoplasm decrease with age. Acellular slime moulds move by streaming a higher volume of cytoplasm towards its desired direction (Nakagaki et al., 2004), thus reduced cytoplasmic streaming in ageing plasmodia may lead to slower movement speed. In addition, decreased growth and slower movement speeds have been observed in many ageing organisms including bacteria and fungi (Nyström, 2007; Ogrodnik et al., 2019), cockroaches and fruitflies (Ridgel and Ritzmann, 2005). Observations of physiological change due to age may also help inform changes in behaviour of *P. polycephalum*.

To investigate the behaviour of ageing *P. polycephalum*, we observed changes in foraging behaviour and network-building ability. Acellular slime mould plasmodia are composed of many small oscillating units, and the oscillation frequency of these units determine the direction of the plasmodium's movement (Ueda et al., 1980). When plasmodia detects food, the units closest to the attractant will increase their oscillation frequency which causes cytoplasm to flow towards those units (Kincaid and Mansour, 1978; Ueda et al., 1980). This simple mechanism of moving cytoplasm between highly oscillating units is called cytoplasmic streaming and it allows acellular slime moulds to forage for food. Plasmodia are also able to connect multiple food sources into a network by concentrating cytoplasm in plasmodial tubes that are in contact with food (Nakagaki et al., 2004). As much of an acellular slime mould's behaviour is determined by the movement of their cytoplasm, we predict that a decrease in an ageing plasmodia's cytoplasmic streaming (Hu et al., 1985) may negatively affect *P. polycephalum*'s ability to forage and build networks.

Thirdly, we used fluorescence microscopy techniques to assess cellular changes in *P. polycephalum*. Cellular changes due to age have been investigated in two species of acellular slime moulds, *Didymium iridis* and *P. polycephalum*. Research primarily focused on nuclei where older plasmodia of both species had a high proportion of large acircular nuclei with ploidy

446 variation (Clark and Mulleavy, 1982; McCullough et al., 1973). In addition, larger nuclei were
447 found in plasmodia before senescence, and removing large nuclei were found to increase the lifes-
448 pan of plasmodia (Clark and Hakim, 1980). Our investigation also focused on nuclei; assessing
449 nuclei circularity, size and density of nuclei in plasmodia as they aged. We expected nuclei in
450 older plasmodia would be acircular and larger in size compared to younger plasmodia, and that
451 density of nuclei would decrease as aged cells would have difficulty replicating their nuclei.

452 Damage to mitochondria has also been hypothesised to drive senescence in ageing *P. poly-*
453 *cephalum*. Abe and colleagues (2000) found that there was an increase in single-stranded break-
454 age of mtDNA in older plasmodia and suggested that in vitro fragmentation of mtDNA may
455 accumulate over time and lead to senescence. Another study also found differences in mtDNA,
456 but in this case they found a plasmid recombined with the mtDNA in a long-lived strain of
457 *P. polycephalum* (Nakagawa et al., 1998), possibly changing mitochondria function. Addition-
458 ally, breakdown in mitochondria function has widely been associated with senescence (Kaushik
459 and Cuervo, 2015; Moger-Reischer and Lennon, 2019). Although mitochondrial function is a
460 relatively understudied facet of acellular slime mould research, analysis of mtDNA of ageing
461 plasmodia may give further insight to cellular changes in *P. polycephalum*.

462 We tested multiple aspects of physiology and behaviour and observed cellular changes in two
463 strains of *P. polycephalum* every 30 days as the plasmodia aged over 330 days. Our study
464 represents the most extensive investigation of ageing in an acellular slime mould species. By
465 investigating ageing from three different angles: physiology, behaviour and cellular changes, we
466 hope to contribute to the understanding of ageing at a fundamental level.

467 1.3 Methods

468 Species and rearing conditions

469 *Physarum polycephalum* is an acellular slime mould, consisting of a large single cell with mul-
470 tiple nuclei. For this study we used the vegetative lifestage of the cell, called a plasmodium.
471 A plasmodium consists of tubes made of specialised cytoplasm that transports food and cell

472 components around the cell using contractile actin filaments (Nakagaki et al., 2004). Due to the
473 multinucleate nature of acellular slime moulds, pieces of the plasmodial cell can be severed from
474 the main cell into plasmodial fragments. These plasmodial fragments become fully functional
475 individuals between 20 - 40 minutes after being severed (Yoshimoto and Kamiya, 1978).

476 We used two strains of *P. polycephalum*, Tu111xAI35-H and TU9xDP89-S, which were created
477 by mating pairs of myxamoebae following the protocol of Moriyama & Kawano (2003). Due to
478 the multiple life-stages of acellular slime mould, it is difficult to determine the true age of each
479 strain. We defined the start point of our ageing study as the first appearance of plasmodia on the
480 malt extract agar, as this is when the cell changes from a haploid myxamaoeba cell to a diploid
481 plasmodial cell. The four parent strains, Tu48.9-111 (Tu111), AI35, Tu9 and DP89 were kept at
482 -80°C and were defrosted before use. We cultured plasmodia on 135 mm diameter Petri dishes
483 filled with malt extract agar at 24°C and kept them in the dark. Once plasmodia had grown
484 to cover the majority of the Petri dish plates, 1cm^2 of the plasmodial search front (the newest
485 growth of the plasmodial cell; Figure 1.1A) was subcultured to new malt extract agar plates.
486 Plasmodia generally took two to five days to cover the majority of the malt extract agar plates.
487 To minimize the effect of external or environmental variables on plasmodia, each strain was
488 cultured across three malt extract agar plates. When plasmodia were subcultured, two pieces
489 of plasmodial search front from different plates were added to each new malt extract agar plate
490 (Figure 1.2). This protocol was used to minimize cell differentiation within the strains between
491 the three malt extract agar plates. If contamination was found on any plate, the plasmodium
492 on the plate would be excluded from the experiment, and only the noncontaminated plasmodia
493 would be subcultured to three new malt extract agar plates.

494 Before each assay, we subcultured plasmodial fragments from each strain into nine 1.75 L
495 containers (500cm^2 surface area) with 1% agar and oat flakes to rapidly increase plasmodial
496 biomass. Plasmodial fragments were always taken from these 1.75 L containers and plasmodial
497 fragments only participated in assays once. We completed the first round of assays when the
498 strains were 39 days old, which was when the plasmodia had stabilised and could grow over a

499 large surface area. We repeated each assay approximately every 30 days until the strains were
500 307 days old, at which point the experiment was stopped. Due to COVID-19 restrictions and
501 loss of access to laboratory facilities, when both strains were 330 days old, the plasmodia were
502 moved and cultured on 1% w/v agar and fed autoclaved whole oat flakes (Woolworths brand)
503 instead of malt extract agar until they died at 628 days old for Tu111xAI35-H, and 621 days
504 old for Tu9xDP89-S.

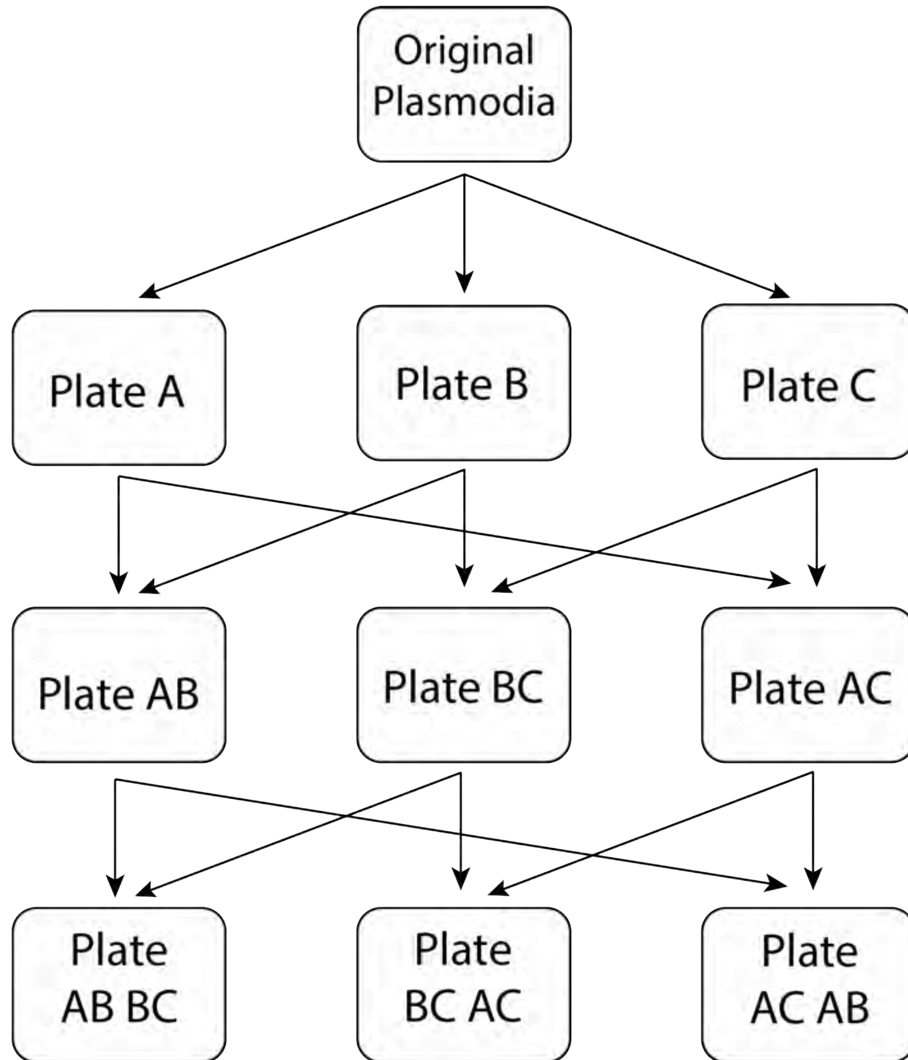

Figure 1.2: Culturing technique to ensure plasmodial cells did not differentiate between the three malt extract agar plates. Plasmodia were moved to new plates when they had completely grown over the malt extract agar nutrient agar (every 3-5 days). Two pieces of plasmodia from separate plates were joined to ensure there was no genetic divergence between three plates. If a plate was contaminated by mould or bacteria, plasmodia from that plate would be removed from the experiment.

Observations of health in culture

Photos were taken every one to five days using a phone camera (Pixel 2 XL, Google) to monitor the health of plasmodia grown on the malt extract agar plates. We developed a qualitative scale of health level guided by observations of cell health and growth over plasmodia's lifespan from Poulter (1969) to assess the health of plasmodia. Photos of plasmodia were rated from 5 (most healthy) to 0 (lysed cell/death) for 330 days (Figure 1.3). Notes were also taken regarding mould or bacterial contamination.

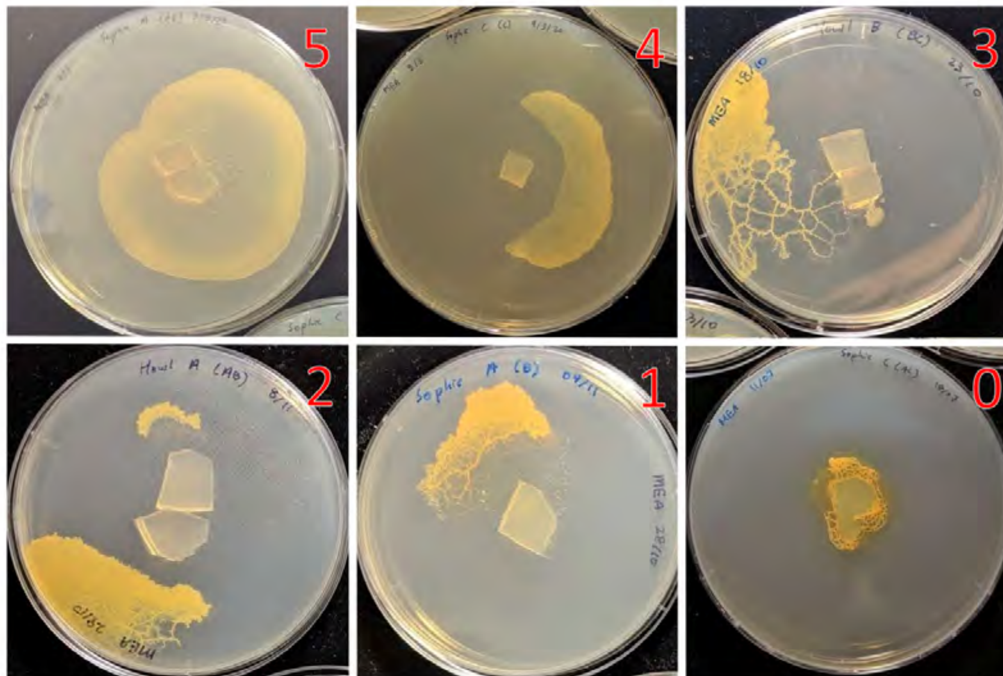

Figure 1.3: Health rating of plasmodia on malt extract agar agar. The plasmodia at health level 5 had strong radial growth. Level 4 plasmodia had strong growth that was directional. Level 3 plasmodia had directional growth with distinct veins in the middle and end sections. Level 2 plasmodia had directional growth but pigmentation of the plasmodia were darker. Level 1 plasmodia had small directional growth with increased intensity in pigmentation. Level 0 plasmodia had no growth, parts of the cell had lysed and pigmentation had leached into the agar.

Growth Assay

To assess growth, we added a plasmodial fragment (0.07 – 0.013 g) to an 85 mm diameter Petri dish filled with 3% oat agar (n = 30 for each strain with exceptions). We kept the plates in the dark at 21°C. We took pictures of each plate after 48 h (DSLR 1000D, Canon). We used ImageJ

(Rasband, 1997) colour thresholding to measure the surface area of each plasmodium. The growth assay was repeated approximately every 30 days for 330 days. For strain Tu111xAI35-H, growth assays were conducted at 34, 59, 90, 122, 153, 185, 212, 239, 273 and 303 days of age. For strain Tu9xDP89-S, the growth assays were conducted at 34, 59, 92, 93, 122, 153, 185, 212, 239, 273 and 303 days of age. Due to a decline in plasmodia health of strain Tu9xDP89-S, we were unable to grow enough plasmodia in the 1.75 L containers at 90 days of age to have 30 replicates. We delayed the growth assay for Tu9xDP89-S and assessed a total of 20 replicates, with 10 replicates at 92 days of age and 10 replicates at 93 days of age. At 185 days of age, there were technical issues that caused both strains to only have 29 replicates. The final sample size for the experiment was 588 replicates in total.

Speed Assay

To measure movement speed, we added a plasmodial fragment (0.07 – 0.013 g) to an 85 mm diameter Petri dish filled with 1% agar (n = 20 for each strain with exceptions). We removed the lids from these plates and kept them in a dark booth at 23°C for 48 h. We used a camera (DSLR 1000D and DSLR 1100D, Canon) with an intervalometer (Captur Timer Kit, Hahnel) to capture timelapse footage of the plasmodia. Every 30 minutes the plasmodia were illuminated for 5 seconds from above to capture an image. We also surrounded the plates with containers of water to prevent the agar and plasmodial cells from drying out.

We repeated the speed assay approximately every 30 days for 330 days. For strain Tu111xAI35-H, the speed assays were conducted at 34, 59, 90, 122, 152, 184, 212, 239, 270 and 303 days of age. For strain Tu9xDP89-S, speed assays were conducted at 34, 59, 93, 122, 152, 184, 212, 239, 272 and 303 days of age. Three replicates of Tu9xDP89-S at 34 days of age did not grow large enough to be measured. Due to issues with growing enough plasmodia of Tu9xDP89-S at 90 days of age, we delayed the speed assay for this strain until 93 days of age but we were only able to grow enough plasmodia for 10 replicates. Tu9DP89-S plasmodia experienced another decline in health at 270 days and we could not grow enough plasmodia in the 1.75 L containers and delayed the speed assays until 272 days of age. The final sample size for the experiment

543 was 387 replicates.

544 We used the ImageJ Manual Tracking plugin (Rasband, 1997) to calculate the speed of each
545 plasmodium over 12 h. We measured the speed from when the plasmodium first grew larger
546 than 20 mm in diameter. In the case of multiple branches, we measured the speed of each branch
547 that was separated by more than 45 degrees. We analysed both physiological and behavioural
548 measurements in the speed assay. We calculated the mean movement speed of each branch that
549 was separated by more than 45 degrees. We also observed behavioural change by noting the
550 number of branches each plasmodium produced in 12 hours.

551 **Network-building Assay**

552 *P. polycephalum* has been shown to connect points using shortest paths (Nakagaki et al., 2004).
553 Acellular slime moulds are able to exploit multiple food sources simultaneously by spreading
554 multiple pseudopods in different directions. *Physarum polycephalum* creates short paths by
555 spreading a fine network of cytoplasm tubes over an area with multiple food sources, then
556 collapsing the tubes that are not in contact with food (Nakagaki et al., 2004). Cytoplasm from
557 collapsed tubes are then re-allocated to strengthen tubes that are in contact with the food and
558 create a stable network (Nakagaki et al., 2004).

559 We measured the length of the networks created by plasmodia between three nodes placed at the
560 vertices of an equilateral triangle to assess network-building ability. We used a 55 mm diameter
561 Petri dish filled with 1.5% agar with an acetate sheet cut out surface to create a circular arena
562 of 25 mm diameter (Figure 1.4). Three plasmodial fragments (0.035 – 0.065 g) were placed in
563 the centre of the arena and acclimated for 4 to 18 h until the fragments had fused to form a
564 single plasmodium of 25 mm diameter. Acclimation time varied widely and 50 plasmodia were
565 prepared for each strain with the aim of having at least 20 replicates for each strain. Plasmodia
566 were selected opportunistically to start experiments (when they had grown to 25 mm diameter),
567 which lead to a range of sample sizes ($n = 20 \pm 5$, Table 1.1) The large range in acclimation time
568 is due to the fluctuating health of plasmodia through the experiment. The acetate sheet was

569 used as an aversive surface to discourage plasmodia from leaving the arena. After plasmodia
570 had fused, we added three 2% w/v oat agar food cubes of 5 x 5 x 3 mm at the vertices of an
571 equilateral triangle. We removed the Petri dish lids and kept the plates in a dark booth at 21°C
572 for 72 h. We used a webcam (Logitech camera) which captured an image every 15 minutes.
573 The plates were illuminated from below for 10 seconds to capture the image.

Table 1.1: Sample size of network-building assay experiments.

Age	Tu111xAI35-H	Tu9xDP89-S
39	0	2
40	20	18
62	18	22
93	21	23
125	22	22
151	22	23
186	22	23
215	24	20
242	23	22
272	22	21
306	0	17
307	15	0

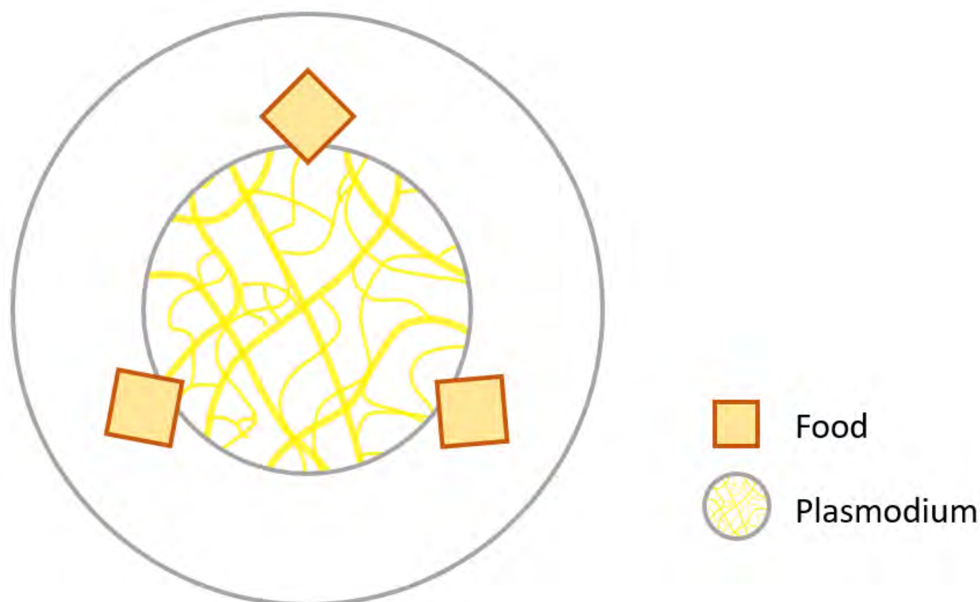

Figure 1.4: Experimental set up of network-building assay. Fragments of plasmodia were grown in the centre of the arena to create a single circular plasmodium. Three food sources were placed at the vertices of an equilateral triangle on top of the circular plasmodium.

Over the course of the experiment, plasmodia consumed the food cubes and created networks between the three points. We defined a network as the first stable configuration that the plasmodium formed for at least three hours. We used ImageJ (Rasband, 1997) to measure the total length of the visible network that plasmodia created between the three food wedges. Network length did not include the food wedges, and followed the path of the plasmodial tubes as closely as possible.

Microscopy

To observe intracellular changes due to age in plasmodia, we measured nuclear size and roundness using fluorescence stained plasmodia. We subcultured fragments of plasmodia from malt extract agar plates onto 1% agar and fed them a small amount of oat flakes. We used plasmodia grown on 1% agar instead of malt extract agar because the cytoplasm tubes of plasmodia on malt extract agar were often too thick and the fluorescent dye could not reliably stain the nuclei. 1cm^2 fragments of both search front and mid-section of both strains were cut to be imaged approximately every 30 days (Figure 1.1). We used Hoechst 33342 (NucBlue Live ReadyProbes Reagent, Invitrogen), a selective nuclei dye that had been diluted in solution of 1 drop per 500 μl mqH2O. 150 μl of the dye solution was added to the surface of the plasmodia (face down), and fixed for 15 minutes. mqH2O was used to rinse off the dye solution five times. The dye was fixed using 4°C 90% ethanol for 10 minutes. The entire fragment, including the agar, was submerged in ethanol, then rinsed three times with mqH2O.

Nuclei were imaged at 405 nm using Nikon A1R confocal microscope at 40X water magnification. Four points of viable material were randomly selected from each sample and imaged using ND2 image acquisition software. For each point, a Z-stack of 5 μm depth at 0.25 μm intervals was used to image the middle of the fragment of a plasmodium. All images in the Z-stacks were deconvolved with Huygens Professional version 21.10 (Scientific Volume Imaging, The Netherlands, http://svi.nl), using the CMLE algorithm, with manual absolute background mode: 15, and 40 iterations. We used FIJI (Schindelin et al., 2012) to create a maximum projection image from the deconvolved Z-stack to maximise the probability of imaging the midsection of nuclei.

601 Gaussian blur at sigma 1.5 was used to filter the maximum projection images. Then we created
602 an auto-threshold montage to compare different methods of thresholding for each maximum pro-
603 jection image. We identified the most suitable auto-threshold method for each sample to create
604 a binary image. Lastly we used the binary watershed function to separate blocks of overlapping
605 nuclei. We used the "Analyze Particles" function to select shapes between 5 - 30 micron^2 and
606 0.70 - 0.999 circularity, this process filtered out non-nuclei artefacts in the images. Circularity
607 of shapes are calculated as 4pi*(area/perimeter^2) and a value of 1.0 indicates a perfect circle
608 (Schneider et al., 2012). We measured the area and circularity of the selected nuclei and also
609 counted how many nuclei could be found within the plasmodial biomass in each image.

610 **mtDNA isolation**

611 **Sample Collection** After growth assays, a random number generator was used to select ten
612 replicates from each strain. Plasmodia from these randomly selected replicates were scraped
613 from the oat agar and placed into 1.5 ml microtubes and snap frozen using liquid nitrogen.
614 Frozen plasmodia samples were kept in a -80°C freezer until further analysis.

615 **mtDNA enrichment and isolation** A novel protocol was developed to isolate mtDNA from
616 frozen plasmodia samples (detailed protocol included in supplementary materials). We studied
617 mtDNA genetic variation in plasmodial replicates across three age-points, 32, 214 and 305 days,
618 for both strains. Briefly, we enriched mitochondria by dounce-homogenising 0.3 g of plasmodial
619 samples (pooled across ten replicates per age point per strain) in mitochondrial isolation buffer.
620 The homogenate was transferred to a fresh 1.5 ml microtube and centrifuged at 1.4 RCF for
621 5 minutes at 4°C. We transferred the supernatant to fresh tubes and further centrifuged the
622 samples at 9 RCF for 10 minutes at 4°C. The resulting pellet containing mitochondria was
623 immediately used for mtDNA isolation following the instructions provided by the QIAamp
624 DNA minikit (QIAGEN). The DNA samples were stored at -80°C until further processing.

625 **Mitochondrial genome sequencing and analysis** The mtDNA samples were sequences
626 using MiSeq v3 2x300bp sequencing chemistry at Ramaciotti Centre for Genomics (NSW, Aus-

627 tralia). We obtained approximately 1 million reads per sample. After quality control of the
628 paired-end reads, we used the de novo assembly method (in Geneious software) to build contigs
629 of predicted mitochondrial genome size of *P. polycephalum*, which is around 65 kb. We then
630 uploaded the assembled mtDNA sequences to the MITOS webserver (http://mitos.bioinf.uni-
631 leipzig.de/index.py) to extract gene annotations using Mold genetic code. We further anal-
632 ysed the mtDNA sequences using comparative genomics approach to estimate pairwise genetic
633 difference betweem strains in Geneious and MEGA v11 bioinformatic software.

634 **Statistical analysis**

635 To analyse cell health level we used a general additive model (GAM) as the data were non-linear.
636 We specified a poisson distribution with logit-link and used cell health level as the response,
637 strain as a fixed effect and individual smooth terms for age for each strain.

638 Similarly, we used a GAM to analyse growth area, specifying a gamma distribution with logit-
639 link as the data were right-skewed and positive. We used the surface area of the plasmodial
640 cell after 48 h of growth as the response variable, strain as a fixed effect and individual smooth
641 terms for age for each strain.

642 For the speed assay we analysed the mean speed and the number of branches produced by
643 a plasmodium. To analyse mean speed we used a linear model with a gaussian distribution.
644 We used mean speed of the branches of a plasmodium as the response, and age and strain as
645 explanatory variables. To analyse the number of branches produced by a plasmodium, we used
646 a generalised linear model (GLM) with a Conway-Maxwell-Poisson distribution as the data were
647 under-dispersed. We used number of branches produced by a plasmodium as the response, and
648 age and strain as explanatory variables.

649 Due to the bimodal distribution of network-building data, we categorised network length of
650 plasmodia into short, medium and long. Networks shorter than 50 mm were classified as short,
651 networks equal to and between 50 to 100 mm were classified as medium, and networks longer
652 than 100 mm were classified as long. To analyse this data we used a multinomial logistic

regression with network length as a categorical response variable, and age and strain as the explanatory variables.

We collected data on nuclei circularity, area and density using fluorescence microscopy images. To analyse nuclei circularity we used a GLM specifying a binomial distribution. We classified nuclei that were between 0.9 to 1.0 in circularity as "regular" and nuclei that were less than 0.9 in circularity as "irregular", and used this as our response variable. We used age, strain and section (search front or mid-section) as our explanatory variables. As we had used a maximum image projection on our image stack, we were able to analyse the widest section of each nucleus. We used the area of the widest point of each nucleus as a measure of nucleus area. To analyse nucleus area we used a GAM and specified a gamma distribution with logit-link as the data were right-skewed and positive. We used nucleus area as the response, strain and section as fixed effects and individual smooth terms for age for each strain. To analyse nucleus density, we calculated the number of nuclei found within each sample. We used a linear model with gaussian distribution to analyse this data, using nucleus density as the response and age, strain and section as the explanatory variables. When testing model fit we identified two outliers which we excluded from the data.

For all statistical analysis we used R (version 4.2.1) and the packages 'mqcv' (Wood and Wood, 2015) for GAM, 'nnet' (Ripley et al., 2016) for multinomial logistic regressions. We used the package 'DHARMa' (Hartig, 2020) to create diagnostic plots for each model, and visually confirmed model assumptions.

1.4 Results

Observations of health in culture

We observed fluctuations in the cell health of plasmodia cultured on malt extract agar (Table 1.2). There were roughly three peaks in cell health as the plasmodia of both strains aged (Figure 1.5). The main difference between the two strains was a slight dip in cell health at approximately 90 days in Tu9xDP89-S which was not seen in Tu111xAI35-H (Figure 1.5). We

679 observed a senescence-like event in Tu9xDP89-S plasmodia cultured on malt extract agar. When
680 plasmodia of Tu9xDP89-S were 87 days of age, we noticed that plasmodia were unable to cover
681 the malt extract agar and the growth forms were small and compact. When the plasmodia
682 were recultured onto new malt extract agar (as prolonged growth on the same nutrient agar
683 can lead to mould or bacterial contamination), plasmodia grew minimally and appeared to
684 completely senesce at 93 days (Figure 1.6). Pigment from the plasmodia appeared to leach out
685 onto the surrounding agar and the plasmodia itself had a pale lace-like appearance (Figure 1.6).
686 After 6 days, at 99 days of age, small plasmodia appeared to grow out of senesced cell matter
687 (Figure 1.6). By 102 days of age, we were able to subculture all plasmodial cells onto fresh malt
688 extract agar agar plates which then continued to grow in a healthy state. This phenomenon of
689 resurrection from senesced cell matter has been observed in a previous study of *P. polycephalum*,
690 where plasmodia were observed cycling between healthy growth and "senescence" multiple times
691 until eventual death (Hu et al., 1985). We cannot confirm whether this senescence-like event
692 was a natural progression of this strain's ageing, or if there was an external stressor that caused
693 this rapid decline in cell health level. Plasmodia from strain Tu9xDP89-S experienced a possible
694 stress event at 60 days of age, where bacterial contamination was found on cultures of plasmodial
695 cells, although healthy growth was observed for over 20 days after contamination and cannot
696 be linked directly to the senescence-like event. In addition, a similar contamination event
697 occurred at 289 days of age, where all plasmodial cell cultures of the strain Tu9xDP89-S were
698 contaminated by mould but no senescence-like event was observed until the death of the strain
699 at 621 days. Although plasmodia of Tu111xAI35-H also experienced decreases in cell health in
700 malt extract agar culture, this strain had no senescence-like events until the death of the strain
701 at 628 days.

702

Table 1.2: Summary of general additive model for cell health level

A. parametric coefficients	Estimate	Std. Error	t-value	p-value
(Intercept)	0.9812	0.0179	54.9567	< 0.0001
B. smooth terms	edf	Ref.df	F-value	p-value
Age:Tu111xAI35-H	8.4444	8.9131	137.7888	< 0.0001
Age:Tu9xDP89-S	8.6216	8.9586	241.5610	< 0.0001

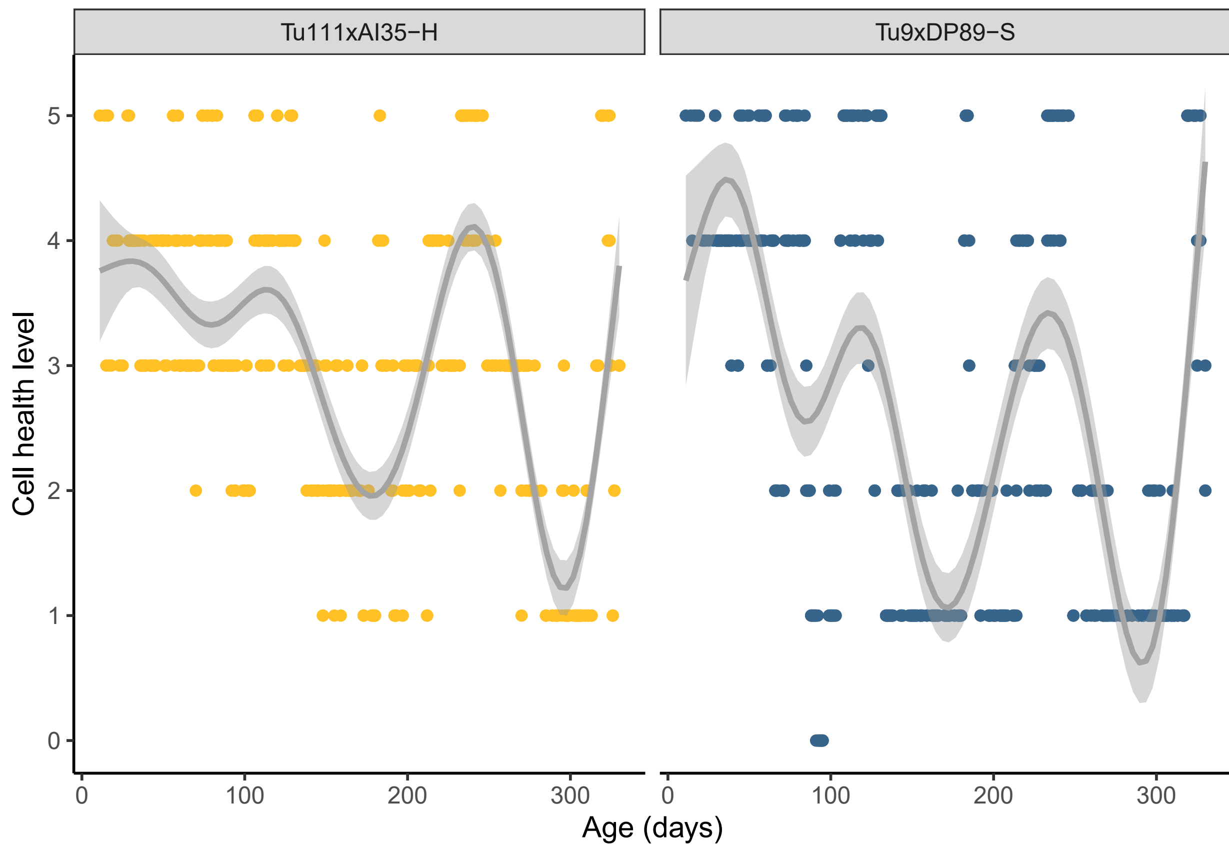

Figure 1.5: Cell health level of plasmodia cultured on malt extract agar. Smooth function fitted with a general additive model and 95 percent confidence interval for each strain are included. N = 1249.

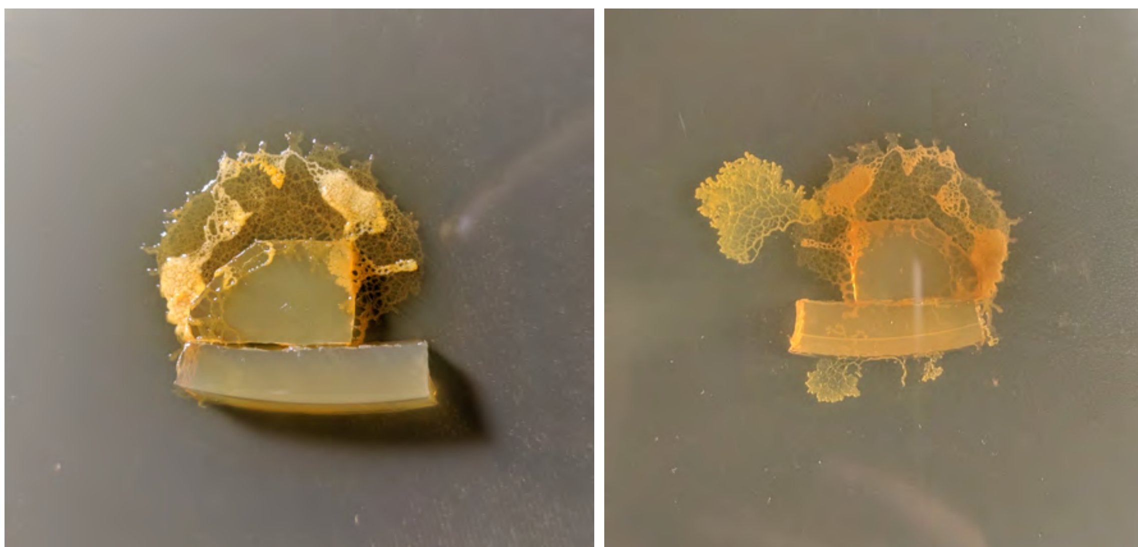

Figure 1.6: Left: Example of senescence in plasmodia of Tu9xDP89-S at 93 days. Right: Example of recovery of plasmodia of Tu9xDP89-S at 99 days.

703 **Growth assay**

704 We found fluctuations in growth area as the strains aged (Table 1.3). Both strains had three
705 peaks where growth area in the assays increased, although the first peak appeared slightly
706 earlier in Tu9xDP89-S (Figure 1.7). The fluctuations in growth area can be contextualised by
707 the fluctuations in cell health level that we observed in plasmodia cultured on malt extract agar
708 (Figure 1.7). Plasmodial cultures on malt extract agar of Tu9xDP89-S had a senescence-like
709 event at 93 days, which may have caused growth area in the assay to decrease earlier than
710 Tu111xAI35-H. We found no difference in growth area between the two strains (Table 1.3).

711 Table 1.3: Summary of general additive model for growth area in growth assay

A. parametric coefficients	Estimate	Std. Error	t-value	p-value
(Intercept)	5.5818	0.0198	282.5178	< 0.0001
B. smooth terms	edf	Ref.df	F-value	p-value
Age:Tu111xAI35-H	8.8009	8.9892	33.8948	< 0.0001
Age:Tu9xDP89-S	8.9129	8.9979	47.4806	< 0.0001

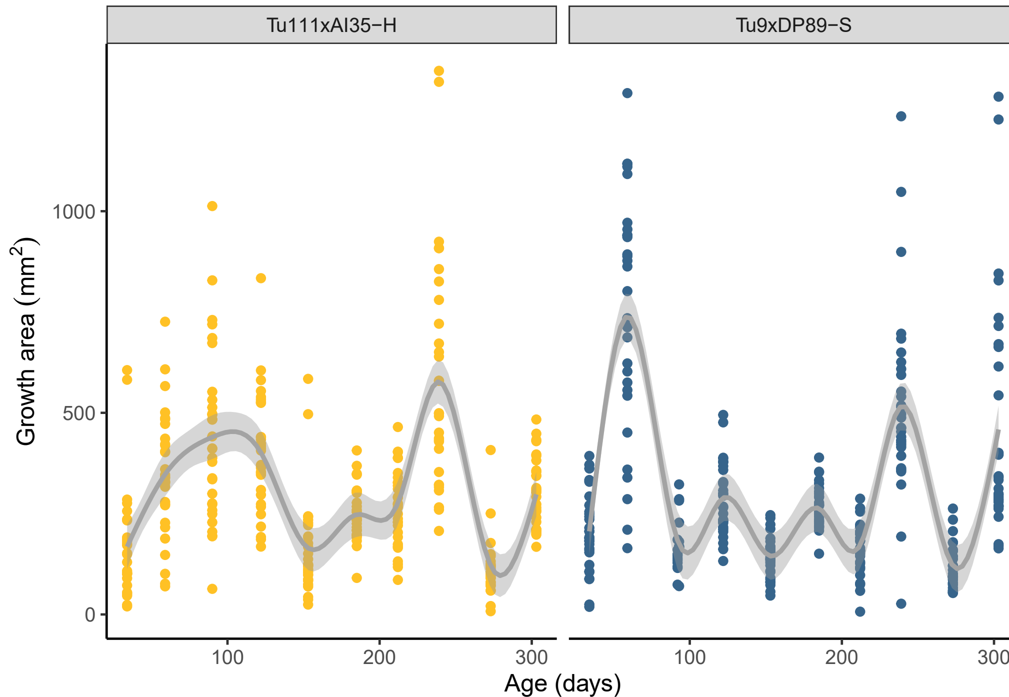

Figure 1.7: Growth area of plasmodia in growth assays. Smooth function fitted with a general additive model and 95 percent confidence interval for each strain are included. N = 588

712 **Speed assay**

713 Contrary to our predictions, we found that the mean speed of both strains became faster with
714 increasing age (estimate ± SE = 0.01 ± 6.97e-04, $P < 0.01$; Figure 1.8). We also found a
715 significant difference in the mean speed between the two strains, with plasmodia of Tu9xDP89-
716 S strain being faster than that of Tu111xAI35-H strain (estimate ± SE = 0.88 ± 0.12, $p<0.01$;
717 Figure 1.8). Interestingly, both strains had a similar mean speed at the beginning of our
718 experiment (age = 34, T-stat = 0.69, $P = 0.50$), but Tu9xDP89-S was significantly faster than
719 Tu111xAI35-H by the end of the experiment (age = 303, T-stat = 6.75, $P < 0.01$; Figure 1.8).

720 We calculated the number of branches the plasmodia produced in the speed assays as a measure
721 of behaviour. We found that plasmodia produced fewer branches with increasing age, although
722 this effect was weak (estimate ± SE = 1.00 ± 1.95e-04, $P < 0.01$; Figure 1.9). This result,
723 in combination with the increase in mean speed, may suggest a switch to an intensive search
724 behaviour as plasmodia become older. We also found a difference between strains where plas-

725 modia of Tu9xDP89-S produced fewer branches compared to Tu111xAI35-H (Estimate ± SE =
726 0.75 ± 2.57e-02, $P < 0.01$; Figure 1.9).

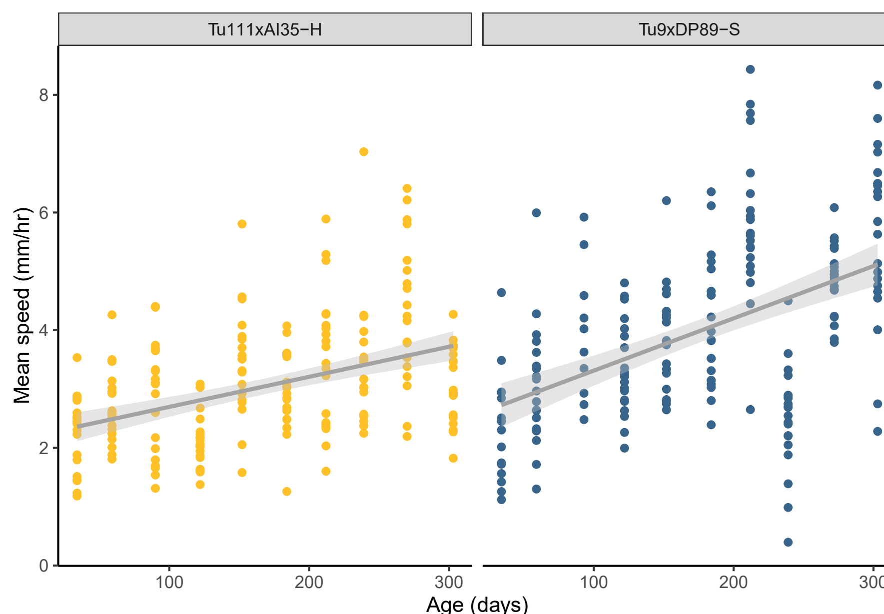

Figure 1.8: Mean speed of plasmodial branches in speed assay for each strain. Linear regression lines with 95 percent CI are included. N = 387.

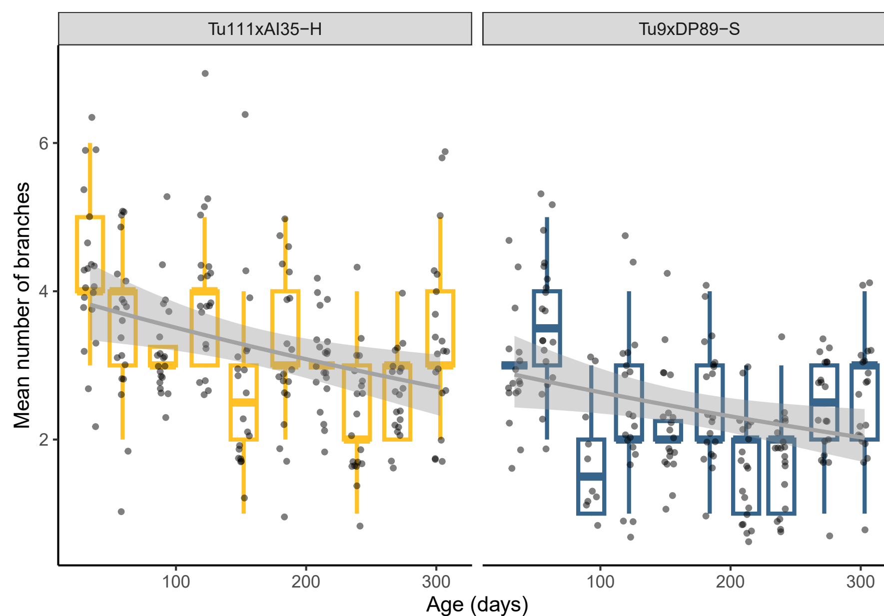

Figure 1.9: Mean number of branches produced by plasmodia in speed assay. Smooth function fitted with a generlised linear model and 95 percent CI for each strain are included. N = 387.

727 **Network-building assay**

728 We found no change in network-building ability with increasing age, where the likelihood that
729 plasmodia would produce a medium or long network over a short network did not change with
730 age (RR = 1.00, P = 0.07; RR = 1.00, P = 0.03; respectively). However, we did find a weak
731 effect of strain on the network length, where plasmodia from strain Tu9xDP89-S were more likely
732 to have a medium network length rather than a short network length compared to plasmodia
733 from strain Tu111xAI35-H (RR = 1.61, P = 0.03).

734 **Microscopy**

735 Due to the nature of plasmodial cells, there were a large number of nuclei imaged in each sample
736 of plasmodia. We determined whether nuclei became more or less circular by splitting the nuclei
737 into two groups based on circularity. We found that the proportion of circular nuclei did not
738 change with increasing age (RR = 1.00, P = 0.68; Figure 1.10). There was a weak effect of
739 strain where Tu9xDP89-S had a lower proportion of circular nuclei (RR = 0.93, 95% CI = 0.89
740 – 0.99, P < 0.01; Figure 1.10). We also found a weak effect of the section of plasmodia that
741 the sample was taken from where there were higher proportion of circular nuclei in plasmodia
742 taken from the search front (RR = 1.17, 95% CI = 1.11 – 1.23, P < 0.01; Figure 1.10). We used
743 area as another measure of nuclei shape, where the area of the widest slice of the nuclei was
744 measured. We found fluctuations in nuclei area (Table 1.4). The fluctuating pattern of nuclei
745 area of Tu111xAI35-H plasmodia had three peaks, similar to the pattern found in growth area
746 and health level (Figure 1.11). In contrast, the pattern seen in Tu9xDP89-S only had one peak
747 at 156 days of age (Figure 1.11). Nuclei density decreased with increasing age, but the trend
748 was linear (Estimate ± SE = -2.79 ± 1.30, P = 0.03; Figure 1.12). We found no effect of strain
749 or section for nuclei density (Estimate ± SE = -219.47 ± 227.38, P = 0.34; Estimate ± SE =
750 -268.08 ± 227.36, P = 0.24; respectively).

751 Table 1.4: Summary of general additive model for nucleus area in microscopy analysis

A. parametric coefficients	Estimate	Std. Error	t-value	p-value
(Intercept)	2.5564	0.0043	600.3274	< 0.0001
sectionSF	0.0260	0.0057	4.5770	< 0.0001
B. smooth terms	edf	Ref.df	F-value	p-value
Age:Tu111xAI35-H	8.7667	8.9829	84.0799	< 0.0001
Age:Tu9xDP89-S	8.7597	8.9826	83.2404	< 0.0001

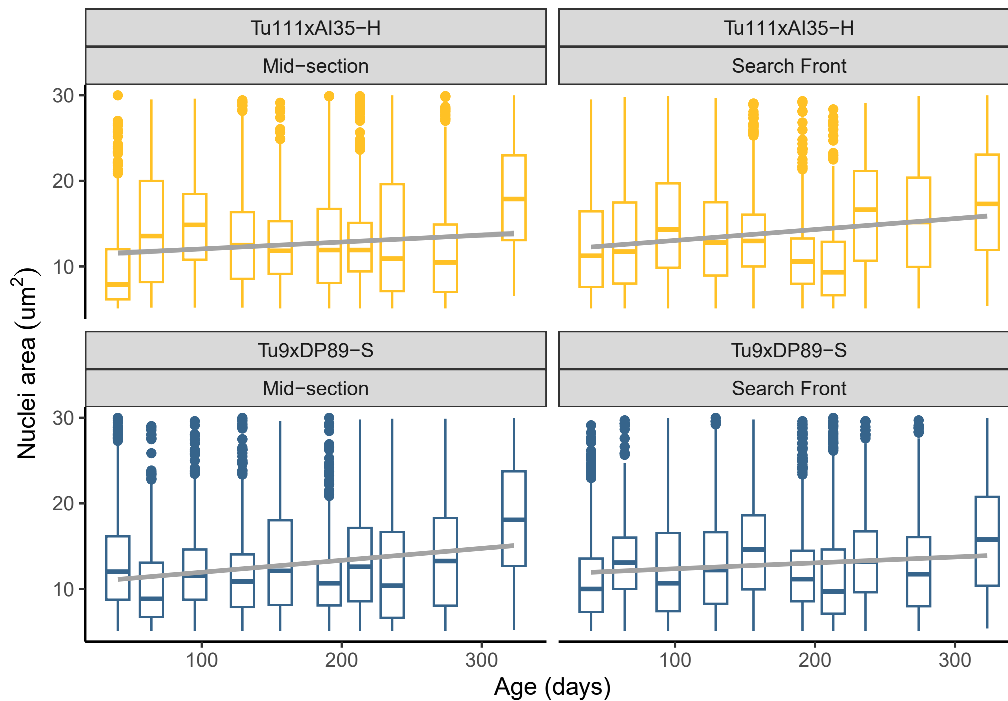

Figure 1.10: Nucleus circularity of ageing plasmodia in microscopy analysis. Smooth function fitted with a linear model and 95 percent confidence interval for each strain and section are included. N = 25862.

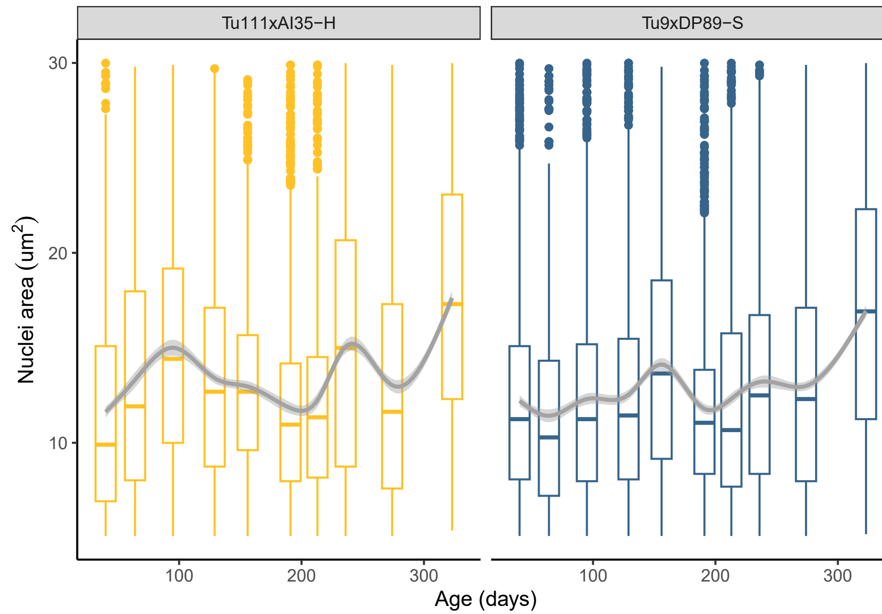

Figure 1.11: Nucleus area of ageing plasmodia in microscopy analysis. Smooth function fitted with a general additive model and 95 percent confidence interval for each strain are included. N = 25862.

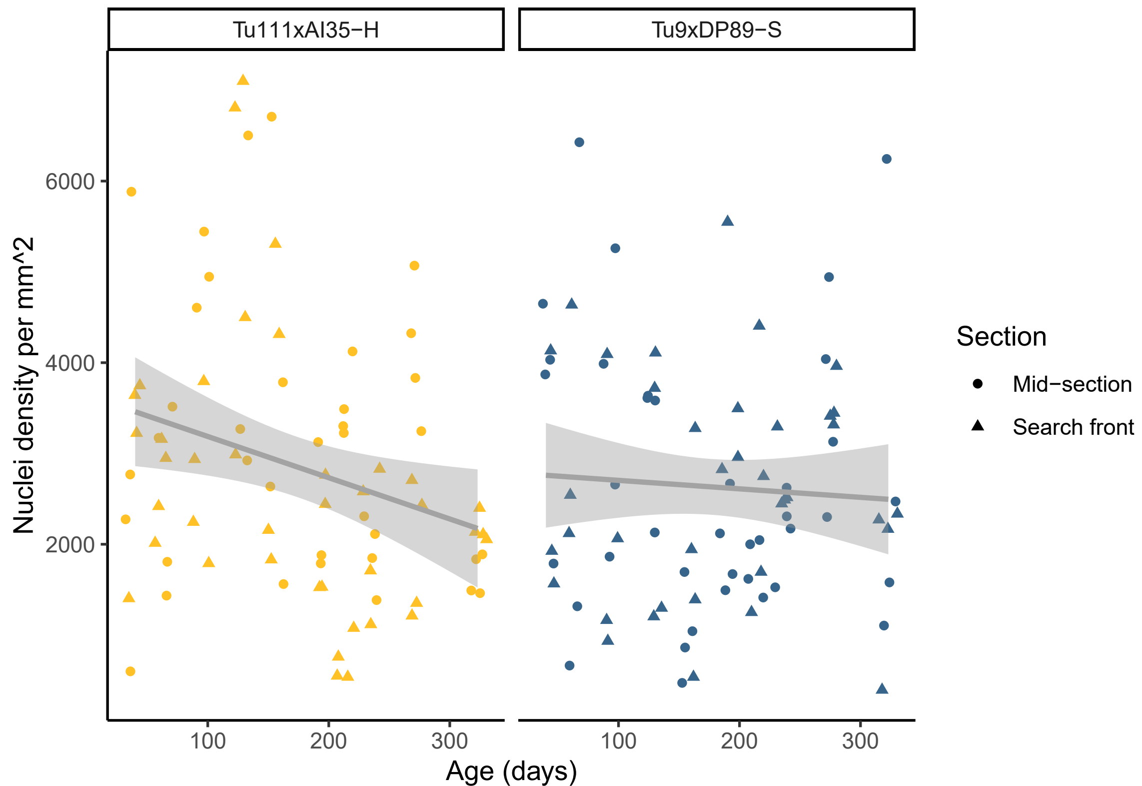

Figure 1.12: Density of nuclei in ageing plasmodia in microscopy analysis. Smooth function fitted with a generlised additive model and 95 percent confidence interval for each strain are included. N = 154.

752 **mtDNA analysis**

753 We predicted that the genetic difference between mtDNA samples would increase as plasmodia
754 age due to accumulation of mutations in mtDNA due to age. We isolated mtDNA from plas-
755 modia at three age-points, 32, 214 and 305 days old. These ages represented young, middle-
756 aged and old plasmodia respectively. We found comparable pairwise genetic distance between
757 young and middle-aged plasmodia and young and old plasmodia of Tu9xDP89-S strain (1.030
758 and 1.029, respectively). Comparatively, the genetic distance between middle-aged and old
759 plasmodia of Tu9xDP89-S was small (0.002). This suggests that mtDNA sequences changed
760 dramatically before Tu9xDP89-S plasmodia were middle-aged and then became stable with few
761 changes after this age point. In contrast, we found fluctuations in genetic difference with age in
762 Tu111xAI35-H where we found comparable pairwise genetic distance between young and middle-
763 aged plasmodia and middle-aged and old plasmodia (1.008 and 1.010, respectively. The genetic
764 distance between young and old plasmodia was comparatively smaller (0.001), suggesting that
765 any genetic changes that occurred in mtDNA sequences between the young and middle-aged
766 plasmodia reverted back when plasmodia of Tu111xAI35-H were old. A possible mechanism
767 may be that mitochondria with different mtDNA sequences exist in different proportions within
768 the cell. When plasmodia recover from a period of low growth, this may select for mitochondria
769 with young mtDNA sequences to be replicated at a higher rate, thus causing the low genetic dif-
770 ference between young and old plasmodia.Further research on genetic changes in mitochondria
771 are necessary to examine this observation.

772 1.5 Discussion

773 Cell health levels of ageing plasmodia were non-linear and fluctuated during the first 330 days
774 of their lifespan. The general additive model showed two drops in cell health level for both
775 strains, followed by periods of improved cell health (Figure 1.5). Notably, Tu9xDP89-S strain
776 had a significant event at 93 days of age, where we observed what appeared to be senescence
777 in all plasmodial cells cultured on malt extract agar (Figure 1.5). At 99 days of age, plasmodia
778 began to grow from the senesced cell fragments and grew vigorously (Figure 1.5). This pattern

of senescence and revival has been observed in a previous study of *P. polycephalum*, where plasmodial cells would fragment into small spherules which would then revive after several days of dormancy to produce new, healthy plasmodia (Hu et al., 1985). Hu and colleagues (1985) observed the cycle of senescence and vigorous growth repeating a number of times before death finally occurred. In our study, this behaviour of cell fragmentation and dormancy was only observed once in plasmodia of Tu9xDP89-S strain, although both strains underwent cyclical periods of vigorous cell growth followed by low cell health levels (Figure 1.5).

In our growth assays, we found fluctuations in growth area (Figure 1.7) which appeared to echo the patterns seen in cell health levels on malt extract agar cultures (Figure 1.7). The main deviation can be observed in Tu9xDP89-S, where cell health level improved after the senescence event at 93 days of age but growth area remained low in growth assays. It is expected to observe similarities in cell health levels and growth area in assays as slower growth rate is one of the key traits used to describe a decrease in cell health due to ageing. We based our descriptions of cell health level on observations made by Poulter (1969), where traits such as smaller growth forms and slower growth rates were used to describe a decrease in health in ageing *P. polycephalum*.

Interestingly, measurements of mean speed of plasmodia in speed assays did not show fluctuations (Figure 1.8). Instead, we found that the mean speed of plasmodia became faster with increasing age. This result contradicts our prediction that plasmodia would slow down with increasing age due to decreased cytoplasmic streaming. Plasmodia move by directing a higher volume of cytoplasm towards their desired direction, often towards a chemical attractant (Durham and Ridgway, 1976). If movement speed was effected by cytoplasmic streaming, we would expect movement speed to slow down when plasmodia are older or have low health levels. We can further contextualise our result of faster movement speed in ageing plasmodia by including our observations of plasmodia behaviour in speed assays.

Age had a significant effect on plasmodia behaviour in speed assays, where plasmodia produced fewer branches with increasing age. Acellular slime moulds can search in multiple directions by growing branches of pseudopods. In the speed assay, older plasmodia produced fewer branches

806 when searching and the mean speed of these branches became faster. In previous experiments of
807 ageing acellular slime moulds, spindly growth was seen when plasmodia were reaching senescence
808 (Poulter, 1969). In our experiments, age-related stress may have caused plasmodia to allocate
809 their limited resources to search in fewer directions at a faster speed, rather than producing
810 many branches that moved slowly to compensate for less efficient movement of cytoplasm or
811 cell components.

812 In our experiment, plasmodia appeared to switch from a slow moving to a fast moving search
813 strategy, which is a pattern often observed in foraging animals. Benhamou (1992) described
814 this pattern as area restricted search, where an animal that finds a patch of food will decrease
815 movement speed and increase their turn rate. This intensive search strategy allows for a more
816 thorough search of an area where food was found previously, increasing the probability of finding
817 more food. After a period of unsuccessful foraging using the intensive search strategy, the
818 animal will switch to an extensive search strategy, which involves faster movement speed with
819 less turning in search of the next patch of food. *Physarum polycephalum* has been observed
820 using intensive and extensive search strategies, where plasmodia that had fed from high quality
821 food used an intensive search strategy for a longer period, whereas plasmodia that fed from
822 low quality food switched to an extensive search strategy faster (Latty and Beekman, 2009).
823 Plasmodia in our speed assay were placed in an empty arena after being fed on oat flakes. It is
824 possible that aged plasmodia were less efficient at foraging on the oat flakes before the assay, or
825 had less energy reserves during the speed assay which caused plasmodia to utilise an extensive
826 search strategy, and search for food at a faster speed and producing fewer branches.

827 Interestingly, there was no affect of age on the network building ability of plasmodia. Under the
828 assumption that age-stressed plasmodia would need to conserve resources, we predicted that
829 ageing plasmodia would make short networks. Although longer networks in our experiments
830 were more resilient, as there are more connections between the food sources, they require more
831 energy and biomass. In comparison, short networks allow plasmodia to feed from multiple food
832 sources while conserving biomass and resources. In actuality, age did not drive differences in

833 whether plasmodia made medium or long networks over short networks. Although the precise
834 physiological mechanism behind network construction in acellular slime moulds is still unclear,
835 perhaps the ability for plasmodia to connect food sources can withstand the pressure of ageing
836 better than the ability to extensively search for food.

837 Notably, our experiments were halted when plasmodia were 330 days old due to disruptions
838 caused by the COVID-19 pandemic. Both strains of *P. polycephalum* had a lifespan of over
839 600 days, which means that our experimental assays were only able to capture the first half of
840 the their lifespan. This may also explain some of our results such as faster mean speed with
841 increasing age or the absence of age effect in the network building assays. It is possible that
842 the pattern of mean speed over the entire lifespan of plasmodia is an inverted U-shape where
843 mean speed becomes faster until a critical point and then begins to slow down as the plasmodia
844 reaches final senescence. In addition, plasmodia may make shorter networks before senescence
845 when the requirement to conserve biomass and resources becomes more vital. We would like to
846 highlight that plasmodial cultures of Tu111xAI35-H died at 628 days old and Tu9xDP89-S died
847 at 621 days old, which is three times longer than the maximum observed lifespan in previous
848 ageing research of this species (Hu et al., 1985). Other strains of *P. polycephalum* have observed
849 lifespans from 85 days up to 235 days (Abe et al., 2000; Hu et al., 1985; McCullough et al.,
850 1973), so the long lifespan of our two strains may point to a genetic difference in lifespan between
851 strains.

852 We were able to observe cellular changes in both strains of *P. polycephalum* in our experiment.
853 We found no changes in nucleus circularity with increasing age, but we did find fluctuations
854 over age in nucleus area using a general additive model (Figure 1.11). Although there are some
855 similarities in fluctuation in the latter half of the experiment, the trends seen in nucleus area do
856 not appear to mirror cell health (Figure 1.5) as closely as growth area trends in our growth assays
857 (Figure 1.6). We also found that nucleus density decreased linearly with increasing age (Figure
858 1.12). Previous studies found that the proportion of non-circular and larger nuclei increased
859 in ageing plasmodia, and that these traits indicate that the nuclei were becoming polyploid

860 (Clark and Mulleavy, 1982; McCullough et al., 1973). Experiments with *D. iridis* showed that
861 filtering larger nuclei from older plasmodia increased their lifespan (Clark and Hakim, 1980).
862 Fluctuations in nucleus area in our experiments may suggest that *P. polycephalum* possess a
863 mechanism that allow them to decrease the proportion of larger nuclei in their plasmodia.

864 A possible mechanism to decrease the proportion of larger nuclei or reduce accumulation of
865 damage in plasmodia is by decreasing growth rate. Plasmodia of *P. polycephalum* have been
866 shown to have an apparently immortal form when kept as microplasmodia in shaken cultures
867 that constantly fragment the cell in liquid media (Hosoda, 1980; Hu et al., 1985). Fragmentation
868 in liquid media allows newer, healthy cell matter to replicate at a faster rate, leading to longer
869 lifespans in shaken cultures (Hu et al., 1985). Plasmodia on axenic cultures may be able to
870 sequester damaged cell components in an unhealthy cell and re-grow the plasmodia rapidly
871 from spherules where only healthy cell components remain (Hu et al., 1985). Furthermore,
872 ageing or stressed plasmodia have been observed splitting into multiple plasmodia (Lee et al.,
873 2018; Poulter, 1969). This may be another mechanism in which acellular slime moulds sequester
874 damaged cell components.

875 Simple cells have been observed sequestering damaged cell components in one half when replicat-
876 ing (Moger-Reischer and Lennon, 2019; Ogrodnik et al., 2019). This asymmetric reproduction
877 can be used as an alternative to cellular repair, where damage can be attached to a specific
878 cellular structure and segregated to one daughter cell, rather than diluting the damage between
879 both daughter cells (Ogrodnik et al., 2019). Such asymmetric reproduction has been shown
880 in other single-celled organisms including bacteria such as *Caulobacter crescentus* (Ackermann
881 et al., 2003), *Escherichia coli* (Stewart et al., 2005) and fungi such as *Schizosaccharomyces*
882 *pombe* (Barker and Walmsley, 1999). It is possible that we observed this mechanism during the
883 senescence-like event of Tu9xDP89-S at 93 days of age. Additionally, fluctuations in growth
884 area and health level may be due to plasmodia temporarily decreasing growth area when dam-
885 aged cell components accumulated in plasmodia, slowing the exponential accumulation of more
886 damage, and then increasing growth rate once healthy cell components had been replicated.

In addition, we found changes in mtDNA sequences of plasmodia at three age-points. Differences in Tu9xDP89-S mtDNA sequences appeared to follow a linear pattern where the genetic distance between mtDNA sequences of young and middle-aged plasmodia were large, but the genetic distance between middle-aged and old plasmodia were small. In contrast, mtDNA sequences of ageing Tu111xAI35-H plasmodia fluctuated, where mtDNA sequences changed greatly between young and middle-aged plasmodia, but then reverted back so that mtDNA sequences between young and old plasmodia were similar. It is possible that mitochondria of *P. polycephalum* also have a repair mechanism that allow mtDNA sequences to regenerate over time. As isolation and analysis of mtDNA in acellular slime moulds is a novel area of research, it is difficult to offer conclusive results on whether the genetic distance between mtDNA sequences of different age-points is due to age-stress. We have found no evidence of accumulation of mutations in the full length mtDNA sequence. Further analysis of specific marker genes within mtDNA may further illuminate how mitochondrial function in plasmodia change with age.

Besides age, we found an affect of strain in many of our physiological and behavioural assays. We found differences between strains in the speed assay such that Tu9xDP89-S produced fewer branches in a plasmodium compared to Tu111xAI35-H. Interestingly, the mean speed of branches in Tu9xDP89-S was faster than Tu111xAI35-H, but Tu111xAI35-H plasmodia produced more branches when searching. The two strains demonstrated two different foraging strategies in the speed assay, where plasmodia of Tu9xDP89-S produced fewer branches that moved quickly, whereas plasmodia of Tu111xAI35-H produced more branches that moved slowly. In addition, we found that the rate of mean speed increase was different between both strains. Both strains started with similar mean speed, but Tu9xDP89-S had a significantly faster mean speed by the end of our experiment. We also found differences between strains in the network-building assay, where Tu9xDP89-S was more likely to build a medium length network over a short network compared Tu111xAI35-H. Intraspecific differences in growth and search strategy has been observed in *P. polycephalum* (Dussutour et al., 2019). Our experiments show another example of behavioural differences between two strains of *P. polycephalum* that had been reared in identical conditions.

In recent years, *P. polycephalum* has been studied extensively as an interesting model for brainless behaviour and problem-solving (Boisseau et al., 2016; Dussutour et al., 2010; Latty and Beekman, 2011b, 2011a, 2010, 2009; Reid et al., 2016; Reid et al., 2012; Saigusa et al., 2008; Smith-Ferguson et al., 2021). Most laboratory experiments re-activate dormant cells of *P. polycephalum* shortly before starting tests, thus the true age of most plasmodial cells are unknown. We have found physiological, behavioural and cellular changes in plasmodia as they age. Future experimental designs should be mindful of plasmodial cell age, especially in long-term experiments, where variability in behaviour may be introduced by age-related stress. Moreover, we have observed non-linear patterns in cell health level, growth area and nuclei area in relation to age in *P. polycephalum*, which suggests that acellular slime moulds have mechanisms of repair for ageing stress. Further investigations on cellular repair mechanisms in single-celled organisms may help develop our understanding of ageing at a fundamental level.

1.6 Acknowledgements

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935 ## 1.7 Supplementary Data

936 ### Protocol for mtDNA enrichment and isolation in *P. polycephalum* plasmodia

937 ### Mitochondria enrichment

938 1. Chill glass Dounce tissue grinder (homogeniser)
939 2. Add 2 ml of mitochondria buffer (referred to as homogenization medium in Dos Santos et
940 al., 2012) to 0.35 g of frozen plasmodia and homogenise
941 3. Transfer homogenised solution into 2 ml tube and centrifuge at 1.4 RCF for 5 minutes at
942 4°C.
943 4. Transfer supernatant to a fresh 2 ml tube and centrifuge at 9 RCF for 9 minutes at 4°C.
944 5. Remove supernatant
945 6. Re-suspend pellet in 200 ul of mitochondria buffer and centrifuge at 9 RCF for 5 minutes
946 at 4°C.
947 7. Remove supernatant and keep on ice
948 8. Make up 1X Cytosol Extraction buffer by mixing 1 ml of the 5X Cytosol Extraction Buffer
949 from abcam Mitochondrial DNA Isolation Kit in 4 ml ddH20.
950 9. Re-suspend pellet in 500 ul of 1X Cytosol Extraction buffer and centrifuge at 9 RCF for
951 9 minutes at 4°C.
952 10. Remove supernatant. The pellet is isolated mitochondria. Keep the pellet on ice.

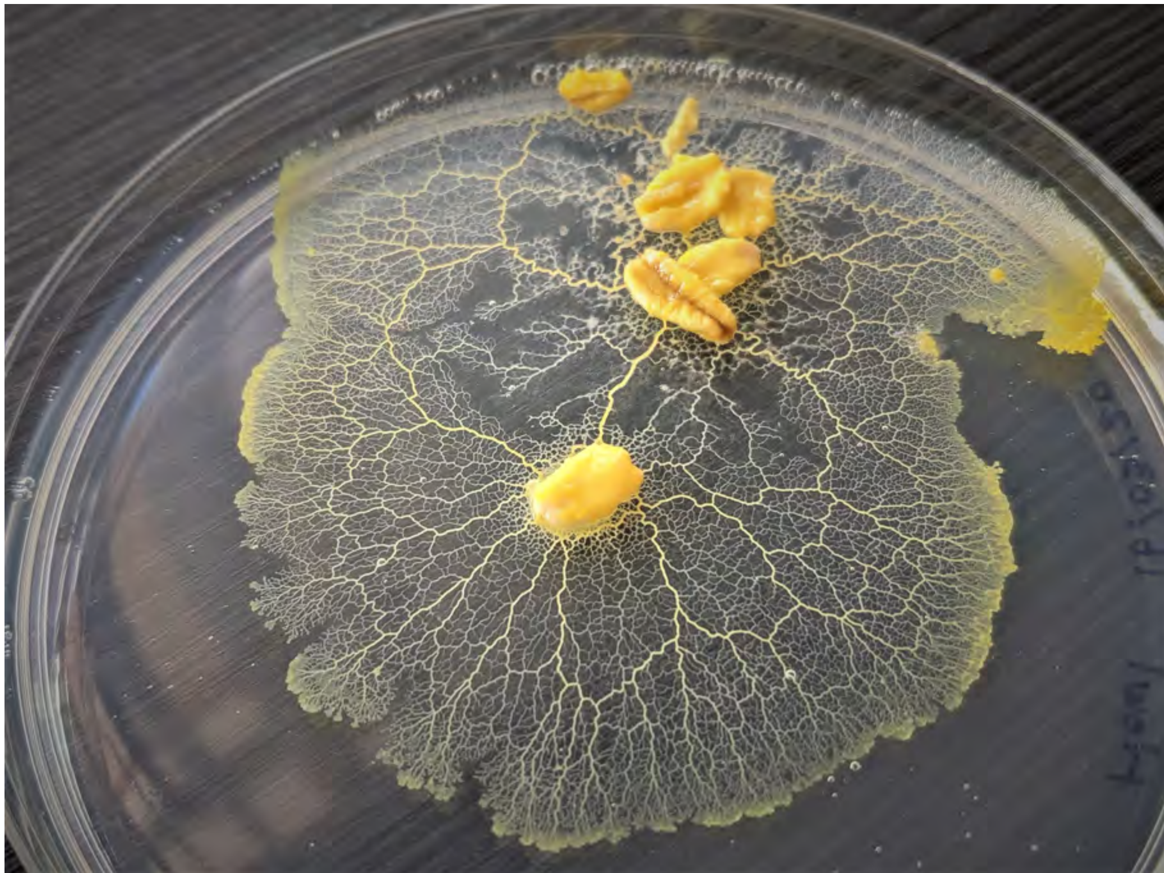
953 ### mtDNA isolation

954 1. Prepare reagents from QIAamp DNA minikit (QIAGEN) as instructed.
955 2. Re-suspend pellet in 180 ul of Buffer ATL and 20 ul of Proteinase K and incubate at 56°C
956 water bath for 15 minutes or until the solution becomes clear.
957 3. Add 2 ul of RNase to the solution and incubate at room temperature for 10 minutes.
958 4. Add 300 ul of Buffer AL to solution, vortex and then incubate at 70°C for 10 minutes
959 5. Add 200 ul absolute ethanol, vortex then briefly centrifuge.
960 6. Pipette the solution into a spin column in a 2 ml collection tube.

7. Add 500 ul Buffer AW1 to spin column and centrifuge at 8 RCF for 1 minute.
8. Transfer spin column into a new 2 ml collection tube and discard old collection tube.
9. Add 500 ul Buffer AW2 to spin column and centrifuge at 24 RCF for 3 minutes.
10. Transfer spin column into a new 2 ml collection tube and centrifuge at 24 RCF for 1 minute.
11. Transfer spin column in a new 1.5 ml tube and pipette 50 ul ultrapure water directly onto the filter.
12. Incubate solution at room temperature for 5 minutes.
13. Centrifuge at 21 RCF for 1 minute and then add another 50 ul ultrapure water directly onto the filter.
14. Incubate solution at room temperature for 1 minute.
15. Centrifuge at 21 RCF for 1 minute then discard spin column.
16. The solution in the 1.5 ml tube is isolated mtDNA. Store at -20°C.

Chapter 2

Speed accuracy trade-offs over the lifespan of *Physarum polycephalum*



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2.1 Abstract

Ageing is an inevitable experience among most organisms, where development and degradation of physical abilities can lead to changes in behaviour. Ageing animals commonly exhibit slower speeds and changes in decision accuracy, and in some animals, the relationship between decision speed and accuracy changes with age. This relationship is called a speed-accuracy trade-off and describes the balancing act of making a quick decision and the ensuing accuracy of that choice. Speed-accuracy trade-offs are not limited to animals and have been observed in brainless organisms such as the acellular slime mould, *Physarum polycephalum*, where faster decision speed was associated with inaccuracy in difficult discrimination tests. Much of the research on behavioural changes due to age focus on animals with brains, even though the majority of life is brainless. *Physarum polycephalum* not only exhibits behaviour such as speed-accuracy trade-offs but also displays physical signs of ageing. We analysed whether speed-accuracy trade-offs changed over the lifespan of two strains of *P. polycephalum.* Over 307 days we repeated a binary discrimination test at two difficulty levels on the *P. polycephalum* strains, Tu111xAI35-H and Tu9xDP89-S, on a monthly basis. SATs were only present at certain ages during the lifespan of the two strains. For Tu111xAI35-H, SATs were present at 62, 215 and 242 days and for Tu9xDP89-S, SATs were present at 125, 215 and 242 days. In contrast to previous research, SATs were only observed in easy tests rather than hard tests. Decision accuracy and decision speed fluctuated over the lifespans of both strains, and the relationship between age and decision accuracy and speed were different between test difficulty and strains. We found a high degree of behavioural variation between two strains of *P. polycephalum* with differences in decision accuracy, decision speed and presence of SATs. In addition, we found that age affected the behaviour of both strains differently. These variations highlight the need for researchers to specify the strain and age of plasmodia used in future behavioural experiments and presents *P. polycephalum* as an exciting model organism to investigate intraspecific behavioural variation in brainless organisms.

2.2 Introduction

Ageing is a fundamental property of most living organisms, and in animals, often causes physiological and cognitive decline (Arking, 2006). Degradation of physiological abilities over lifespan can also lead to changes in behaviour, such as inability to nest effectively in mice (Nolte et al., 2019), less efficient web building in orb-web spiders (Anotaux et al., 2014) and lower response to alarm calls in yellow-bellied marmosets (Moya-Larano, 2002). Conversely, ageing can have positive impacts, for example on foraging behaviour. Foraging abilities improve with age in social wasps (Mandal and Brahma, 2019) and desert ants (Wehner et al., 2004). By accumulating information of their foraging environment over their lifetime, both species are able to improve foraging efficiency as they age (Mandal and Brahma, 2019; Wehner et al., 2004).

Animals foraging for food must often make trade-offs as there are typically multiple sources of food in the environment. Animals must balance tasks, such as gathering information about food quality, consuming food, searching for more food, or finding shelter. Gathering information takes time and although having more information will likely lead to a more nutritionally rewarding decision (Bogacz et al., 2010), the time spent deciding might be better used on other tasks. This relationship between decision time and quality of choice is called a speed-accuracy trade-off (SAT) (Bogacz et al., 2010; Chittka et al., 2009). In humans, physiological and cognitive declines due to age cause shifts in SATs (Salthouse, 1979). Through the adult lifespan, processing speed steadily slows (Deary and Der, 2005; Der and Deary, 2006; Salthouse, 2000) although accuracy either improves or stays the same (Salthouse, 1979). The effect of age on processing speed is further compounded when tasks are difficult, as the brain has to manage and execute more steps to complete a complex task (Hasher and Zacks, 1988; Salthouse, 1996). In mice, accuracy of decision-making changes throughout their lifespan (Gür et al., 2020). When given a perceptual decision-making task, young mice were significantly more accurate than old mice (Gür et al., 2020).

Despite the vast majority of organisms on Earth being brainless, most ageing research focuses on behavioural change caused by neurological decline. Behavioural change is not always caused

1036 by a decline in neurological mechanisms and can often be due to physiological decline instead.
1037 There is evidence that brainless organisms exhibit physiological decline. In old plants, the rate
1038 of root and shoot formation decreases, lowering the ability to intake resources such as nutrients
1039 and light (Gatsuk et al., 1980). In bacteria such as *Escherichia coli*, older cell components are
1040 sequestered to one "mother" cell during binary fission, which leads to slower growth rate in the
1041 mother cell (Gomez, 2010; Moger-Reischer and Lennon, 2019; Stewart et al., 2005).

1042 The acellular slime mould *Physarum polycephalum*, also experiences physiological changes as
1043 they age. Acellular slime moulds are composed of a large multinucleate cell called a plasmodium.
1044 Plasmodia have been studied for their ability to exhibit a range of behaviour such as decision-
1045 making (Dussutour et al., 2010; Latty and Beekman, 2010; Nakagaki et al., 2004; Reid et al.,
1046 2016, 2013; Reid et al., 2012; Reid and Beekman, 2013), habituation (Boisseau et al., 2016;
1047 Vogel and Dussutour, 2016) and speed-accuracy trade-offs (Dussutour et al., 2019; Latty and
1048 Beekman, 2011b). As the acellular slime mould cell ages, growth rate and cytoplasmic streaming
1049 decline, ultimately leading to a loss of yellow pigment and fragmentation as it reaches senescence
1050 (Nakagawa et al., 1998). As cytoplasmic streaming is heavily utilised in the communication of
1051 chemical signals and decision-making of an acellular slime mould (Beekman and Latty, 2015;
1052 Durham and Ridgway, 1976; Ueda et al., 1980), the relationship between speed and accuracy
1053 may also change throughout their lifespan.

1054 In this study, we investigated whether the decision-making behaviour of a brainless organism
1055 changes throughout their lifespans. We used the acellular slime mould, *Physarum polycephalum,*
1056 as a model organism to investigate whether SATs would change over 307 days of their lifespan.
1057 SATs have been demonstrated in acellular slime moulds (Dussutour et al., 2019; Latty and
1058 Beekman, 2011b). In previous studies, acellular slime moulds exhibited SATs in difficult food
1059 discrimination tasks when hunger stressed, where they were more likely to be inaccurate if they
1060 took less time to make a decision (Latty and Beekman, 2011b). Previous longevity experiments
1061 of *P. polycephalum* have shown that lifespans can range from as little as 20 days to up to
1062 200 days (Hu et al., 1985; McCullough et al., 1973; Poulter, 1969). We expected 365 days of

experimentation would be sufficient time to investigate behavioural changes over the acellular slime mould's entire lifespan, but due to the COVID-19 pandemic, we were restricted to 307 days of observations which was still 100 days longer than the previously recorded lifespan. Investigating how age affects foraging behaviour such as decision speed and accuracy in acellular slime moulds will give us insight on age-related behavioural changes in other brainless organisms, which consist of the greater part of organisms on Earth. Furthermore, observing foraging behaviour in more basic organisms may also help us understand the fundamental mechanisms of ageing in animals with brains.

2.3 Methods

Species and rearing conditions

Physarum polycephalum is an acellular slime mould in the Protist kingdom. During its vegetative life stage it exists as a large multinucleate cell, called a plasmodium, that consists of a network of channels that rhythmically stream cytoplasm (Durham and Ridgway, 1976). Contractile actomyosin filaments within the channels are regulated by chemical oscillations that direct the cytoplasm stream towards stimuli via structures called pseudopods (Kobayashi et al., 2006). The cell can be understood as many small oscillating pieces, often called coupled non-linear oscillators, with each piece oscillating at a frequency determined by local energy inputs and feedback conditions (Durham and Ridgway, 1976). The pieces can also be affected by their neighbours, which allows for chemical signals to be transferred in a wave-like manner throughout the cell (Durham and Ridgway, 1976). This positive feedback loop allows acellular slime moulds to accurately choose high quality food (Latty and Beekman, 2011b, 2010), balance their nutritional intake (Dussutour et al., 2010) and change foraging strategies based on their environment (Latty and Beekman, 2009).

We used the same study specimens tha were used in Chapter 1 of this thesis. These two strains of *P. polycephalum*, Tu111xAI35-H and Tu9xDP89-S, were created by mating pairs of myxamoebae following the protocol of Moriyama & Kawano (2003). The four parent strains,

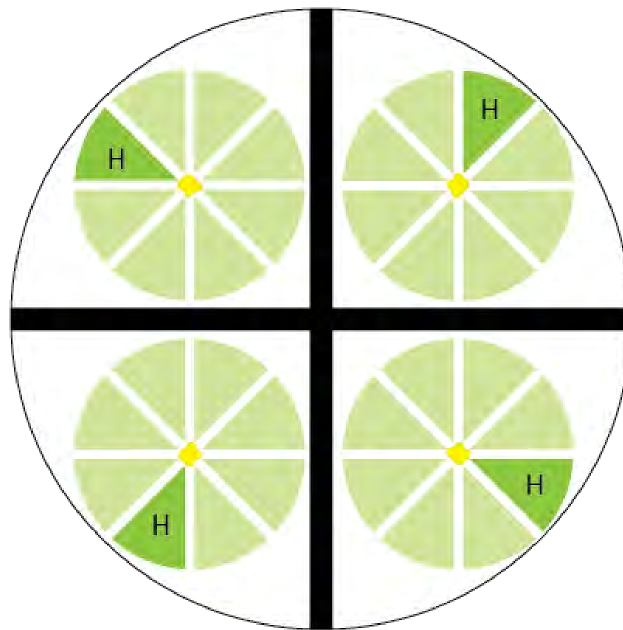
1089 Tu48.9-111 (Tu111), AI35, Tu9 and DP89 were kept at -80°C and were defrosted before use.
1090 We maintained these plasmodia that we created on malt extract agar at 24°C in the dark. Due
1091 to the multinucleate nature of acellular slime moulds, pieces of a single plasmodium can be split
1092 into multiple plasmodial fragments which become fully functional plasmodia between 20 - 40
1093 minutes after being severed (Jones et al., 2020).

1094 We maintained main plasmodial cells on malt extract agar for the duration of our study. Before
1095 each speed-accuracy test, we subcultured large plasmodial fragments from the main plasmodial
1096 cells of each strain and grew them in 1.75 L containers (base surface area = 500 cm^2) with 1%
1097 agar and fed the plasmodia oat flakes to rapidly increase plasmodial biomass. We took 20 plas-
1098 modial fragments for each treatment from the 1.75 L containers for use in speed-accuracy tests
1099 (fragments weighed 0.0035 - 0.0065 g). Once speed-accuracy tests were completed, plasmodia
1100 grown from the plasmodial fragments were destroyed and not returned to fuse back with the
1101 main plasmodial cell grown on malt extract agar. Speed-accuracy tests were completed approx-
1102 imately 30 days apart. Tu111xAI35-H speed-accuracy tests were completed on 39, 62, 93, 125,
1103 155, 186, 215, 242, 270, 306 and 307 days. Due to poor growth of Tu111xAI35-H plasmodia
1104 in 1.75 L containers, tests were split over two days at 306 and 307 days. Tu9xDP89-S speed-
1105 accuracy tests were completed on 39, 62, 93, 125, 155, 186, 215, 242, 272 and 306 days. Due to
1106 poor growth of Tu9xDP89-S plasmodia, speed-accuracy tests were meant to be completed at 270
1107 days at the same time as Tu111xAI35-H strain, but was delayed for two days and completed at
1108 272 days instead. Due to COVID-19 restrictions and loss of access to laboratory facilities, when
1109 both strains were 330 days old, the plasmodial cells were moved and maintained on 1% w/v
1110 agar and autoclaved whole oat flakes (Woolworths brand, Bella Vista) instead of malt extract
1111 agar until they died at 628 days old for Tu111xAI35-H, and 621 days old for Tu9xDP89-S.

1112 Speed-accuracy test

1113 We measured the decision-making ability of the acellular slime moulds by giving plasmodia
1114 (grown from a plasmodial fragment) a choice between one high concentration and seven low
1115 concentration foods. *Physarum polycephalum* can differentiate between different concentrations

1116 of oat agar, and it prefers high concentrations of oat agar over lower concentrations (Latty
1117 and Beekman, 2011b, 2010). The oat agar was made by mixing different concentrations of
1118 autoclaved ground oat flakes into 2% agar. We used an 85 mm diameter Petri dish filled with
1119 1% agar that was cut into quadrants that were separated by 5 mm channels. This allowed us to
1120 have four replicates per plate, as plasmodia were repelled by the dry plastic of the Petri dish.
1121 Each quadrant had seven wedges of the same low concentration food and one wedge of high
1122 concentration food arranged in a circle surrounding a plasmodium (0.005 ± 0.0015 g, Figure
1123 2.1).



1124

1125 Figure 2.1: Experimental set-up of speed-accuracy test. A Petri dish filled with 1% agar was
1126 cut into quadrants. Dark black lines show areas where the agar was removed. Each quadrant
1127 contained one replicate. The wedge labelled H was the high concentration food choice (6% w/v
1128 oat agar) and the remaining seven wedges were all low concentration food choices (2% w/v oat
1129 agar in easy tests, 4% w/v oat agar in hard tests). The configuration shown in the figure was
1130 kept constant for all replicates. The yellow blob in the centre is the plasmodial fragment.

1131 We defined difficulty levels in this experiment by changing the concentration difference between
1132 the low concentration and high concentration food. Plasmodia of *P. polycephalum* have more
1133 difficulty discerning between food that have similar concentrations (Latty and Beekman, 2011b).
1134 Easy treatments used 2% w/v oat agar for the low concentration food and 6% w/v oat agar as
1135 the high concentration food. Hard treatments used 4% w/v oat agar for the low concentration
1136 food and 6% w/v oat agar as the high concentration food. This meant that easy treatments
1137 had a concentration difference of 300% between the two food options and hard treatments had
1138 a concentration difference of 150% between the two food options. Twenty replicates of each
1139 strain were used for both levels of difficulty.

1140 We placed the set-up Petri dishes without lids in a dark booth at 24°C for 48 h. We used cameras
1141 (Canon DSLR 1000D and 1100D) with an intervalometer (Captur Timer Kit, Hahnel) to capture
1142 time-lapse footage of the plasmodia. Every 30 minutes, the plasmodia were illuminated for five
1143 seconds from above to capture an image. We also placed containers of water in the dark booth
1144 to prevent the agar and food wedges from drying out.

1145 We analysed the time-lapse footage using FIJI in ImageJ (Rasband, 1997; Schindelin et al.,
1146 2012). When feeding, plasmodia will initially contact all food wedges. Cytoplasm then flows
1147 towards the preferred food, causing the channels connected to non-preferred food to collapse
1148 and the whole plasmodial cell to retract (Latty and Beekman, 2011a). To ensure that plas-
1149 modia had recovered from being severed, we defined the start point of the experiment as the
1150 time that plasmodia had grown large enough to contact one food wedge (5 mm diameter). A
1151 plasmodium was considered to have made a choice when it had covered 75% of a food wedge,
1152 as the amoeboid nature of plasmodia allow them to initiate searching while in the process of
1153 consuming food (Latty and Beekman, 2009). We calculated decision time from the start point
1154 of the experiment until the time when plasmodia had covered 75% of a food wedge. We recorded
1155 whether plasmodia had chosen low concentration or high concentration food, decision time, and
1156 the number of food wedges plasmodia were in contact with at the choice point.

1157 Three replicates were removed as the food wedges had shifted out of place during set up, and

1158 four replicates failed to grow and cover a food wedge during the allotted time (48h; n = 793).

1159 Statistical Analysis

1160 We used R (ver. 4.2.1) (R Core Team, 2021) for all analyses.

1161 Due to the morphology of plasmodia, acellular slime moulds were able to make contact with
1162 multiple food sources at the same time. We classified plasmodia that were in contact with
1163 multiple food wedges at the choice point as making a split decision. We found that 50.69%
1164 of plasmodia made a split decision. We used a generalised linear model (GLM) with binomial
1165 distribution to analyse the effect of age on whether plasmodia made a split decision. We used
1166 split decision as the binary response variable and age, strain and test difficulty as explanatory
1167 variables and calculated the odds-ratios (OR) by exponentiating the coefficients of the model.

1168 We used a generalised additive model (GAM) to analyse the effect of age on decision accuracy as
1169 the data were non-linear. We specified a binomial distribution with logit-link using food choice
1170 (high concentration or low concentration) as the response variable, whether the plasmodia split
1171 as a fixed effect, and individual smoother terms for age for each combination of strain and test
1172 difficulty.

1173 We analysed the effect of age on decision time using a GAM as the data were non-linear.
1174 We specified a gamma distribution with logit-link as the data were right-skewed and positive,
1175 and used decision time as the response, whether the plasmodia split as a fixed effect, and
1176 individual smooth terms for age for each combination of strain, test difficulty and food choice.
1177 We calculated the difference between smooth terms of groups that chose high concentration
1178 food and low concentration food. This showed us that speed-accuracy trade-offs were present
1179 at certain age groups. We used GLMs with binomial distribution to analyse the effect of food
1180 choice on decision time for each age group at both test difficulty levels. We used food choice (low
1181 or high quality) as the binary response variable and decision time as the explanatory variable.

1182 For all three models, we used the R package DHARMa was used to produce residual diagnostics

for models and ensure all assumptions for the models were met (Hartig, 2020).

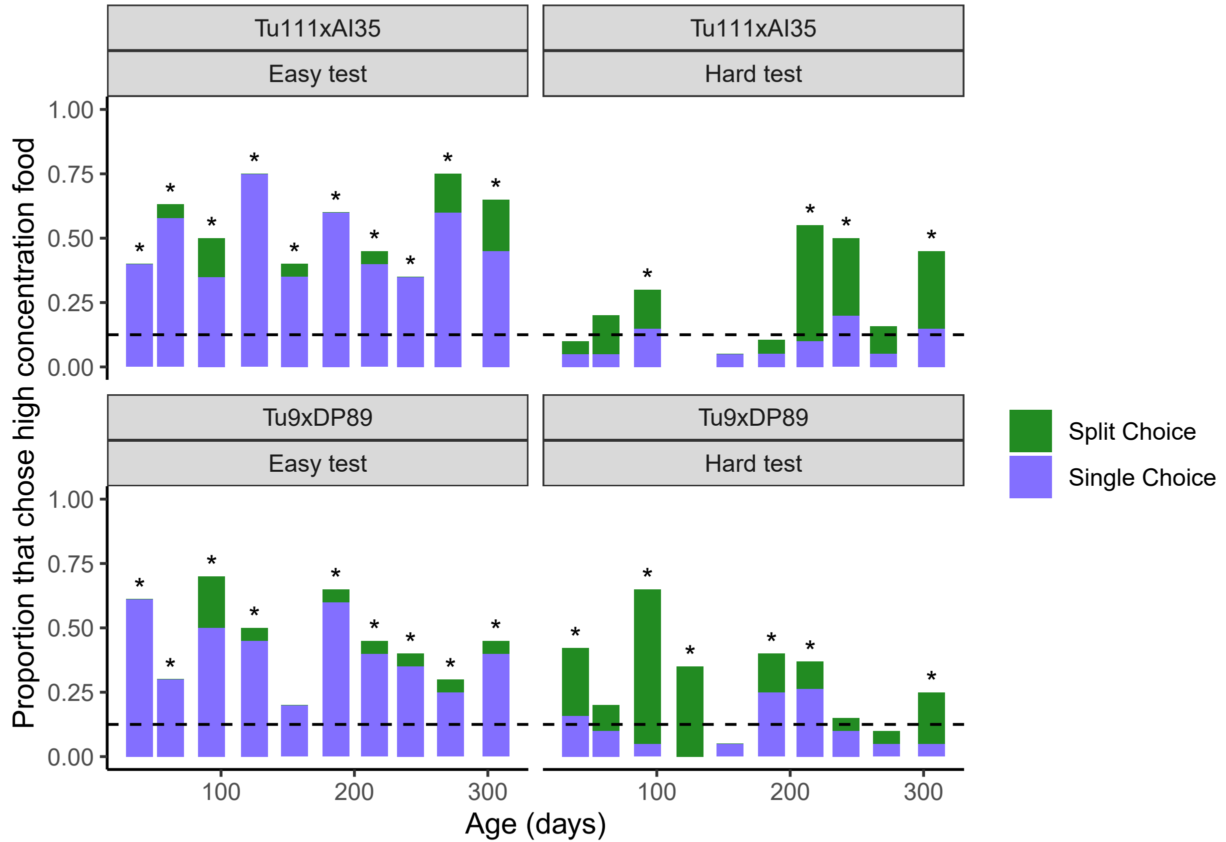
2.4 Results

After fragmentation, plasmodia took a mean ($\pm$ SE) of 1.69 $\pm$ 0.06 h to recover and begin to move. Plasmodia grew radially, with pseudopods usually contacting all food wedges before moving their biomass towards a select few food wedges. A portion of plasmodia made split decisions, where they were in contact with multiple food sources at the choice time point. Age did not affect the likelihood of plasmodia making split decisions, but strain had a significant effect where Tu9xDP89-S plasmodia were less likely to make split decision (OR = 0.74, 95% CI: 0.55 - 0.99). Test difficulty also had a significant effect on split decisions with 64.39% of plasmodia making split decision in the hard test, compared to 37.02% in the easy tests (OR = 3.10, 95% CI: 2.32 - 4.15).

Predictably, accuracy was higher in easy tests compared to hard tests. For Tu111xAI35-H, the mean accuracy of plasmodia over the whole experiment was 54.77% in easy tests and 24.75% in hard tests. For Tu9xDP89-S, the mean accuracy over the whole experiment was slightly lower than Tu111xAI35-H in easy tests at 45.45% but slightly higher in hard tests at 29.29%. Our expectations were that age would affect our variables linearly, but our results showed that accuracy fluctuated with age (Figure 2.2 & Figure 2.3). Only Tu111xAI35-H plasmodia in easy tests were not affected by age (Table 2.1). In the hard tests for Tu111xAI35-H and both test difficulties for Tu9xDP89-S, there were two peaks in accuracy over age and a large drop in accuracy at 155 days (Figure 2.2 & Figure 2.3). When analysing the accuracy of plasmodia at each age, we found that Tu111xAI35-H in easy tests chose the high concentration food over random choice at every age (Figure 2). Tu9xDP89-S in easy tests was not able to choose the high concentration food over random choice at 155 days (Figure 2.2).

In hard tests, it was more common for plasmodia to fail to accurately choose the high concentration food. Tu111xAI35-H plasmodia in difficult tests did not choose high concentration food over random chance at 39, 62, 125, 155, 186 and 270 days of age. Tu9xDP89-S were accurate in

1209 more age groups in difficult tests as plasmodia did not choose high concentration food over ran-
1210 dom chance at only 62, 155, 242 and 272 days of age. We also found an effect of split decisions,
1211 where split plasmodia were less likely to choose high concentration food over low concentration
1212 food (Table 2.1).



1213

1214 Figure 2.2: Proportion of plasmodia that chose high concentration food. If a choice is random,
1215 the expected probability of choosing a high concentration food is 0.125 (dashed line). Two
1216 colours indicate whether plasmodia made a split decision (purple chose a single food and green
1217 was in contact with multiple food). Asterix shows whether age group chose high concentration
1218 food with a probability significantly higher than random. N = 793.

Table 2.1: Summary of general additive model for accuracy over age

A. parametric coefficients	Estimate	Std. Error	t-value	p-value
(Intercept)	0.4130	0.1122	3.6813	0.0002
Split Choice	-2.0089	0.1867	-10.7577	< 0.0001
B. smooth terms	**edf**	**Ref.df**	**F-value**	**p-value**
Tu111xAI35:Easy	2.8271	3.5124	4.8889	0.1967
Tu111xAI35:Hard	6.5594	7.7243	36.7514	< 0.0001
Tu9xDP89:Easy	6.6099	7.7777	17.2699	0.0470
Tu9xDP89:Hard	7.8998	8.7020	49.3991	< 0.0001

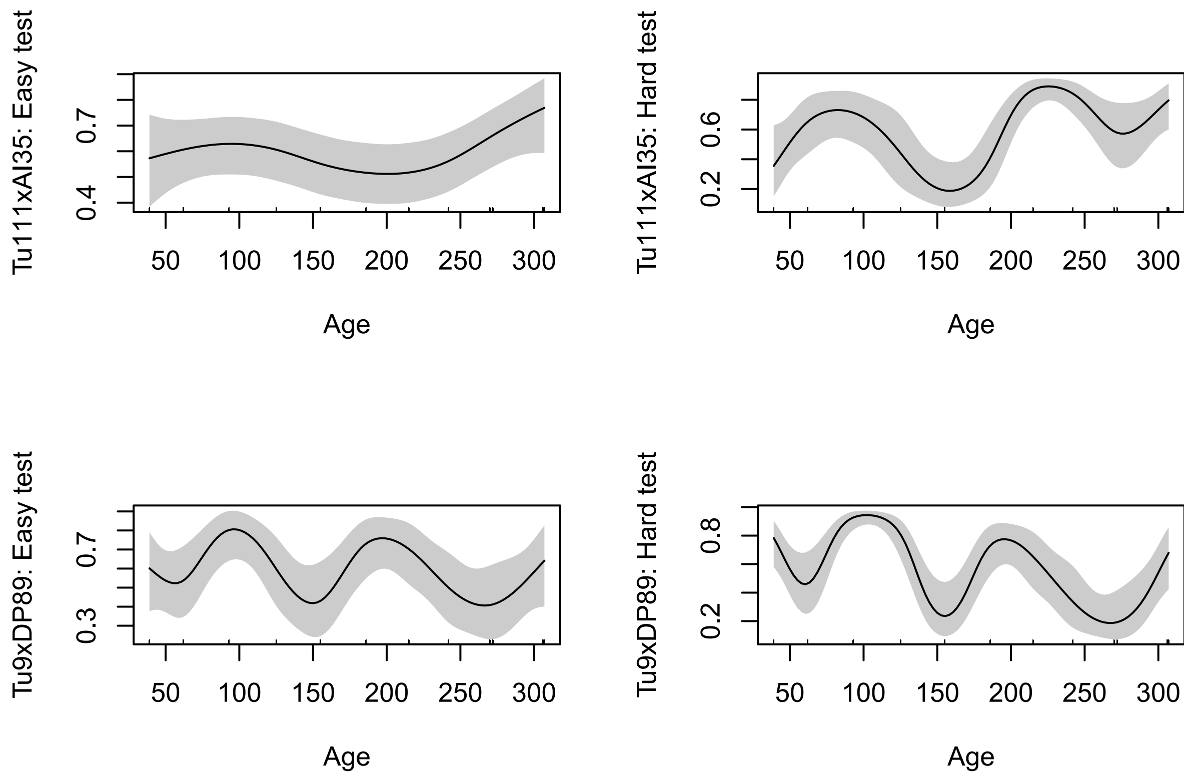


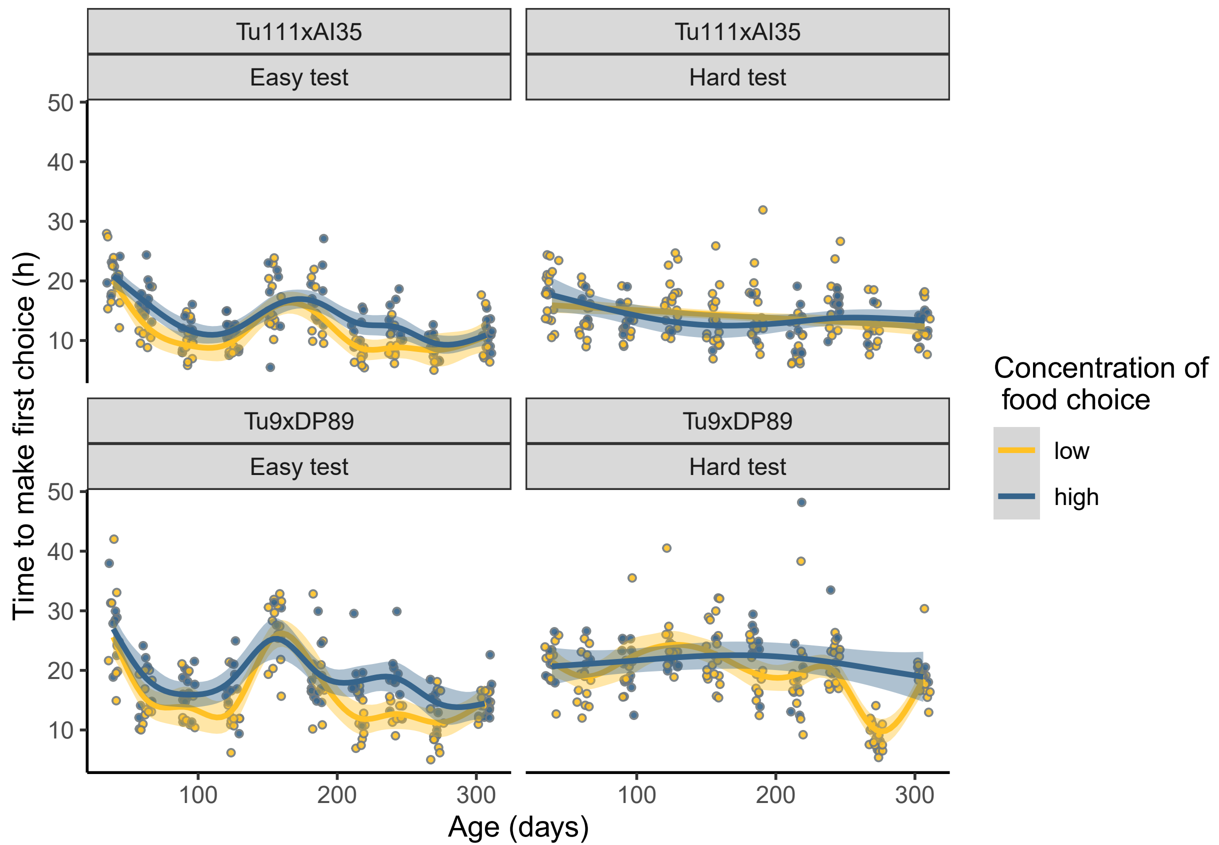
Figure 2.3: Smooth functions for general additive model of decision accuracy (y-axis) over age (x-axis) with 95 percent confidence intervals.

Decision time also fluctuated with age (Table 2.2). In easy tests, both Tu111xAI35-H and Tu9xDP89 decision time has one main peak of longer decision time and two valleys where decision time was shorter (Figure 2.4). In hard tests, age affects Tu111xAI35-H decision time

linearly, with decision time becoming shorter with increasing age (Figure 2.4). Decision time of Tu9xDP89-S in hard tests also fluctuated, where decision time was longer in younger plasmodia but drops dramatically at 272 days of age (Figure 2.4). In a speed-accuracy trade-off, we would expect plasmodia to have a longer decision time when choosing high concentration food. However, in our experiment we found that the decision time of plasmodia that chose high concentration food and low concentration were generally similar at each age. SATs were only present at certain ages in easy tests and no SATs were found in hard tests. In Tu111xAI35-H we found SATs when plasmodia were 62 (OR = 1.63, 95% CI: 1.13 - 2.84), 215 (OR = 2.72, 95% CI: 1.37 = 11.14) and 242 (OR = 1.81, 95% CI: 1.20 - 3.61) days of age. SATs appeared slightly later in Tu9xDP89-S at 125 (OR = 1.41, 95% CI: 1.09 - 2.10), 215 (OR = 1.58, 95% CI: 1.15 - 2.66) and 242 days of age (OR = 1.96, 95% CI: 1.27 - 4.09).

Table 2.2: Summary of general additive model for decision speed over age

A. parametric coefficients	Estimate	Std. Error	t-value	p-value
(Intercept)	2.7599	0.0169	163.2673	< 0.0001
Split Choice	-0.0156	0.0240	-0.6490	0.5165
B. smooth terms	**edf**	**Ref.df**	**F-value**	**p-value**
Tu111xAI35: Easy - Choice = Low Quality	6.3991	7.5827	9.0042	< 0.0001
Tu111xAI35: Easy - Choice = High Quality	6.1535	7.3172	8.5440	< 0.0001
Tu111xAI35: Hard - Choice = Low Quality	1.0012	1.0024	6.0728	0.0139
Tu111xAI35: Hard - Choice = High Quality	1.0069	1.0137	4.3520	0.0373
Tu9xDP89: Easy - Choice = Low Quality	7.6762	8.5695	10.9033	< 0.0001
Tu9xDP89: Easy - Choice = High Quality	4.6169	5.6309	5.0087	0.0001
Tu9xDP89: Hard - Choice = Low Quality	7.9951	8.7405	10.9930	< 0.0001
Tu9xDP89: Hard - Choice = High Quality	7.7472	8.5763	4.4994	< 0.0001



1238

1239 Figure 2.4: Decision speed of plasmodia over age. Smooth function fitted with a general
1240 additive model and 95 percent confidence intervals are included. SATs are present when the
1241 average time to choose a low concentration food is faster than the time taken to choose a high
1242 concentration food.

1243 ## 2.5 Discussion

1244 We found that *P. polycephalum* exhibited SATs only in easy tests. Our result contradicts
1245 previous experiments where SATs were only found in plasmodia that participated in difficult
1246 discrimination tasks (Dussutour et al., 2019; Latty and Beekman, 2011b). In fact, SATs are
1247 most often found in animals when tasks are difficult (reviewed by Chittka et al., 2009). For our
1248 experiment, we used 6% w/v oat agar for the high concentration for both the easy and hard
1249 tests, changing the low concentration food to create different difficulty levels. This design meant
1250 that there was a larger total availability of food in the hard test as the average concentration of
1251 the food wedges were 4.25% w/v agar compared to 2.5% average concentration of food wedges

in the easy test. Plasmodia have previously been shown to make faster decisions when the value of food sources increase, possibly as a method to reduce decision deadlocks (Dussutour et al., 2019). It is possible that when the average food available was high in hard tests, plasmodia may have used the same speed to feed from any food source, prioritising food value over accuracy and leading to a lack of SATs.

SATs were only present at certain ages during the acellular slime mould's lifespan and these ages differed between strains. In Tu111xAI35-H, SATs were first exhibited early in the lifespan at 62 days. In contrast, SATs were first exhibited in Tu9xDP89-S at 125 days, more than double the age of Tu111xAI35-H. Both strains exhibited SATs again later in their lifespan, at 215 and 242 days, but did not continue to exhibit SATs in discrimination tests after these ages. We observed fluctuations in cell health of plasmodial cell cultures maintained on malt extract agar, with periods of slow growth followed by sudden recovery (Chapter 1). It is possible that periods of poor health in our strains may explain the non-sequential occurrence of SATs observed in our experiments. We also found fluctuations in decision accuracy and decision time in relation to age.

The relationship between decision accuracy and age was non-linear in the strain Tu9xDP89-S. Decision accuracy fluctuated in both easy and hard tests with the most pronounced drop in accuracy at 155 days of age. This was the only age point where plasmodia of Tu9xDP89-S were unable to choose the high concentration food over random chance in the easy test. Interestingly, the relationship between decision time and age was also non-linear, with decision time in easy tests being the longest at 155 days. At this age point, Tu9xDP89-S plasmodia appear to make inaccurate decisions despite spending a long time making their decision. Tu9xDP89-S plasmodia had poor cell health at 155 days (Figure 1.5, Chapter 1). It is possible that plasmodia in poor health lose the ability to accurately discriminate between high and low quality food.

The relationship between age and decision accuracy and time were different in Tu111xAI35-H strain compared to Tu9xDP89-S. Age had no effect on decision accuracy in easy tests, with Tu111xAI35-H successfully discriminating between low and high concentration food at every age.

However, in hard tests, Tu111xAI35-H plasmodia were inaccurate more often than Tu9xDP89-S, being unsuccessful at discriminating between low and high concentration food at six of the ten age points, in comparison to Tu9xDP89-S plasmodia which were only unsuccessful in four out of ten age points. Like Tu9xDP89-S, the relationship between age and decision time of Tu111xAI35-H fluctuated in easy tests. However, in hard tests the relationship between age and decision time was linear, with Tu111xAI35-H making marginally faster decisions with increasing age. Behavioural variation between the two strains also extended to whether their plasmodia made split decisions, where Tu111xAI35-H plasmodia were more likely to make split decisions compared to Tu9xDP89-S plasmodia.

Behavioural variations between strains of acellular slime moulds have been previously reported, including differences in foraging behaviour, movement speed and decision accuracy (Dussutour et al., 2019; Vogel et al., 2018; Zabzina et al., 2014). We have demonstrated strain level variation in behavioural changes due to age. In previous experiments, strains of plasmodia are obtained from biological supply houses as sclerotia, which are the dormant life stage of acellular slime moulds. This makes it difficult to determine the exact age of plasmodia used in experiments, as sclerotia can survive in their dormant state for up to three years (Anderson, 1992; Gehenio, 1944). Our strains of plasmodia have been reared from myxamoebae in identical conditions from the beginning of their life stage as plasmodia. This means that it is more likely that the behavioural variation we have observed are due to intrinsic differences between strains rather than variation driven by environmental variables such as time spent in the resistant sclerotia life stage, bacterial or fungal contamination, or culturing techniques at biological supply houses.

The high intraspecific variability in foraging strategies between strains of *P. polycephalum* provides a promising model for studying behavioural variation. Differences in foraging strategies have been observed between different species and groups of organisms but are becoming more apparent within species (Bolnick et al., 2003; Ceia and Ramos, 2015; Chang et al., 2017). There is evidence of intraspecific variation in 93 species over a broad range of taxonomic groups (Bolnick et al., 2003). *Physarum polycephalum* is a useful model organism as they are easy to manipu-

late and grow well in laboratory environments. The brainless nature of *P. polycephalum* also allows researchers to quantify behavioural mechanisms and create biologically inspired models that mimic *P. polycephalum* behaviour (Tero et al., 2010; Tero et al., 2006). Observations of the differences between *P. polycephalum* strains at the cellular pathway level could show what is driving different foraging strategies in this decentralised organism, and may lead to clearer understandings of the development of different foraging strategies in higher-level organisms.

In addition, due to Covid-19 restrictions we were only able to assess the behaviour of plasmodia over 307 days. We continued to culture plasmodia through Covid-19 restrictions and both strains had a lifespan of over 600 days, three times longer than previously recorded lifespans of *P. polycephalum* which ranged from as little as 20 days up to 200 days (Hu et al., 1985; McCullough et al., 1973; Poulter, 1969). Most researchers only continue culturing plasmodia for the duration of their experiment, which is a few months at maximum. In our experiments we observed variations in behaviour as early as 62 days of age, where only Tu111xAI35-H plasmodia showed SATs in easy tests. Our findings have significant implications on behavioural research of acellular slime moulds as individual level variation in behaviour that may have previously been dismissed as noise, may in actuality be due to the age of plasmodia in experiments. Researchers should be mindful of the age of plasmodia they are using when designing experiments for acellular slime mould behaviour.

In summary, we found evidence of SATs at non-sequential age points in easy discrimination tests. We found differences between strains in on all aspects of behaviour, including decision speed, decision accuracy, and likelihood of split decisions. We also found differences in the effects of age on behaviour between two strains. This highlights the need for researchers to specify the strain and age of *P. polycephalum* that are being used in experiments, and also presents *P. polycephalum* as an exciting model organism to investigate behavioural variation in brainless organisms. By looking at intraspecific variation in behaviour and the underlying genetic differences driving behaviour in brainless organisms, we can further understand the mechanisms behind how decision-making behaviour evolved in more complex organisms.

2.6 Acknowledgements

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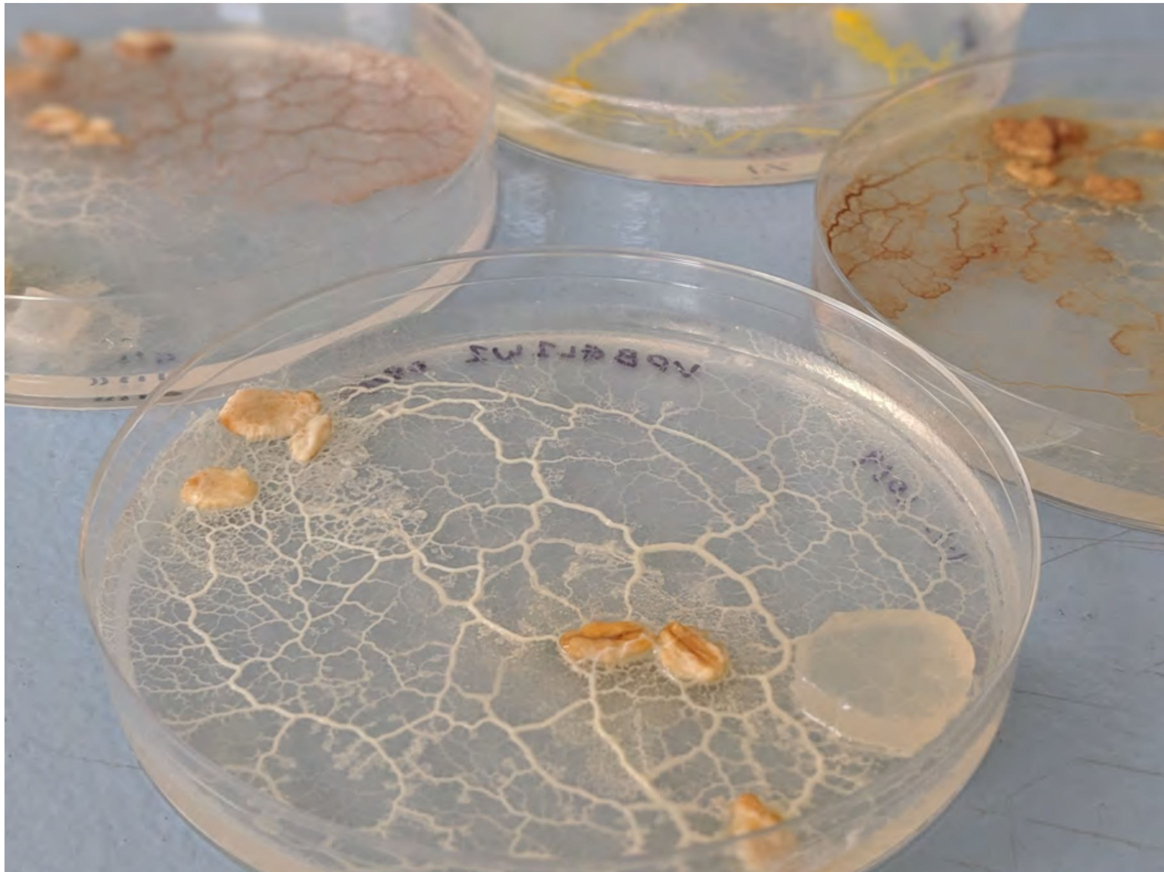
Chapter 3

Make Peace Not War: Facilitative interactions in acellular slime moulds



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Research article prepared for submission to Oikos.

Author contributions: AH, CR and TY contributed to the study design. AH conducted the experiment. AH collected and analysed the data. CR and TY provided valuable feedback and editing for the article prepared by AH.

3.1 Abstract

Interspecific interactions play an incredibly important role in the decomposition process as organic matter are broken down sequentially by different species in the ecosystem. Protists are important predators in the decomposition system that free nutrients from decomposers and convert nutrients into more accessible forms. However, little is known about interactions between protists in decomposition systems. We studied the interactions between three species from the protist group, acellular slime moulds, *Didymium iridis, Physarum polycephalum* and *Physarum melleum*. Acellular slime moulds have a vegetative life stage called a plasmodium, which is a large, multinucleate, single cell consisting of a network of tubes made of cytoplasm. When plasmodia move, they leave behind a trail of extracellular secretions that protect the plasmodia from environmental damage and can act as an externalised spatial memory system. We first characterised the foraging strategy of each species in two different foraging environments; a homogeneous environment where food was spread evenly and a heterogeneous environment where food was spread in four distinct patches. We then observed direct interactions between pairs of species in the two foraging environments. Lastly, we observed indirect interactions by testing how species react to extracellular secretions of themselves and the other species. We found distinct differences in the foraging behaviour of the three species. We observed facilitation between species where the presence of one species increased the food consumed by another species. We found indirect interactions where *D. iridis* and *P. melleum* were attracted to the extracellular secretions of the other. Interestingly, we found no evidence of negative interactions or competition between species. This may be because acellular slime moulds are part of the decomposition system, where facilitation is more common due to the sequential nature of organic matter breakdown. Further investigations on interactions between protist species will help us understand diversity and functioning in decomposition systems.

3.2 Introduction

Interspecific interactions are pervasive in all ecological systems. Interactions between organisms drive diversity and community structure of ecosystems. These interactions can be negative, positive or neutral. Negative interactions include competition, predation and parasitism where one species directly benefits while harming another species. In competition, species compete for a shared limiting resource. Species can also negatively affect others indirectly by releasing chemicals that suppress growth or establishment of nearby species in a process called allelopathy (Wardle et al., 2011). Negative interactions between species drives diversity and community structure, as exclusion from resources such as nutrition or habitats can reduce the realised niche of a species (Bruno et al., 2003; Thorpe et al., 2011). Positive or neutral interactions are often termed facilitation, where at least one species benefits while causing no harm to other species. Facilitation includes both mutualism, where both species benefit, and commensalism, where one species benefits while another is unaffected. Contemporary research has highlighted facilitation as an important driver of diversity and community structure as facilitation allows a species to access resources outside of their fundamental niche (Bruno et al., 2003; Cardinale et al., 2002; Lortie et al., 2004; Soliveres et al., 2015; Thorpe et al., 2011; Valiente-Banuet and Verdú, 2007). For example, partnerships between fungal mycelial networks and plant roots allow plants to access nutrients from an extended range (Peay, 2016). Sheltering by established trees allows vulnerable plants to grow in harsh conditions (Gómez-Aparicio et al., 2004; Valiente-Banuet and Verdú, 2007). In decomposition systems, small invertebrates physically break down detritus which increases the surface area available for chemical decomposition by microbes (Swift et al., 1979).

The decomposition system involves a high proportion of positive interspecific interactions as detritus and organic matter are broken down sequentially by different species (Wall and Moore, 1999). Although small invertebrates facilitate decomposers by physically breaking down detritus, often this organic matter has been colonised by other species of bacteria and fungi which modify it to be more palatable for these small invertebrates (Swift et al., 1979). Another major facilitative interaction is between decomposers and plants, where decomposers transform nutri-

1400 ents, including nitrogen and carbon, that are bound in dead organic matter into forms that can
1401 be used by plants (Gessner et al., 2010; Tiunov and Scheu, 2005). Negative interspecific inter-
1402 actions also play a role in decomposition systems. The initial breakdown and decomposition are
1403 done by bacteria and fungi, who are then consumed by microbial predators in a complex food
1404 web (Hättenschwiler et al., 2005). Predators in the decomposition system free nutrients from
1405 decomposers and often convert nutrients into more accessible forms. Although often overlooked
1406 in decomposition studies, protists occupy a key position in the food web as predators of bacteria,
1407 fungi and other small eukaryotes (Geisen et al., 2018).

1408 Protists are an incredibly diverse group of organisms, constituting the majority of eukaryotic
1409 life (Geisen et al., 2018). Protists have been shown to increase soil nitrogen availability to plants
1410 (Clarholm, 1985) and protist species richness can affect rates of decomposition (Hünninghaus
1411 et al., 2017). Little is known about whether protist interactions drive biodiversity and further
1412 investigations of how species-species interactions affect the processes that occur during seques-
1413 tration and mineralization of organic matter in decomposition ecosystems are needed (Gessner
1414 et al., 2010; Hättenschwiler et al., 2005). A group of protists, acellular slime moulds, have
1415 been identified as catalysts of microbial litter breakdown (Geisen et al., 2021) and present an
1416 interesting model to study interspecific interactions in decomposition systems.

1417 Acellular slime moulds are microbial predators found primarily on decaying matter (Martin
1418 and Alexopoulos, 1969). There are approximately 1000 known species of acellular slime moulds
1419 (Lado, 2001) and a high abundance have been found in grassland and agricultural soil (Feest
1420 and Madelin, 1985). Various factors that affect acellular slime mould biodiversity have been
1421 identified, including temperature, moisture, soil pH and substrate type (Martin and Alexopou-
1422 los, 1969; Stephenson, 1989). In addition, species richness of acellular slime moulds tend to be
1423 linked to the diversity and biomass of vascular plants in their habitat (Stephenson et al., 2008).
1424 Multiple species are often found to co-exist in the same microhabitat (Hosokawa et al., 2019;
1425 Rojas and Stephenson, 2021; Stephenson et al., 2008) and there is evidence that different species
1426 use different foraging strategies (Yip et al., 2014). These observations lead us to believe that

interactions between species are inevitable, but direct interactions between different acellular slime mould species have not been investigated.

Signs of allelopathic interactions between acellular slime mould species have been shown through chemical signalling via extracellular secretions. Acellular slime moulds have a vegetative life stage called a plasmodium, which is a large multinucleate single cell consisting of a network of tubes made of specialised cytoplasm that rhythmically contract. Sections of the network in close proximity to attractants contract at a higher frequency creating a pressure gradient that allows cytoplasm within the tubes to flow towards the attractant and plasmodia to extend its network (Nakagaki et al., 2004). Plasmodia are covered in an extracellular sheath which allow them to move over surfaces and their extracellular secretions have been shown to act as an externalised spatial memory to aid in navigating the environment (Reid et al., 2012). The species, *P. polycephalum* avoids its own extracellular secretions but follows the secretions of another species *D. bahiense* (Reid et al., 2013).

Allorecognition has been found in the species *P. rigidim,* where individuals use chemical signals from extracellular secretions to determine whether to fuse with other compatible individuals (Masui et al., 2018). Chemical composition of extracellular secretions, such as the concentration of calcium, may also affect the behaviour of individuals. Calcium appears to be an attractant for the species *P. polycephalum*, and individuals that secrete higher concentrations of calcium are able to find high quality food sources more accurately (Dussutour et al., 2019). Individuals of *P. polycephalum* have been shown to avoid areas previously occupied by a stressed individual of the same strain (Briard et al., 2020). Types of stressors included light stress, hunger stress and chemical stress, with hunger stress eliciting the strongest repellent response (Briard et al., 2020). As positive and negative signals can be detected in the extracellular secretions of plasmodia within species, it is possible that acellular slime moulds can use extracellular secretions to affect the foraging behaviour of other neighbouring species.

For this study, we investigated interspecific interactions between three species of acellular slime mould. Studies comparing the foraging behaviour of acellular slime mould species are scarce,

1454 therefore our first aim was to characterise the foraging strategy of our acellular slime mould
1455 species. We used two types of foraging environments with food spread in a homogeneous or het-
1456 erogeneous pattern. Previous comparisons of acellular slime mould foraging behaviour showed
1457 that patch quality affected foraging strategy differently between species (Latty and Beekman,
1458 2015; Yip et al., 2014). The network structure of plasmodia are similar to plant roots, as they
1459 can exploit multiple food sources simultaneously. Plant root foraging is affected by soil nutrient
1460 heterogeneity (Campbell et al., 1991; Casper and Jackson, 1997; Fransen et al., 2001; Mommer
1461 et al., 2012; Rubio et al., 2003). For example, *Festuca rubra* is more successful in homogeneous
1462 environments due to their dense and extensive root system (Fransen et al., 2001). In com-
1463 parison, the sparser root system of *Anthoxanthum odoratum* allowed for higher root plasticity
1464 which was more successful in heterogeneous environments (Fransen et al., 2001). We observed
1465 acellular slime mould behaviour in both homogeneous and heterogeneous environments to de-
1466 termine whether different species performed better or adapted their foraging behaviour based
1467 on nutrient heterogeneity, similarly to plant root systems.

1468 Our second aim was to observe direct interactions between species of acellular slime moulds
1469 by observing their behaviour in a shared environment. We observed every pair-combination of
1470 our three acellular slime mould species in both homogeneous and heterogeneous environments.
1471 Direct interactions between species of acellular slime moulds have rarely been observed, and
1472 it is unknown whether they have the ability to directly inhibit or promote growth of other
1473 acellular slime moulds as seen in bacteria or fungi (Geisen et al., 2021). Competition between
1474 strains of *P. polycephalum* have been observed, where fast moving strains have the advantage
1475 in consuming high quality food (Dussutour et al., 2019). We may observe similar interactions
1476 between species of acellular slime moulds that have different foraging behaviour. In addition,
1477 we were interested to observe whether species-species interactions were affected by nutrient
1478 heterogeneity. We quantified foraging success as the number of food sources plasmodia were
1479 able to cover. If a species covered more food when paired with another we would classify
1480 the interaction as facilitation. If a species foraging success decreased, we would classify the
1481 interaction as competition.

Thirdly, we aimed to observe whether species of acellular slime moulds could be affected purely through extracellular secretions, with no other slime mould directly present. We used binary preference tests to observe whether species showed attraction or avoidance towards extracellular secretions of other species. This third test allowed us to infer possible mechanisms driving the direct interactions between species of acellular slime moulds.

We predict that we will find evidence of facilitation between our acellular slime mould species as all three species are found in leaf litter microhabitats. Additionally, acellular slime moulds are part of the decomposition system where facilitation is the predominant type of interspecific interaction. Alternatively, as acellular slime moulds are microbial predators, they may compete for shared food resources and use different foraging strategies based on nutrient heterogeneity to co-exist in the same microhabitat. Through our investigation on the interactions between acellular slime mould species we hope to expand current knowledge on interspecific interactions in decomposition systems.

3.3 Methods

Study species

We studied three species of acellular slime moulds, *Physarum polycephalum*, *Didymium iridis* and *Physarum melleum*. All three species are from the Physarale family of true slime moulds. The species *P. polycephalum* is used frequently for behavioural experiments and samples can be purchased from scientific supply stores. We re-activated a sclerotia sample of *P. polycephalum* that we purchased from Southern Biological (Victoria, Australia). Sclerotia are the dormant life stage of acellular slime moulds and become plasmodia when introduced to a moist environment. For the remaining two species, we collected leaf litter from various parks in Greater Sydney, Australia and used the moist chamber method to isolate wild acellular slime mould species (described in Stephenson and Stempen, 1994). We cultured the acellular slime moulds species that we found in the moist chambers on 1.5% agar and fed them a diet of oat flakes (Woolworths, Bella Vista NSW), which are the preferred food of *P. polycephalum*. We selected

two species that appeared to most readily consume oat flakes and had a relatively fast growth rate. *Didymium iridis* was collected from Sir Joseph Banks Park, in the suburb Botany which is located approximately 10 km south of the city of Sydney. *Physarum melleum* was collected from Reserve 742 in the suburb Blacktown which is located approximately 35 km west of the city of Sydney. Acellular slime moulds were cultured on 1.5% agar, kept in the dark at 23°C and fed oat flakes before experiments.

For this study we used the vegetative life stage of the cell, called a plasmodium. The majority of behavioural experiments on acellular slime moulds have been conducted on plasmodia. A plasmodium consists of tubes made of specialised cytoplasm that transports food and cell components around the cell using contractile actin filaments (Nakagaki et al., 2004). Due to the multinucleate nature of acellular slime moulds, pieces of the plasmodial cell can be severed from the main cell into plasmodial fragments that become functioning individuals after some time (Yoshimoto and Kamiya, 1978).

Experimental setup

We used 75 mm diameter Petri dishes filled with 1.5% agar to create arenas for this experiment. We had two environment types which both had a total of 12 food sources of 7 mm diameter spread through the arena. The homogeneous environment had the food sources spread evenly through the arena and the heterogeneous environment had four patches of three food sources placed in the arena (Figure 3.1). A custom designed 3D printed mould was used to cut wells in the arenas and a mixture of 3% w/v oat and 2% w/v agar was pipetted into the wells as the food sources. Rectangles that were 15 x 25 mm were cut from the top and bottom of the arena and plasmodia were placed in this space. Plasmodia were placed alone or paired with each of the other species in the opposite rectangle thus creating six possible groupings. All six groupings were tested on both levels of environment type equaling 12 unique treatments (Table 3.1). As the two wild species of acellular slime mould have not been observed in a laboratory environment before, we also observed the behaviour of all three species in blank arenas of 1.5% agar (n = 10 for each species). We cut 15 x 25 mm rectangles of plasmodial search front (the

1535 leading edge of the slime mould, Figure 3.2) of each species and placed them in the experimental
1536 arenas. Images were taken every 10 minutes for 48 hours to observe the behaviour of plasmodia
1537 in the experiment. A light illuminated the arenas from below for five seconds each time an
1538 image was captured, otherwise the plasmodia were kept in the dark.

1539 We created a time-lapse video from the images captured during the experiment to observe
1540 acellular slime mould behaviour. We recorded the latency of plasmodia, first contact to food, the
1541 total number of food sources covered and the maximum number of food sources that plasmodia
1542 could cover with their biomass. We defined latency as the time it took for cut fragments of
1543 plasmodial search front to extend a pseudopod into the experimental arena. We calculated first
1544 contact to food as the time it took for a pseudopod to contact a food source after it had entered
1545 the experimental arena. We also observed events including if a plasmodium failed to contact
1546 any food, sporulation (plasmodia developing into the reproductive life stage), food sharing and
1547 food avoidance. Food avoidance was an unexpected behaviour that we observed only in *P.*
1548 *melleum*, where plasmodia appeared to avoid food sources completely, often preferring to cover
1549 the extracellular secretions of the other species if available (Supplementary video 3.1). We also
1550 measured the time it took for plasmodia to cover 12 food sources if they were able to.

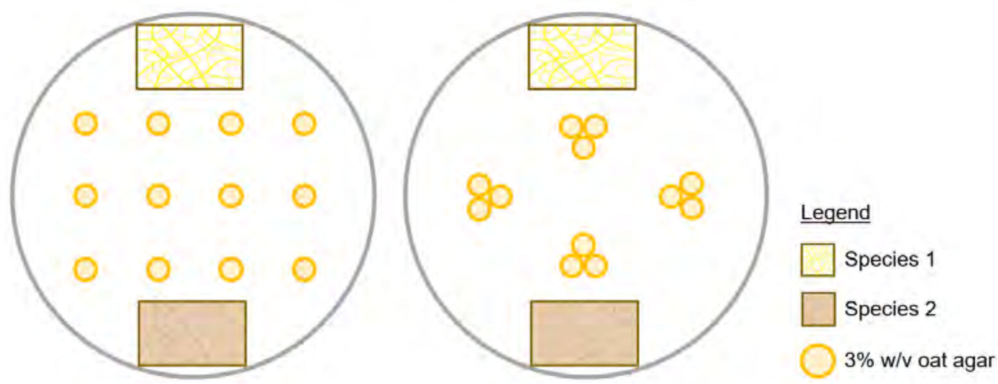


Figure 3.1: Experimental set-up with two environment types, homogeneous (left) and heterogeneous (right). Species 2 section was empty in alone groupings.

Environment Type	Species 1	Species 2	N
Blank	*D. iridis*	-	15
Blank	*P. polycephalum*	-	20
Blank	*P. melleum*	-	10
Homogeneous	*D. iridis*	-	40
Homogeneous	*P. polycephalum*	-	30
Homogeneous	*P. melleum*	-	20
Homogeneous	*D. iridis*	*P. melleum*	30
Homogeneous	*D. iridis*	*P. polycephalum*	*30*
Homogeneous	*P. polycephalum*	*P. melleum*	30
Heterogeneous	*D. iridis*	-	40
Heterogeneous	*P. polycephalum*	-	39
Heterogeneous	*P. melleum*	-	30
Heterogeneous	*D. iridis*	*P. melleum*	25
Heterogeneous	*D. iridis*	*P. polycephalum*	*40*
Heterogeneous	*P. polycephalum*	*P. melleum*	30

Table 3.1: Sample size of interaction experiments.

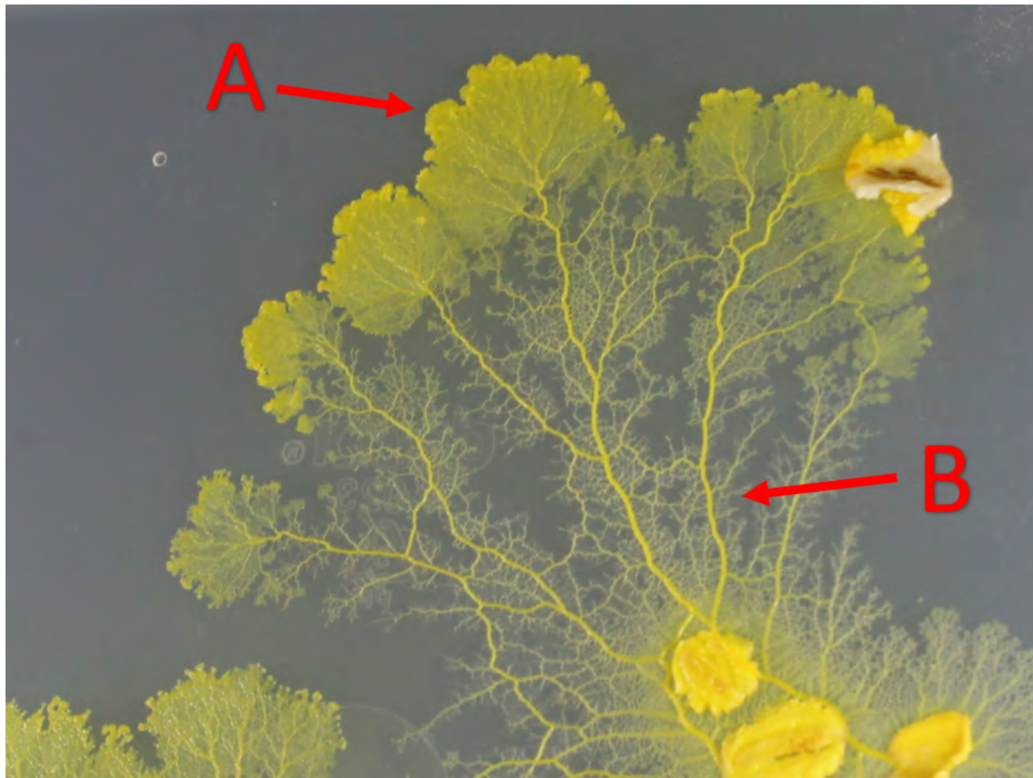


Figure 3.2: Section of plasmodium of *P. polycephalum.* A) Search front (or pseudopod) of plasmodia

Binary preference test

We conducted binary preference tests to investigate the behaviour of each species towards extracellular secretions. The extracellular secretions of some acellular slime moulds contain chemical signals that plasmodia use as an externalised spatial memory (Reid et al., 2013) or for allorecognition between strains (Masui et al., 2018). We set up y-mazes where plasmodia chose between two 2% agar bridges to reach a food source; one bridge was blank and the other bridge was covered in extracellular secretions. We placed 15 x 15 mm squares of plasmodial search front of each species on empty 75 mm Petri dishes. Two 15 x 25 mm bridges of 2% w/v agar were placed in direct contact on perpendicular edges of the plasmodial search front square. At the end of each bridge was a 15 x 15 mm square made of 3% w/v oat and 2% w/v agar (Figure 3.3). One bridge was always composed of 2% w/v agar and the second bridge would be covered in the extracellular secretions of one of the three acellular slime mould species. The order of these two bridges were determined randomly. We observed the behaviour of the plasmodia using time-lapse photography with images taken every 10 minutes for 18 hours. A light illuminated plasmodia for five seconds for each image but otherwise experiments were kept in darkness. We used ImageJ (Rasband, 1997) to analyse the choices of the plasmodia. Plasmodia chose a bridge if they were in contact with the associated food source at the end of 18 hours. If plasmodia were in contact with both food sources their choice would be classified as a split decision.

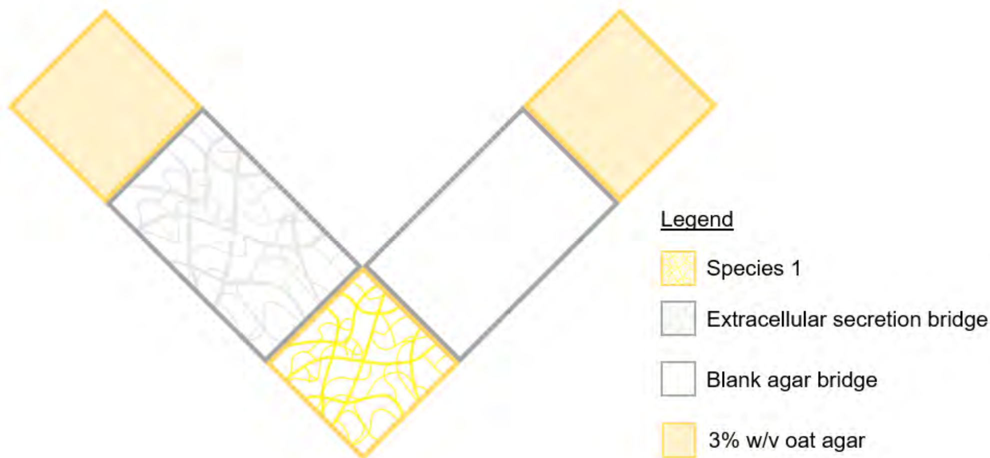


Figure 3.3: Experimental set-up with binary preference test

1570 We aimed to have 30 replicates of each species for every treatment in the Y-maze experiment.
1571 Unfortunately, our culture of *P. polycephalum* had degenerated to the extent that we were
1572 unable to produce enough biomass and only had 10 replicates for each treatment in this species.
1573 As a result, treatments that involved using *P. polycephalum* extracellular secretions were also
1574 limited to 10 replicates. The behaviour of *P. polycephalum* towards extracellular secretions have
1575 been previously studied and we will be using this previous research to guide our discussion for
1576 this species (Reid et al., 2013). We were able to maintain 30 replicates for each treatment in
1577 *D. iridis* and *P. melleum* with a total of 71 replicates and 70 replicates respectively.

1578 **Statistical analysis**

1579 **Comparing foraging behaviour between species** When comparing foraging behaviour
1580 between species, we only analysed data of plasmodia that were alone. We used a Kruskal-
1581 Wallis test to analyse differences in latency between species as the residuals of the data were
1582 non-normal. We used latency of plasmodia to begin moving as the response variable and species
1583 as the independent variable. We analysed the difference between first contact of food between
1584 species separately for homogeneous and heterogeneous environments. We used a Kruskal-Wallis
1585 test as residuals of the data were non-normal. We used time for plasmodia to first contact food
1586 as the response variable and species as the independent variable. To compare differences in total
1587 number of food sources covered by each species, we used a generalised linear model (GLM) with
1588 poisson distribution using total number of food sources covered as the response variable and
1589 species and environment type as independent variables. To compare differences in maximum
1590 food sources plasmodia could cover with their biomass between species, we used a GLM with
1591 poisson distribution using maximum number of food sources covered as the response variable
1592 and species and environment type as independent variables.

1593 **Analysing interactions between species** We used two-way ANOVAs without replication
1594 to analyse differences in latency for each species. We used environment type (blank arena,
1595 homogeneous environment or heterogeneous environment) and grouping (alone or with one of
1596 the other species) as the two independent variables. We used Levene's test to assess the equality

of variances in these models and we removed one outlier from the ANOVA for *P. melleum* so that the data would meet the assumptions of the model. Removing this outlier had no effect on the outcome of the ANOVA. We used Tukey's test to assess the significance of differences between pairs of group means as a post-hoc analysis (Abdi and Williams, 2010).

When analysing the difference of first contact to food we used a Scheirer-Ray-Hare test which is a non-parametric method used for two-way data. We used a non-parametric model as our data did not meet the assumptions of normality and heterescedascity of variances required for a two-way ANOVA without replication. We made a separate model for each species and used environment type and grouping as the two independent variables. We used Dunn's test to determine which groups were significantly different as a post-hoc analysis after the Scheirer-Ray-Hare test.

We used separate models for each species when analysing the total number of food sources covered by plasmodia. For data on *P. melleum,* we used a GLM with a zero-inflated poisson distribution as we noticed overdispersion of residuals in this data. We used total number of food sources covered by plasmodia as the response variable, and environment type and grouping as independent variables. For *D. iridis* and *P. polycephalum* data plasmodia were more likely to cover all 12 food sources, which lead to a left-skewed distribution of data. To be able to analyse these two species using a Poisson distribution, we created a separate variable for the total number of food sources that plasmodia *failed* to cover. We used a GLM with Conway-Maxwell-Poisson distribution as the residuals of both species' data were underdispersed. For *D. iridis* data, we used total number of food sources plasmodia *failed* to cover as the response variable, and environment type and grouping as independent variables. For *P. polycephalum*, we used the total number of food sources plasmodia *failed* to cover as the response variable, and environment type, grouping and an interaction term between environment type and grouping as independent variables.

When analysing the maximum number of food sources that plasmodia could cover with their biomass, we used a GLM with Poisson distribution for *D. iridis* and *P. polycephalum* data. For *D. iridis* data we used maximum number of food sources covered as the response variable,

1624 and environment type and grouping as the independent variables. For *P. polycephalum* data
1625 we used maximum number of food sources covered as the response variable, and environment
1626 type, grouping and an interaction term between environment type and grouping as independent
1627 variables. For *P. melleum* data we used a GLM with Conway-Maxwell-Poisson distribution,
1628 as the residuals in this data were underdispersed. We used maximum number of food sources
1629 covered as the response variable, and environment type, grouping and date the experiment was
1630 conducted as the independent variables.

1631 We further analysed a subset of the samples for the time it took plasmodia to contact all 12
1632 food sources. We used two-way ANOVAs without replication to analyse differences in time to
1633 contact all food sources for each species, with environment type and grouping as the independent
1634 variables. We did not assess *P. melleum* as plasmodia from this species only covered all 12 food
1635 sources when alone. We used Levene's test to assess the equality of variances in the model.

1636 We used binary variables to assess observed behaviours, with the value of 1 used for if plasmodia
1637 did exhibit the behaviours and the value of 0 used if plasmodia did not exhibit the behaviours.
1638 The behaviours we observed were: plasmodium failed to contact any food, sporulation, food
1639 co-habitation and food avoidance. Only two replicates from *P. melleum* and *P. polycephalum*
1640 failed to contact any food, therefore we did not statistically analyse this observation. We used
1641 a GLM with binomial distribution to assess sporulation in *D. iridis*, as this was the only species
1642 that sporulated during the experiment. We used the binary variable of sporulation as the
1643 response, and environment type and grouping as the independent variables. We calculated the
1644 proportion of plasmodia that co-habitated on a food source by visually assessing whether two
1645 species covered a food source at the same time. Only *P. melleum* plasmodia exhibited food
1646 avoidance, and we used a GLM with binomial distribution with food avoidance as the binary
1647 response variable and environment type and grouping as the independent variables.

1648 To analyse the model fit and assess the dispersion of our models we used the R package DHARMa
1649 (Hartig, 2020).

We calculated the preference of acellular slime mould species in the Y-maze experiment using binomial probability (P of random choice = 0.5).

3.4 Results

Characterising species foraging behaviour

We found a significant difference between the latency of all three species ($\chi^2 = 82.59$, df = 2, $P < 0.01$; Figure 3.4), with latency defined as the time taken for cut fragments of plasmodial search front to start moving in the arena. *Didymium iridis* had the fastest latency with a median time of 40 minutes, *P. polycephalum* had a slower latency than *D. iridis* with a median time of 70 minutes and *P. melleum* had the slowest latency with a median time of 200 minutes. We also found no effect of environment type on the latency of all three species (*D. iridis*: P = 0.06, *P. polycephalum*: P = 0.24, *P. melleum*: P = 0.98).

A similar pattern was found for first contact time (the time taken for plasmodia to first contact the food sources), where *D. iridis* was the fastest with a median time of 115 minutes in homogeneous environments and 130 minutes in heterogeneous environments. In homogeneous environments, *P. polycephalum* had a similar first contact time as *D. iridis,* with a median time of 120 minutes. *Physarum melleum* was significantly slower to contact food in homogeneous environments compared to the other species ($\chi^2 = 21.17$, df = 2, $P < 0.01$) with a median time of 450 minutes to first contact food. In heterogeneous environments, both *P. polycephalum* and *P. melleum* had significantly slower first contact time than *D. iridis* ($\chi^2 = 11.49$, df = 2, $P < 0.01$) with a median time of 300 minutes and 350 minutes, respectively. Environment type had no effect on first contact time of *D. iridis* or *P. melleum*, but did effect *P. polycephalum* where plasmodia took longer to first contact the food sources in the homogeneous environment compared to the heterogeneous environment (*D. iridis*: P = 0.13, *P. melleum*: P = 0.71, *P. polycephalum*: H-stat = 17.65, $P < 0.01$; Figure 3.5).

Total food contacted by plasmodia were significantly different between species (Figure 3.6). *Physarum polycephalum* had higher total food contacted than *P. melleum* (OR = 3.24, 95%

1676 CI: 2.70 - 3.92) but similar total food contacted as *D. iridis* (OR = 1.12, 95% CI: 1.0 - 1.25).
1677 *Didymium iridis* also had a higher total food contacted than *P. melleum* (OR = 2.90, 95%
1678 CI: 2.41 - 3.51). Environment type had no effect on total food contacted for *D. iridis* (Table
1679 3.2). For *P. melleum,* the environment type drove the difference where total food contacted
1680 by *P. melleum* was higher in the heterogeneous environment compared to the homogeneous
1681 environment (Table 3.3). The effect of environment type for *P. polycephalum* depended on the
1682 presence of other species, and will therefore be discussed further below (Table 3.4).

1683 We also analysed the amount of food plasmodia could contact at a time in a variable called
1684 maximum food contacted (Figure 3.7). We found a significant difference between species where
1685 *D. iridis* had a higher maximum food contacted than *P. polycephalum* (OR = 1.60, 95% CI: 1.38
1686 - 1.85) and *P. melleum* (OR = 3.79, 95% CI: 3.04 - 4.78). *Physarum polycephalum* also had a
1687 higher maximum food contacted than *P. melleum* (OR = 2.36, 95% CI: 1.87 - 3.02). We found
1688 no effect of environment type on maximum food contacted by *D. iridis* (Table 3.5). However,
1689 maximum food contacted by *P. melleum* and *P. polycephalum* were higher in the heterogeneous
1690 environment compared to homogeneous environment (Table 3.6 & 3.7; Figure 3.7).

1691 **Direct interactions between species**

1692 We found that latency was shorter when *D. iridis* was paired with both *P. polycephalum* (Type
1693 III ANOVA: F-stat = 3.57, df = 2, P = 0.03) and *P. melleum* (Type III ANOVA: F-stat =
1694 3.28, df = 2, P = 0.04; Figure 3.4). Latency of *D. iridis* was not affected by the presence of
1695 other species (P = 0.09). We also found no effect of environment type on the latency of all
1696 three species (*D. iridis*: P = 0.06, *P. polycephalum*: P = 0.24, *P. melleum*: P = 0.98).

1697 First contact time (the time taken for plasmodia to first contact the food sources) did not
1698 differ between plasmodia regardless of grouping in all three species, but first contact time of *P.*
1699 *melleum* were slower when plasmodia were paired with *D. iridis* compared to when plasmodia
1700 were paired with *P. polycephalum* (*D. iridis*: P = 0.91, *P. polycephalum*: P = 0.24, *P. melleum*:
1701 H-stat = 10.89, P < 0.01; Figure 3.5).

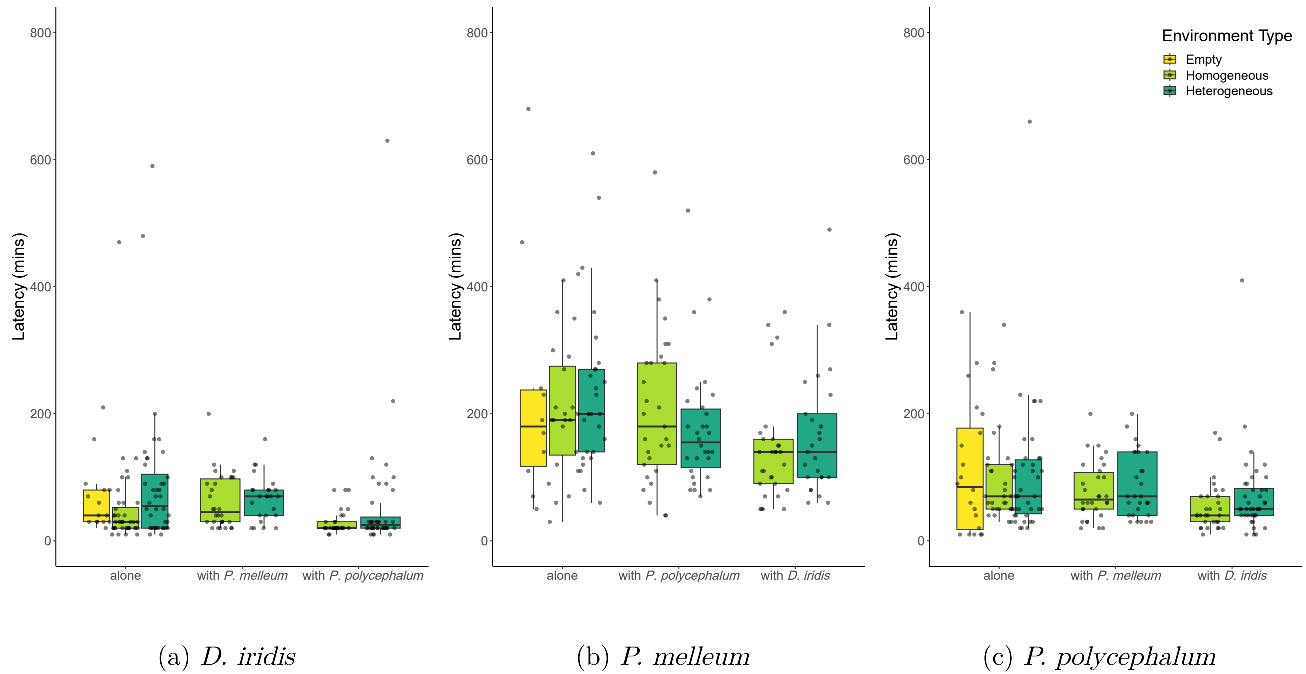
Total food contacted by *D. iridis* plasmodia increased when paired with *P. polycephalum* but not when paired with *P. melleum* (Table 3.2). The presence of *D. iridis* however, had no effect on the total food contacted by *P. polycephalum* (Table 3.2). Instead, total food contacted by *P. polycephalum* increased when paired with *P. melleum* (Table 4). We also found an interaction between environment type and grouping, where the effect of environment type on the total food contacted by *P. polycephalum* depended on whether plasmodia were paired with *P. melleum* or *D. iridis*. Total food contacted by *P. polycephalum* plasmodia were fewer in homogeneous environments when plasmodia was paired with *P. melleum*, but when *P. polycephalum* were paired with *D. iridis* plasmodia, total food contacted were higher in homogeneous environments. Total food contacted by *P. melleum* was not affected by the presence of either *D. iridis* or *P. polycephalum.* Maximum food contacted by *D. iridis* increased when paired with *P. polycephalum* (Table 3.5). Maximum food contacted by *P. polycephalum* also increased when paired with *D. iridis* (Table 3.6). Once again, both *D. iridis* and *P. polycephalum* had no effect on maximum food contacted by *P. melleum* (Table 3.7).

We analysed observations of plasmodia behaviour during the experiment, specifically of sporulation, food co-habitation and food avoidance. There was no effect of environment type or grouping on sporulation of *D. iridis* (Table 3.8). We found that *D. iridis* and *P. polycephalum* co-habited at least one food source in 97.14% of tests. We found fewer cases of food co-habitation with *P. melleum*, where *P. melleum* co-habited a food source in 48.33% of tests when paired with *P. polycephalum*, and 41.82% of tests when paired with *D. iridis.*

Physarum melleum demonstrated a unique behaviour where plasmodia avoided food sources (Supplementary video 3.1). Food avoidance decreased in *P. melleum* when paired with *P. polycephalum* (Table 3.9). There was no effect of environment type on observations of plasmodia food avoidance behaviour (Table 3.9).

1726 **Allelopathic interactions between species**

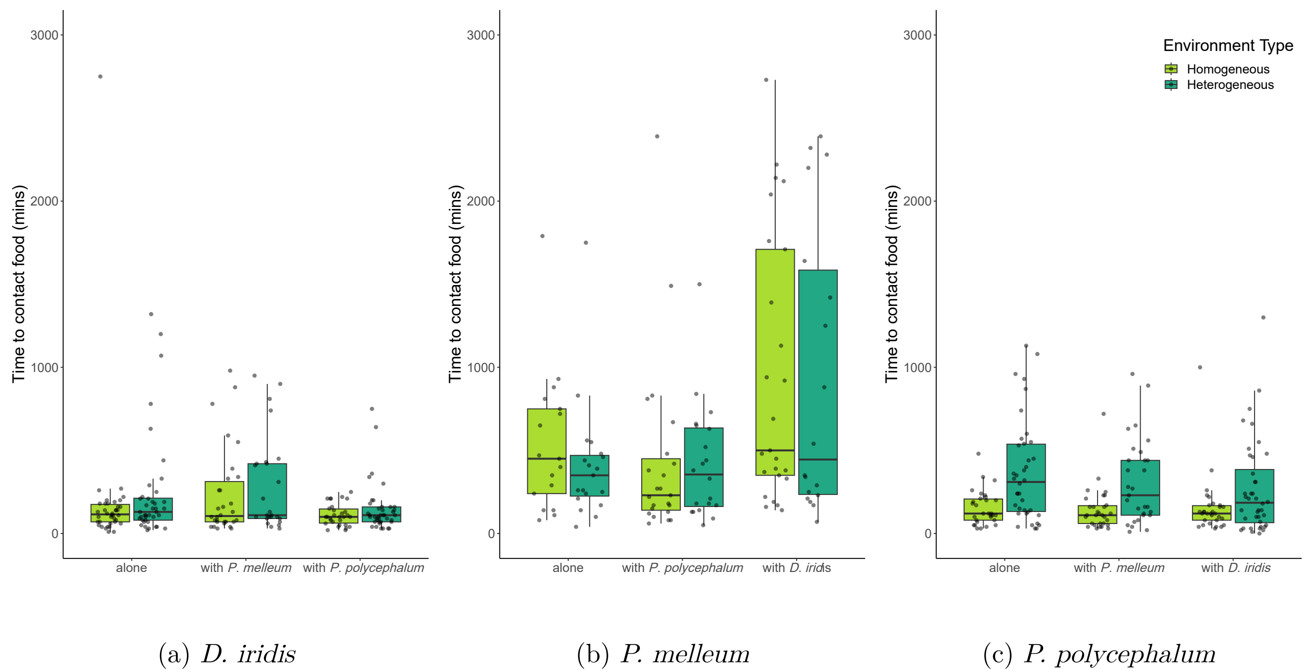
1727 We tested whether plasmodia preferred travelling on extracellular secretions or blank agar using
1728 binary tests (Supplementary data 3.2). We found that *D. iridis* preferred travelling on the
1729 extracellular secretions of *P. melleum* over blank agar (Binomial test (probability of 0.5): P
1730 < 0.01, $N = 31$). Additionally, *D. iridis* plasmodia also preferred travelling on their own
1731 extracellular secretions over blank agar (Binomial test (probability of 0.5): $P < 0.01$, $N =$
1732 30). *Physarum melleum* plasmodia preferred to travel on *D. iridis* extracellular secretions,
1733 with 73.9% of plasmodia travelling on the *D. iridis* bridge to access the food (Binomial test
1734 (probability of 0.5): $P < 0.01$, $N = 30$). Unlike *D. iridis*, *P. melleum* plasmodia avoided
1735 their own extracellular secretions with 100% of plasmodia using the blank agar bridge to access
1736 food (Binomial test (probability of 0.5): $P < 0.01$, $N = 30$). *Physarum melleum* also showed
1737 a preference for the blank agar bridge over a bridge covered with the extracellular secretions
1738 of *P. polycephalum* (Binomial test (probability of 0.5): $P = 0.04$, $N = 10$). As *P. melleum*
1739 exhibited food avoidance in our experiments, we also assessed whether plasmodia would prefer
1740 to travel over a bridge covered in the extracellular secretions of *D. iridis* or to immediately
1741 consume a portion of 3% w/v oat agar. We found that only 5.26% of *P. melleum* plasmodia
1742 traveled on *D. iridis* extracellular secretions and the majority of plasmodia chose the 3% w/v
1743 oat agar instead (Binomial test (probability of 0.5): $P < 0.01$, $N = 20$). *Physarum polycephalum*
1744 appeared ambivalent to the extracellular secretions of the other species, with 50% travelling on
1745 the arm with *P. melleum* extracellular secretions and 25% travelling on the arm with *D. iridis*
1746 extracellular secretions. It should be noted that sample size of both of the *P. polycephalum*
1747 binary preference tests were low, at 10 replicates, as the health of *P. polycephalum* plasmodia
1748 had diminished by the time of this experiment.



(a) *D. iridis* (b) *P. melleum* (c) *P. polycephalum*

Figure 3.4: Latency of plasmodia (mins). N = 429.

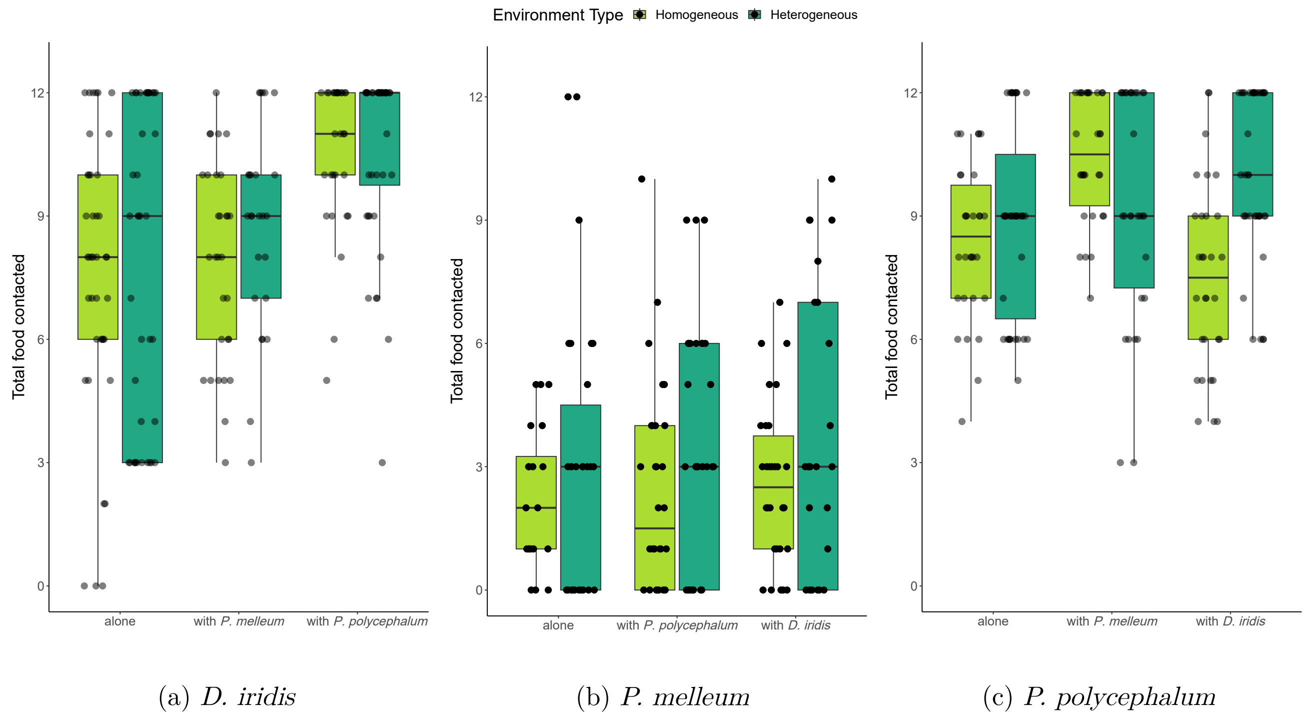
1749



(a) *D. iridis* (b) *P. melleum* (c) *P. polycephalum*

Figure 3.5: Time for plasmodia to first contact food (mins). N = 384.

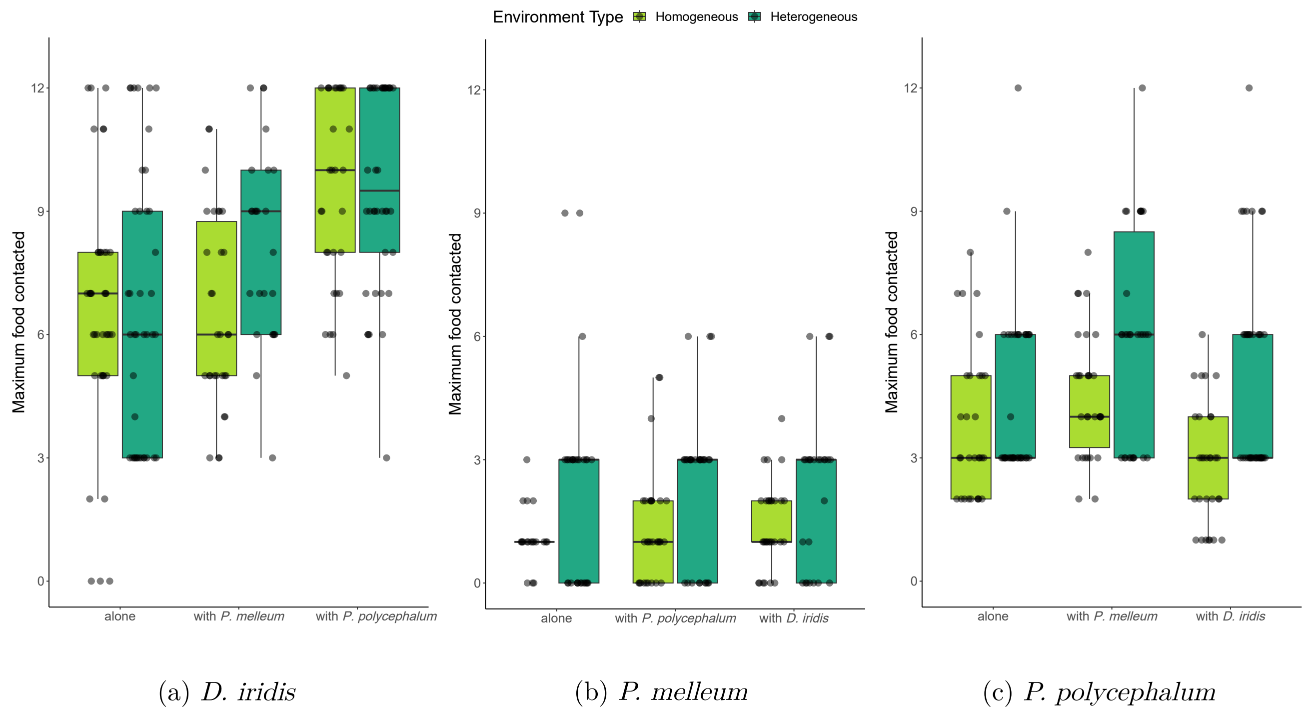
1750



(a) *D. iridis* (b) *P. melleum* (c) *P. polycephalum*

Figure 3.6: Total food contacted by plasmodia. N = 384.

1751



(a) *D. iridis* (b) *P. melleum* (c) *P. polycephalum*

Figure 3.7: Maximum food contacted by plasmodia. N = 384.

1752

1753 Table 3.2: Model summary of total food that *D. iridis* failed to contact

Fixed Effects

Parameter	Coefficient	SE	95% CI	z	p
(Intercept)	1.51	0.12	(1.28, 1.73)	12.94	< .001
Environment (Heterogeneous)	-0.10	0.13	(-0.36, 0.16)	-0.76	0.444
Grouping (*P. melleum*)	-0.15	0.15	(-0.45, 0.15)	-0.98	0.328
Grouping (*P. polycephalum*)	-1.09	0.17	(-1.42, -0.76)	-6.42	< .001

1754 Table 3.3: Model summary of total food contacted by *P. melleum*

Parameter	Coefficient	SE	95% CI	z	p
(Intercept)	331.73	105.37	(125.21, 538.26)	3.15	0.002
Environment (Heterogeneous)	0.62	0.10	(0.42, 0.82)	6.10	< .001
Grouping (*P. polycephalum*)	-0.04	0.12	(-0.29, 0.20)	-0.36	0.719
Grouping (*D. iridis*)	0.03	0.12	(-0.21, 0.26)	0.21	0.831
Date	-0.02	5.52e-03	(-0.03, -6.50e-03)	-3.14	0.002

1755 Table 3.4: Model summary of total food that *P. polycephalum* failed to contact

Parameter	Coefficient	SE	95% CI	z	p
(Intercept)	1.29	0.13	(1.03, 1.55)	9.64	< .001
Environment (Heterogeneous)	-0.16	0.18	(-0.52, 0.20)	-0.86	0.388
Grouping (P. melleum)	-0.84	0.23	(-1.29, -0.40)	-3.71	< .001

Parameter	Coefficient	SE	95% CI	z	p
Grouping (*D. iridis*)	0.21	0.18	(-0.14, 0.57)	1.18	0.239
Interaction: Environment x Grouping (*P. melleum)*	0.70	0.30	(0.12, 1.29)	2.35	0.019
Interaction: Environment x Grouping (*D. iridis)*	-0.68	0.26	(-1.20, -0.16)	-2.57	0.010

1756 Table 3.5: Model summary of maximum food contacted by *D. iridis*

Parameter	Log-Mean	SE	95% CI	z	p
(Intercept)	1.85	0.05	(1.75, 1.95)	36.55	< .001
Environment (Heterogeneous)	0.07	0.05	(-0.03, 0.17)	1.32	0.186
Grouping (*P. melleum*)	0.10	0.07	(-0.03, 0.23)	1.55	0.122
Grouping (*P. polycephalum*)	0.37	0.06	(0.25, 0.48)	6.32	< .001

1757 Table 3.6: Model summary of maximum food contacted by *P. melleum*

Parameter	Coefficient	SE	95% CI	z	p
(Intercept)	0.17	0.17	(-0.16, 0.50)	1.01	0.311
Environment (Heterogeneous)	0.59	0.15	(0.29, 0.88)	3.91	< .001
Grouping (*P. polycephalum*)	0.12	0.18	(-0.23, 0.47)	0.66	0.511
Grouping (*D. iridis*)	0.12	0.18	(-0.24, 0.48)	0.65	0.514

1758 Table 3.7: Model summary of maximum food contacted by *P. polycephalum*

Parameter	Log-Mean	SE	95% CI	z	p
(Intercept)	1.34	0.09	(1.15, 1.51)	14.25	< .001
Environment (Heterogeneous)	0.15	0.12	(-0.09, 0.39)	1.23	0.218
Grouping (*P. melleum*)	0.18	0.13	(-0.07, 0.43)	1.39	0.165
Grouping (*D. iridis*)	-0.29	0.14	(-0.58, -0.01)	-2.05	0.041
Interaction: Environment x Grouping (*P. melleum*)	0.12	0.17	(-0.21, 0.45)	0.72	0.469

Parameter	Log-Mean	SE	95% CI	z	p
Interaction: Environment x Grouping (*D. iridis*)	0.42	0.18	(0.08, 0.77)	2.40	0.017

1759 Table 3.8: Model summary of sporulation of *D. iridis*

Parameter	Log-Odds	SE	95% CI	z	p
(Intercept)	-1.87	0.76	(-3.73, -0.59)	-2.46	0.014
Environment (Homogeneous)	1.22	0.81	(-0.20, 3.14)	1.50	0.134
Environment (Heterogeneous)	0.82	0.82	(-0.61, 2.74)	1.00	0.316
Grouping (*P. melleum*)	-0.06	0.39	(-0.83, 0.69)	-0.16	0.872
Grouping (*P. polycephalum*)	-0.11	0.37	(-0.84, 0.60)	-0.31	0.758

1760 Table 3.9: Model summary of food avoidance of *P. melleum*

Parameter	Log-Odds	SE	95% CI	z	p
(Intercept)	1.71	0.43	(0.92, 2.59)	4.01	$< .001$
Environment (Heterogeneous)	-0.50	0.35	(-1.20, 0.17)	-1.45	0.148
Grouping (*P. polycephalum*)	-1.52	0.44	(-2.43, -0.68)	-3.42	$< .001$
Grouping (*D. iridis*)	-0.57	0.47	(-1.52, 0.33)	-1.23	0.220

3.5 Discussion

We found distinct differences in foraging behaviour between the three different acellular slime mould species. *Didymium iridis* had a fast behavioural type, with the shortest latency and and a fast first contact time. *Didymium iridis* also had the highest maximum food contacted. Behaviour of *D. iridis* did not vary between homogeneous and heterogeneous environments, as none of our observations were affected by environment type. Our result is interesting as previous behavioural tests of *D. iridis* found that this species had a more variable approach for different foraging environments (Yip et al., 2014). In an experiment conducted by Yip and colleagues (2014) acellular slime mould search strategies were assessed after contact with food of different qualities. Our experiment observed the behaviour of acellular slime moulds in environments with different spatial configurations rather than food quality. Perhaps the foraging strategy of *D. iridis* depends on the concentration of nutrients in the food that they consume rather than the spatial configuration of food patches.

Physarum polycephalum had similar first contact time and number of total food contacted as *D. iridis* but a lower maximum number of food contacted. This demonstrates how both species have a similar ability in finding food sources but have different foraging styles. The high maximum contact of *D. iridis* shows how this species spreads through the environment while creating a larger network, whereas *P. polycephalum* moves around the environment making less extensive networks. Environment type affected *P. polycephalum* behaviour where maximum food contact was higher and first contact speed was slower in heterogeneous environments. This result further corroborates our finding that *P. polycephalum* makes less extensive networks, as it is easier to connect multiple food sources in a heterogeneous environment due to the fact that each patch has three food sources. Slower first contact speed of *P. polycephalum* in heterogeneous environments may suggest that this species has lower accuracy in finding food as food sources in homogeneous environments are evenly spaced making it more likely to contact a food source by random chance over the heterogeneous environment.

Search behaviour of *P. melleum* was the most different from other species as their behaviour

was not affected by the presence of other species but was strongly affected by environment type. In heterogeneous environments, total and maximum food contacted were higher for *P. melleum*. Interestingly, the time to first contact food was not different between homogeneous and heterogeneous environments. These results indicate that *P. melleum* has a higher accuracy in finding food sources, as they were able to contact a food source in a patchily distributed environment in a similar time as an evenly distributed environment. Higher total and maximum food contacted in heterogeneous environments suggest that *P. melleum* plasmodia do not create an extensive network and instead consume adjacent food patches. *Physarum melleum* was the slowest acellular slime mould species, with the longest latency and slow first contact time (Figure 3.4 & Figure 3.5). In addition, this species contacted less total food sources than both *D. iridis* and *P. polycephalum* (Figure 3.6). Higher accuracy in finding food sources in *P. melleum* may be an adaptation for survival as a less dominant species. In plants, subordinate species use high precision foraging by making fine adjustments to leaf and root distribution to compete in heterogeneous environments (Campbell et al., 1991).

Of the three species, only *D. iridis* sporulated during the experiment. Acellular slime mould sporulation involves the creation of thousands of spores in specialised structures (Martin and Alexopoulos, 1969). Each spore can become a viable myxamoeba, a microscopic amoeba often found in soil, and will eventually fuse with another myxamoeba to grow into a plasmodium (Martin and Alexopoulos, 1969). Ideal sporulation conditions are relatively unknown and may differ between species, but sporulation appears to occur in brighter light conditions (Hosoda, 1980). We cannot be certain whether the sporulation of *D. iridis* was a stress response to our experimental set-up, although the ability of this species to successfully cover many food sources may indicate that sporulation is a positive response to having consumed sufficient nutrients during our experiment.

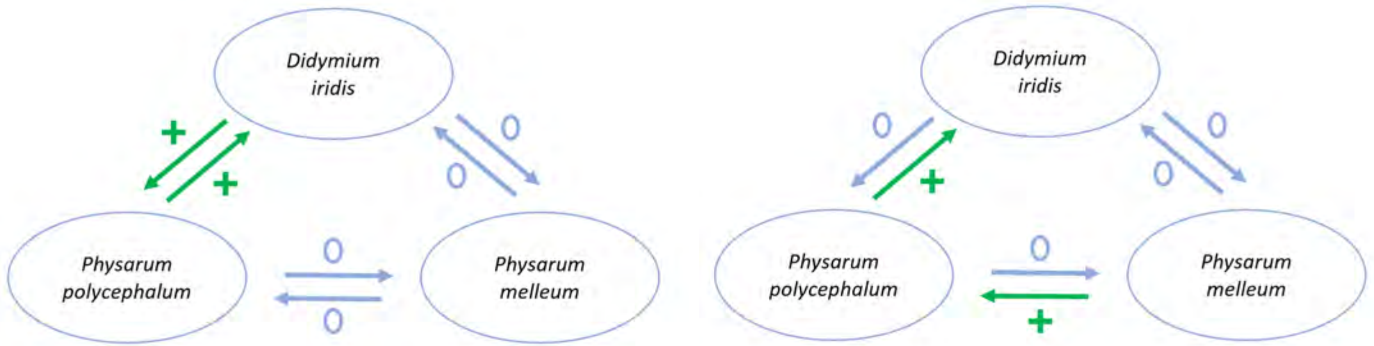


Figure 3.8: Interspecific interactions between species. Left: Maximum food contacted. Right: Total food contacted

We found evidence of facilitation in interspecific interactions, where the presence of another species increased the amount of food consumed (Figure 3.8). Maximum food contacted by *D. iridis* and *P. polycephalum* increased when paired with each other, demonstrating a mutualistic interaction. When we looked at the total food contacted by plasmodia, we found commensalism where the total food contacted by *D. iridis* increased in the presence of *P. polycephalum*, but the total food contacted by *P. polycephalum* was not affected. Instead, total food contacted by *P. polycephalum* increased when in the presence of *P. melleum*. The interaction between *P. polycephalum* and *P. melleum* was commensalism, as the total food contacted by *P. melleum* did not change when paired with *P. polycephalum*. The mechanisms of how one species facilitates another were not obvious through visual observations of the experiment. Do *D. iridis* and *P. polycephalum* use extracellular secretions of other species to increase their food consumption ability? Results from tests on extracellular secretion preference were inconclusive.

Despite being facilitated by *P. polycephalum*, *D. iridis* plasmodia were not attracted to *P. polycephalum* extracellular secretions. This suggests that *D. iridis* were not following cues from *P. polycephalum* to find more food. Allelopathic inhibition by *P. polycephalum* through repellent cues from extracellular secretions were also unlikely, as both species had a high frequency of cohabitation on food. Our experimental set-up did not allow us to observe the amount of nutrition plasmodia were able to gain from the food sources and we could only observe how many food sources each plasmodium contacted. Perhaps the nutrition in food shared between *D. iridis* and *P. polycephalum* was depleted faster, causing *D. iridis* to move on to the next

1832 food source more rapidly and contacting a higher total number of food sources. This would
1833 indicate that *P. polycephalum* actually had a negative effect on *D. iridis*. Two observations
1834 cast doubt on this theory. We recorded the time taken for *D. iridis* to contact all 12 food
1835 sources. If pressure from food sharing with *P. polycephalum* caused *D. iridis* to move to new
1836 food more rapidly, the time taken to contact all 12 food sources should be shorter when with
1837 *P. polycephalum*. Our results showed the contrary, where there was no effect of the presence
1838 of another species or of the environment type on the time taken to contact all 12 food sources.
1839 The second observation that makes this theory unlikely, is the higher maximum food contacted
1840 by *D. iridis* in experiments with *P. polycephalum*. Maximum food contacted indicates network
1841 size, capturing a plasmodium's ability to exploit multiple food sources at once. If *D. iridis*
1842 plasmodia were gaining less nutrition from food cohabited with *P. polycephalum*, they should
1843 have less available biomass to allocate across their network, leading to a smaller network size.
1844 In fact, *D. iridis* had a larger network when paired with *P. polycephalum* compared to when
1845 they were alone, suggesting that they were gaining more nutrition when in the presence of *P.*
1846 *polycephalum*. *Physarum polycephalum* also had higher maximum contact when paired with *D.*
1847 *iridis*, suggesting that both species had a positive effect on network size of each other.

1848 A similar puzzling relationship was observed between *P. polycephalum* and *P. melleum*. Again,
1849 total and maximum food contacted by *P. polycephalum* increased when in the presence of *P.*
1850 *melleum*. In binary preference tests, *P. polycephalum* appeared ambivalent to extracellular se-
1851 cretions from *P. melleum*, which suggests that they were not using cues from *P. melleum* to
1852 find more food. *Physarum melleum* were repelled by extracellular secretions of *P. polycephalum*,
1853 which may have allowed *P. polycephalum* to access more food in experiments where they were
1854 paired with *P. melleum*. If the relationship between *P. polycephalum* and *P. melleum* was exclu-
1855 sionary in nature, one would expect *P. melleum* to contact fewer food sources when paired with
1856 *P. polycephalum*. In actuality, our results found no effect of the presence of both *P. polycephalum*
1857 or *D. iridis* on the total and maximum food contacted by *P. melleum*. Additionally, *P. melleum*
1858 exhibited food avoidance behaviour, where plasmodia would move around food sources rather
1859 than covering them directly. We found that this behaviour decreased when *P. melleum* was in

the presence of *P. polycephalum*. Despite some indications of a negative relationship between *P. polycephalum* and *P. melleum*, such as repellent behaviour towards extracellular secretions of *P. polycephalum*, we found no evidence of direct competitive interactions between these two species.

Interestingly, we found no evidence of competitive interactions between any species-species pairs in our experiment. All effects in our experiment that lead to positive outcomes for acellular slime mould species did not negatively affect others. Besides direct increases in total and maximum food contact, we also found positive effects in other observations. The first was a significantly faster latency time for both *P. polycephalum* and *P. melleum* when in the presence of *D. iridis*. We also found that food avoidance behaviour in *P. melleum* decreased when paired with *P. polycephalum*. Neither of these observations lead to an increase in food contact in affected species. Other observations that we expected would lead to increases in food contact also had no effect. In the binary experiment, *P. melleum* plasmodia were attracted to extracellular secretions of *D. iridis*, despite being repelled by their own extracellular secretions and that of *P. polycephalum*. As *D. iridis* had high total contact of food, one might expect *P. melleum* to follow *D. iridis* extracellular secretions and cover more food sources, but total and maximum food contacted of *P. melleum* were not affected by *D. iridis* presence. Alternatively, *P. melleum* may be extremely attracted to *D. iridis* extracellular secretions which might cause them to avoid contacting food sources altogether. If this was the case, in experiments paired with *D. iridis*, we would expect a higher incidence of food avoidance in *P. melleum* and lower total and maximum food contact. Rates of food avoidance were the same when *P. melleum* were alone and paired with *D. iridis*. Additionally, in binary preference experiments we presented *P. melleum* with the choice between directly consuming a food source and travelling on a bridge with *D. iridis* extracellular secretions, and 94% of plasmodia chose the food source.

There is a possibility that acellular slime mould plasmodia have other mechanisms to indirectly affect the behaviour of other species apart from extracellular secretions. Results from an investigation between clones of *P. polycephalum* tentatively suggest that plasmodia may be able to

sense the satiation of others without the use of extracellular secretions (Stirrup and Lusseau, 2019). Starved plasmodial clones were faster at finding food when paired with a satiated clone compared to another starved clone (Stirrup and Lusseau, 2019). The interactions between clones of an acellular slime mould are unique as clones have the ability to fuse together into a single cell and it is possible that *P. polycephalum* plasmodia may have allorecognition mechanisms similar to those observed in *P. rigidum* (Masui et al., 2018) . In contrast, allorecognition behaviour has not been observed between different species of acellular slime mould, and further investigations are required to elucidate the mechanisms plasmodia may use to indirectly affect other species.

We found evidence of facilitation between three species of acellular slime moulds but no evidence of competition. Due to the sequential nature of decomposition, there is a large potential for facilitative interactions to arise among microbial decomposers and litter organisms (Gessner et al., 2010). Facilitation helps to mitigate harsh environmental conditions and may help maintain local maladapted ecotypes within a habitat (Soliveres et al., 2015; Valiente-Banuet and Verdú, 2007). Additionally, there is evidence that facilitative interactions could help preserve less adapted lineages to recent environmental conditions (Soliveres et al., 2015; Valiente-Banuet and Verdú, 2007), highlighting the importance of facilitative interactions in a rapidly changing environment. Facilitative interactions in plants increase along a stress gradient, with facilitation increasing with increased abiotic and biotic stress while competitive interactions decrease (He et al., 2013; Valiente-Banuet and Verdú, 2007). Species interactions are one of the drivers that influence ecosystem properties, increasing biodiversity and driving local evolution (Hooper et al., 2005; Jensen and Ehlers, 2010; Valiente-Banuet and Verdú, 2007). In our small sample, we have found evidence of facilitative interactions between acellular slime mould species. In addition, we have identified three distinct foraging behaviours in the species that we observed. Acellular slime moulds are only a sub-section of the larger protist kingdom, but this high variability of behaviour and interactions seen in our experiment points to the large diversity of functional characteristics within acellular slime moulds and perhaps of protists as a whole. Our knowledge of the role of protists in decomposition systems is growing (Adl and Gupta, 2006; Clarholm, 1985; Geisen et al., 2021, 2018; Hünninghaus et al., 2017) and further investigations

1915 of functional characteristics of protist species will increase our understanding of the role these
1916 species play in the structure and functioning of our ecosystems.

1917 ## 3.8 Supplementary Data

1918 ### Supplementary video 3.1

1919 Food avoidance behaviour in *P. melleum*

1920 Youtube video link: https://youtu.be/R65ilxpPm_0

1921 ### Supplementary data 3.2

Binomial probability of plasmodia choosing bridge with extracellular secretions (ECS)

Species	Bridge 1	Bridge 2	N	Prop. that chose ECS	pBin
P. melleum	Blank agar	*P. melleum* ECS	25	0	1
P. melleum	Blank agar	*P. polycephalum* ECS	8	0.125	0.965
P. melleum	Blank agar	*D. iridis* ECS	23	0.739	0.00531
P. melleum	3% oat agar	*D. iridis* ECS	19	0.053	1
P. polycephalum	Blank agar		2	0.5	0.25
P. polycephalum	Blank agar		8	0.25	0.855
D. iridis	Blank agar		28	0.821	9e-05
D. iridis	Blank agar	*P. polycephalum* ECS	5	0.2	0.812
D. iridis	Blank agar	*D. iridis* ECS	19	0.737	0.00961
D. iridis	3% oat agar	*P. melleum* ECS	17	0.059	1

Chapter 4

Thesis under emergency conditions: Covid-19 Impacts

Arisa Hosokawa, Chris Reid and Tanya Latty

Chapter detailing planned experiments that could not be completed due to laboratory and travel restrictions caused by the Covid-19 pandemic.

Author contributions: AH, MB, CR and TY contributed to the study design. AH conducted pilot experiments. CR and TY provided valuable feedback and editing.

4.1 Behaviour of acellular slime moulds after entering resistant life stages.

Introduction

Many soil protists can transform into resistant structures to survive adverse environmental conditions (Geisen et al., 2018). Free-living amoebae from the genus *Acanthamoeba* can develop into resistant cysts, with some species able to re-emerge from dormancy after 20 years (Sriram et al., 2008). *Protosiphon botryoides*, a green algae protist, can survive 43 years as a cyst and re-emerge from dormancy (Lewis and Trainor, 2012). Parasitic soil protists, such as *Entamoeba sp.* and *Giardia sp.* are also able to form resistant cysts (Adam, 1991; McConnachie, 1969). Protists from the genus *Giardia* are often parasites of mammals and become resistant cysts when they enter the acidic environment of their hosts stomach (Adam, 1991). A diverse range of protist cysts found in permafrosts have also been re-activated from dormant and resistant states (Shatilovich et al., 2009), including ciliates (Shatilovich et al., 2015) and amoebozoa (Shmakova et al., 2016). Resistant structures are an important mechanism for survival in soil protists, as it allows them to survive frequent and cyclical dry periods in the soil (Adl and Gupta, 2006).

Plasmodia of acellular slime moulds are able to enter a dormant life stage called a sclerotium (Figure 4.1). Sclerotia form when plasmodia encounter adverse environmental conditions such as low temperature, dry conditions or harmful chemicals (Jump, 1954). Before a sclerotium develops, the flow of cytoplasm in plasmodia slows down and nucleus distribution becomes more uniform. Plasmodia then segment into smaller spherules with an outer wall and nuclei within these spherules shrink to 50% of their original diameter. Then, the whole structure becomes brittle and hard as it forms a sclerotium. When sclerotia are rehydrated, they can return to active plasmodia. First, the spherules formed during sclerotia development begin to swell and expand, then nuclei become enlarged and mitochondria become fully formed (Anderson, 1992). Pseudopodial growths are formed which rupture the walls of spherules, allowing cytoplasm to stream between spherules and create a network. Once this network is formed, cell components

begin to scatter more widely throughout the plasmodium. When transforming into sclerotia and then back to plasmodia, the structure of nuclei and mitochondria change dramatically. One of my planned research aims was to investigate whether physiological, behavioural and cellular traits would be preserved in plasmodia that become sclerotia and then were re-activated.

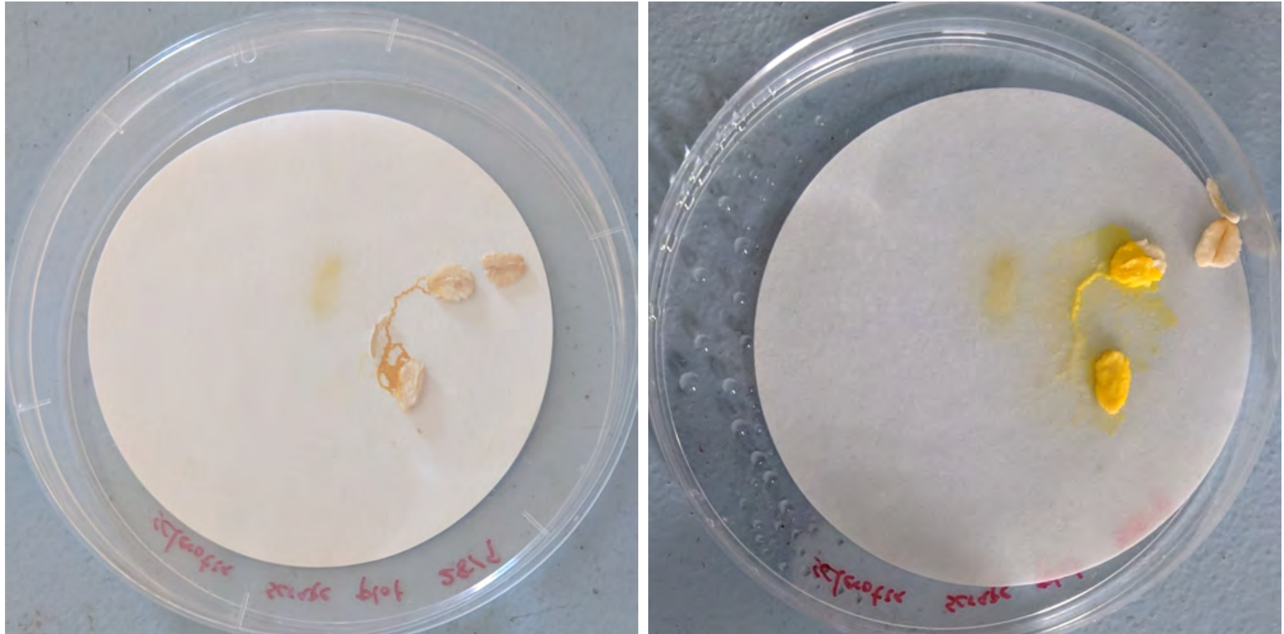


Figure 4.1: Left: Sclerotium with four oat flakes. Right: Plasmodium growing from moistened sclerotium.

Methods

My research into behaviour of post-sclerotised plasmodia was planned as an extension of experiments performed in Chapter 1 and Chapter 2. Five samples of sclerotia were prepared by taking plasmodial fragments from plasmodia grown in 1.75 L containers filled with 1% agar and oat flakes and growing them in Petri dishes lined with filter paper that had been moistened with distilled water. The filter paper gradually dried over five days which caused plasmodia to form sclerotia. Sclerotia from strain Tu111xAI35-H were prepared at 39, 59, 90, 122, 151, 186, 212, 239, 270, 303 days of age. Sclerotia from strain Tu9xDP89-S were prepared at 36, 59, 93, 122, 151, 186, 212, 239, 270, 303 days of age. Plasmodia successfully developed into sclerotia at every age point.

I planned to run this experiment approximately 60 days after the final sclerotia were prepared,

to allow for an adequate period of dormancy for all sclerotia. Sclerotia of both strains at 36/39, 151 and 303 days of age would be re-activated and post-sclerotised plasmodia would undergo physiological, behavioural and nuclei imaging assays as described in Chapter 1 methods and foraging behaviour investigation as described in Chapter 2 methods. Previous research has shown that re-activation of sclerotia of *P. polycephalum* is greatly reduced after 8-13 months in storage (Hodapp, 1942), and sclerotia viability is completely lost after two to three years (Anderson, 1992; Gehenio, 1944). Due to restrictions to laboratory access caused by the Covid-19 pandemic, I was unable to complete this experiment before the viability in my sclerotia samples were lost.

Contribution to thesis

A comprehensive comparison of physiology, behaviour and cellular traits pre- and post-sclerotisation has never been conducted in acellular slime moulds. Plasmodia of acellular slime moulds are most active on rotting logs and in the leaf litter; environments that are prone to changes in moisture and temperature. It is likely that acellular slime moulds cycle between plasmodia and sclerotia while exploring their environment. Results from my planned experiment may have revealed whether behaviourial, physiological or cellular traits changed or remained constant after sclerotia were formed.

It is possible that behavioural, physiological or cellular traits in plasmodia change post-sclerotisation, as the structure of nuclei and mitochondria change between plasmodia and sclerotia life stages. In addition, the development of spherules when plasmodia transform into sclerotia may be similar to the spherules that I observed during the senescence-like event in Tu9xDP89-S plasmodia (see Chapter 1). After plasmodia of Tu9xDP89-S recovered from the senescence-like event, cell health rapidly improved. This indicates that a similar renewing process may occur after plasmodia become sclerotia, as the reduction in biomass during the development of sclerotia may decrease the proportion of damaged cellular components in the cell.

1998 This planned experiment would have made an important contribution to my thesis, as it would
1999 have provided observations of behavioural variability within individuals that had undergone
2000 dormancy. In addition, I may have observed species-level variation as changes in behaviour
2001 post-sclerotisation may have differed between strains of *P. polycephalum*.

2002 4.2 Interspecific interactions in different geographies.

2003 Introduction

2004 My second planned experiment would have investigated the relationship between habitats that
2005 acellular slime moulds species are found and the interspecific interactions between these species.
2006 These experiments would have been an extension of the interspecific interactions experiment
2007 that I conducted in Chapter 3.

2008 The diversity of acellular slime mould species varies widely depending on their habitat. Diversity
2009 can vary at small scales, such as between different microhabitats, which are small and highly
2010 specialised sections of habitat such as tree bark, litter, dung, soil or inflorescences (Stephenson
2011 (1989); reviewed by Liu et al. (2015); Figure 4.2). For example, litter microhabitats have been
2012 found to have high species abundance (Costa et al., 2014; Ndiritu et al., 2009). Acellular slime
2013 mould species can also be highly specialised, with some species only found in microhabitats
2014 such as dung (Bezerra et al., 2008; Calaça et al., 2020; Eliasson, 2013; Stephenson et al., 2022)
2015 or snowbanks (Dagamac et al., 2021; Schnittler et al., 2015; Stephenson and Shadwick, 2009).
2016 Diversity can also vary at large scales, such as between climate regions. Species distribution
2017 can be highly dependent on climate regions. For example in tropical or sub-tropical regions,
2018 species from the *Physarum* and *Didymium* genus are predominant (Stephenson et al., 1993).
2019 In temperate coniferous regions, species from the geneus *Cribaria* and *Trichia* are predominant
2020 (Stephenson et al., 2000). Patterns of diversity in microhabitats also differ between climate
2021 regions. In tropical regions, aerial microhabitats (dead leaves and flowers that are still attached
2022 to vegetation) have the highest diversity of acellular slime moulds whereas in temperate regions
2023 the highest diversity is found in forest floor microhabitats (Stephenson, 2011). As there are

2024 distinct differences in patterns of species diversity in different climate regions, I wanted to
2025 investigate whether there was behavioural variation between species of acellular slime moulds
2026 from different climate regions. In addition, I wanted to investigate differences in behaviour at
2027 small scales, by comparing the behaviour and interspecific interactions found in species from
2028 different microhabitats.

(a) *Arcryria* sp. on the bark of a living tree.

(b) Yellow plasmodium on bark in a rotting log.

(c) White plasmodium on leaf litter.

(d) Brown plasmodium on leaf litter.

Figure 4.2: Acellular slime moulds found on various microhabitats

2029 Methods

2030 This planned experiment would have followed the experimental design detailed in Chapter 3.
2031 I planned to repeat this experiment in two other geographical regions: Lethbridge in Alberta,

2032 Canada (which has a semi-arid climate) and Tokyo in the Kanto region, Japan (which has a
2033 sub-tropical climate). I planned to sample litter microhabitats in these additional geographical
2034 regions, to compare differences in interspecific interactions between climate regions. In addition,
2035 I planned to sample different microhabitats in the Greater Sydney bio-region to compare differ-
2036 ences in interspecific interactions between microhabitat types. Due to travel restrictions caused
2037 by the Covid-19 pandemic, I was unable to complete my experiments in other geographical
2038 regions. In addition, I resided in Sydney, Australia during my PhD, which had strict restric-
2039 tions during the Covid-19 pandemic. Movement further than a 5 km radius from my place of
2040 residence was heavily restricted during periods of the pandemic. Due to this, I was unable to
2041 complete further investigations into interspecific interactions of acellular slime mould species in
2042 different microhabitats.

2043 **Contribution to thesis**

2044 My experimental design involved observing the foraging behaviour of acellular slime mould
2045 species in two environments with food placed in different spatial configurations (see Chapter 3
2046 for methods). I observed foraging behaviour in both homogeneous environments, where food
2047 was placed evenly around the arena, and heterogeneous environments where food was placed in
2048 patches. Results from this planned experiment may have shown acellular slime mould species
2049 from different microhabitats using different foraging strategies. Food distribution in litter mi-
2050 crohabitats are usually more heterogeneous than bark microhabitats, as litter usually consists
2051 of plant debris from different tree species. I may have found that acellular slime mould species
2052 from litter microhabitats could successfully contact more food sources in the heterogeneous for-
2053 aging environment compared to the homogeneous environments. In comparison, acellular slime
2054 mould species found in homogeneous microhabitats, such as aerial litter or bark, may have been
2055 less successful in the heterogeneous foraging environment. In addition, I could observe new
2056 foraging techniques that are optimised for certain environment types.

2057 In Chapter 3, I found only positive interactions between species of acellular slime moulds. I
2058 may have observed different interspecific interactions between acellular slime moulds in different

2059 climate regions. The Stress Gradient Hypothesis proposes that communities with high abiotic
2060 or biotic stress have a high proportion of facilitative interactions, whereas communities with
2061 fewer stressors have competitive interactions (He et al., 2013). In plant communities, facili-
2062 tation increases in communities where climate is more stressful, possibly because facilitation
2063 ameliorates harsh environments or because facilitation can make resources that were previously
2064 inaccessible available to other species (Valiente-Banuet and Verdú, 2007). Plasmodia of acellu-
2065 lar slime moulds require humidity to actively forage therefore low humidity or temperature can
2066 be a limiting abiotic factor for their survival. I may have seen more facilitative interactions in
2067 species found in semi-arid climates compared to sub-tropical climates.

2068 Observing the differences in foraging behaviour between species of acellular slime moulds from
2069 different microhabitats or climate regions would develop our understanding of the drivers of be-
2070 havioural variation in acellular slime moulds. This planned experiment would have contributed
2071 to my thesis by incorporating environmental factors that could explain behavioural variability
2072 between species.

Discussion

Protists are extremely common and are present in every biome on Earth. Protists are incredibly diverse and are responsible for a range of ecological functions such as primary production (Jassey et al., 2015; Ward and Follows, 2016), element cycling (Aoki et al., 2007; Puppe et al., 2014; Sommer et al., 2013) and predation of bacteria and other eukaryotes (Berney et al., 2013; Foissner, 1999; Geisen et al., 2016; Glücksman et al., 2010; Hess et al., 2012; Rosenberg et al., 2009; Sherr et al., 1983; Yeates and Foissner, 1995). Protists are especially abundant in soils and decomposition systems and make up an estimated 31% of biomass and 69% of respiration in soil ecosystems (Wu et al., 2022). Despite this, protist behaviour and ecology are critically under-studied. One group of protists, the acellular slime moulds, are commonly found in leaf litter and decomposition systems and have been identified as catalysts of microbial litter breakdown (Geisen et al., 2021).

Studies of acellular slime mould behaviour have focused on investigating the problem-solving abilities of the model species, *Physarum polycephalum*. This research has uncovered a wide range of abilities in these brainless organisms, from maze-solving abilities (Nakagaki et al., 2000; Reid et al., 2012), decision-making (Latty and Beekman, 2015, 2011b; Reid et al., 2016), to irrationality (Latty and Beekman, 2011a) and even a basic form of learning (Boisseau et al., 2016; Vogel and Dussutour, 2016). Less is known about the behavioural variability among and within species of acellular slime moulds and whether the behaviour found in *P. polycephalum* is representative of acellular slime moulds. There are approximately 1000 known species of acellular slime mould (Lado, 2001) and many species can be found in close proximity to each other. It is essential to understand the diversity and variability in acellular slime mould behaviour to begin to understand how this group of protists may affect ecosystem functions such as decomposition or drive species diversity in the ecosystems that they inhabit.

My thesis aimed to find behavioural variation of acellular slime moulds at the species, strain and individual level through the following investigations.

In Chapter 1, I investigated variability at the individual level by observing physiological, behavioural and cellular changes in the acellular slime mould species, *P. polycephalum*, as they aged over their lifespan. I used two strains in this experiment, Tu111xAI35-H and Tu9xDP89-S, and I observed behavioural variability at the strain level as well as differences in the effect of age on physiological and cellular measurements between the strains.

In Chapter 2, I investigated behavioural variability at both the individual and strain level by assessing the decision-making ability of two strains of *P. polycephalum* as they aged. I repeated a binary discrimination test approximately every 30 days, where plasmodia chose between a high quality food source and seven low quality food sources. The nutrient concentration of the low quality food sources were different depending on the difficulty level of the test. In easy tests all seven low quality food sources were 2% w/v oat agar and in hard tests all seven low quality food sources were 4% w/v oat agar. The nutrient concentration of the high quality food source remained constant at both difficulty levels at 6% w/v oat agar. I observed differences in both accuracy and decision speed between the two strains as well as variation in behaviour as they aged.

In Chapter 3, I observed the behaviour of three different acellular slime mould species to assess behavioural variability at the species level. My experimental design involved observing the behaviour of the species in two different environment types, which allowed me to assess behavioural variation at the individual level as well. I also assessed the behaviour of the species in paired experimental setups, to observe whether behaviour of the species varied when interacting with other species.

Finally, in Chapter 4 I detailed planned investigations that could not be completed due to restrictions caused by the Covid-19 pandemic. The first of these investigations would have provided further information on behavioural variation at the individual level, by comparing the behaviour of acellular slime moulds before and after entering a dormant life stage. The second investigation would have provided further information on behavioural variation at the species level, by observing the foraging behaviour of acellular slime mould species from different climate

regions and microhabitats. Although these investigations could not be completed, they present promising ideas for future investigations.

Species Variation

All three of my study species are classified in the family Physarales, but each species had distinct behaviour and foraging strategies (Chapter 3). Plasmodia of *Didymium iridis* recovered rapidly from fragmentation, found their first food quickly and could cover large quantities of food sources at once. Plasmodia of *P. polycephalum* found their first food as quickly as *D. iridis* but covered fewer food sources at a time. Despite covering fewer food sources, *P. polycephalum* were as effective at foraging as *D. iridis*, as both species contacted a similar quantity of food sources in the duration of the experiment. In contrast to these two species, *Physarum melleum* were the slowest to recover from fragmentation and to find their first food, covered fewer food sources and contacted the lowest amount of food in the experiment. Plasmodia of *P. melleum* may have been highly selective about which food sources to consume as I observed unique behaviour where plasmodia avoided food sources, possibly in search of a higher quality food option. Only *D. iridis* sporulated during the experiment, suggesting that the factors that cause sporulation differ between species. All of my study species were found in leaf litter microhabitats and both *D. iridis* and *P. melleum* were found in the same sub-tropical climate (Sydney, Australia). Despite this, all three species showed differences in behaviour, demonstrating the diversity of acellular slime mould behaviour even in closely related species found in similar environments.

The majority of research on acellular slime mould behaviour focuses on the model species, *P. polycephalum*. The wide variation of behaviour observed in the closely related species in my experiment highlights the need to study the behaviour of a diverse range of acellular slime mould species, as it is highly likely that the behaviour of *P. polycephalum* is not representative of acellular slime moulds. The behaviour of acellular slime mould species from different climate regions may differ greatly from behaviour found in our three species. This pattern of behavioural variability between species found in different climate regions has been observed in animal species. For example, tropical medaka fish species exhibit different mating behaviour

2153 compared to temperate medaka fish species, where males will chase other males more often
2154 during the mating season (Sumarto et al., 2020). House wrens found in tropical regions were
2155 found to invest less in annual reproduction, where they would make fewer feeding trips to the
2156 nest and produce fewer chicks compared to house wrens found in temperate regions (Tieleman
2157 et al., 2006). Acellular slime mould species from different climate regions may also exhibit
2158 differences in physiology, which may drive behavioural variation. For example, soybean strains
2159 that originate from tropical climates have higher seed yield and biomass accumulation compared
2160 to strains that originate from temperate regions (Saryoko et al., 2018). This variation may be
2161 driven by physiological differences between the strains such as lower gas exchange activity in
2162 temperate soybean strains (Saryoko et al., 2018). Most investigations on foraging behaviour of
2163 acellular slime moulds have focused on species in the *Physarum* and *Didymium* genus (Latty
2164 and Beekman, 2015; Yip et al., 2014), which are commonly found in tropical regions. There
2165 are no known studies investigating the foraging behaviour of acellular slime mould species that
2166 specialilse in temperate regions. Further research studying foraging strategies and behaviour
2167 of acellular slime mould species from different climate regions may uncover different behaviour
2168 that acellular slime mould species have evolved to survive in different environmental conditions.

2169 **Interactions between species**

2170 I also found variation in interspecific interactions between acellular slime mould species. I
2171 only observed positive interactions between species, and the same interactions were not present
2172 between all species. I observed mutualism between *D. iridis* and *P. polycephalum* where both
2173 species increased the number of food sources that they covered at once when in the presence of
2174 each other. I also observed commensalism between species of acellular slime moulds. The total
2175 number of food sources that *D. iridis* contacted in the experiment increased when paired in
2176 an environment with *P. polycephalum.* The total number of food sources that *P. polycephalum*
2177 contacted was not affected by the presence of *D. iridis,* but did increase when paired in an
2178 environment with *P. melleum.* The foraging behaviour of *P. melleum* was not affected through
2179 direct interactions with the other species.

I found variations in behaviour when assessing allelopathic interactions between species through reactions to extracellular secretions. Previous research showed that *P. polycephalum* was repelled by their own extracellular secretions and used this mechanism as an externalised spatial memory to navigate complex environments (Reid et al., 2012). In my experiments, *P. melleum* was repelled by their own extracellular secretions, however *D. iridis* was attracted to their own extracellular secretions. I found allelopathic interactions between species where *P. melleum* was attracted to the extracellular secretions of *D. iridis* plasmodia, and *D. iridis* was attracted to the extracellular secretions of *P. melleum* plasmodia. Both species were not attracted to extracellular secretions of *P. polycephalum* plasmodia, and *P. polycephalum* had no clear attraction to the extracellular secretions of both *D. iridis* and *P. melleum.* This shows that chemical signalling mechanisms that affect other species of acellular slime moulds, are species specific. My findings show a diverse range of direct and indirect interactions between three species of acellular slime moulds and demonstrate that interactions between species are variable and complex.

Future research investigating interspecific interactions between species from different climate regions and microhabitats may uncover different types of interactions between acellular slime mould species. Negative interactions such as competition may be more common in environments that are less stressful, as the stress gradient hypothesis (He et al., 2013) suggests that competitive interactions decrease along a gradient of increasing environmental stress. In addition, it may be interesting to investigate whether species diversity of acellular slime moulds differ between microhabitats where competitive or facilitative interactions are high, as facilitation has been shown to drive diversity by helping to mitigate harsh environmental conditions.

Strain Variation

While studying variation in behaviour due to age (Chapter 1 & 2), I found distinct differences in behaviour between the two strains of *P. polycephalum*, Tu111xAI35-H and Tu9xDP89-S. The strains had distinct differences in search behaviour where the plasmodia of strain Tu111xAI35-H produced many branches that moved slowly and the plasmodia of Tu9xDP89-S produced fewer branches that moved quickly. Network-building ability was also different between the two

strains where Tu9xDP89-S was more likely to build a medium network over a short network than Tu111xAI35-H. This shows that Tu9xDP89-S plasmodia were more likely to allocate their biomass over several branches to connect the food sources, whereas Tu111xAI35-H made fewer branches between food sources. I also found differences in decision-making ability between the strains. Tu9xDP89-S were more accurate than Tu111xAI35-H in hard tests, and Tu111xAI35-H plasmodia were more likely to make split decisions than Tu9xDP89-S plasmodia. Additionally, I found differences in individual variation between the two strains where although both strains had similar movement speed when they were young, plasmodia of Tu9xDP89-S became faster with increasing age at a higher rate than Tu111xAI35-H. Although studies on the behavioural variation between strains of *P. polycephalum* have been conducted previously (Dussutour et al., 2019; Vogel et al., 2015; Zabzina et al., 2014), the plasmodia in my research were cultured from myxamoebae at the same time and were reared in identical conditions. This means that behavioural variation between Tu111xAI35-H and Tu9xDP89-S were not caused by differences in culturing conditions such as quality of food, exposure to environmental stress or the period of time the strains were kept as dormant sclerotia. Behavioural variation between strains of *P. polycephalum* highlights the need for researchers to note which strains they use when designing their experiments and when drawing conclusions about *P. polycephalum* behaviour. It also presents *P. polycephalum* as a model species for studying behavioural variation in brainless organisms.

Cellular or genetic differences may drive variation in physiology and behaviour between strains of *P. polycephalum.* Within bacterial colonies, genetic differences between individuals can lead to variation in morphology or resistance to toxic chemicals. For example, individuals that are resistant to mercury in populations of the marine bacterium, *Pseudomonas stutzeri*, have been found to have different genes that encode mercury ion transportation proteins (Zheng et al., 2018). In biofilms, a small percentage of individuals in the colony can exhibit morphological changes over time. These changes can be linked to specific differences in genetic loci (reviewed in Stewart and Franklin, 2008). Further experiments involving a large array of *P. polycephalum* strains with a gradient of genetic differences may help to dissect whether cellular differences

between strains are directly related to variation in the behaviour and physiology of *P. polycephalum*.

Individual Variation

Variations in ageing individuals

I studied the individual variation of acellular slime mould species by observing changes in plasmodia over age (Chapter 1 & 2) and in different foraging environments (Chapter 3). In Chapter 1, I observed physiological, behavioural and cellular changes in *P. polycephalum* plasmodia with increasing age. I found non-linear patterns in measurements of physiology, cellular changes and behaviour over their lifespan. Cell health of plasmodia fluctuated with cyclical periods of poor health followed by rapid recovery. In one strain, Tu9xDP89-S, plasmodia appeared to completely senesce but then revived after a period of 10 days and continued to be cultured for the remainder of the experiment. A physiological measurement, growth area, also fluctuated over age. As growth area of plasmodia is linked to cell health, this similarity was expected. Nucleus area also fluctuated over age. Increase in nucleus area is associated with increasing mutations within nuclei (Clark and Hakim, 1980; Clark and Mulleavy, 1982; McCullough et al., 1973). This result suggests that mutations in nuclei were increasing and then decreasing throughout the duration of this experiment. Patterns found in genetic differences in mtDNA appear to follow a fluctuating pattern where mtDNA of Tu111xAI35-H of young plasmodia were less similar to the mtDNA of middle-aged plasmodia, but the mtDNA of old plasmodia was very similar to young plasmodia. This result suggests that mtDNA in old plasmodia of Tu111xAI35-H underwent a period of renewal or selection to become more similar to the mtDNA of young plasmodia. In contrast, mtDNA of middle-aged and old plasmodia of Tu9xDP89-S were very similar to each other, but were not similar to young plasmodia, suggesting that mtDNA mutations accumulated in this strain with a linear relationship to age.

I found non-linear patterns in behaviour, where decision accuracy of Tu111xAI35-H in hard tests fluctuated with age (Chapter 2). In plasmodia of Tu9xDP89-S decision accuracy fluc-

2261 tuated in both easy and hard tests with age. Interestingly, the occurrence of speed-accuracy
2262 trade-offs (SAT) also varied with age. Previous research demonstrated that SATs were found
2263 in *P. polycephalum* when test difficulty was hard (Latty and Beekman, 2011b). In my experi-
2264 ment, plasmodia only demonstrated SATs in easy tests and SATs did not occur at every age.
2265 The first age that SATs occurred were different between strains where Tu9xDP89-S plasmodia
2266 demonstrated SATs at 125, 215 and 242 days of age and Tu111xAI35-H plasmodia demonstrated
2267 SATs at 62, 215 and 242 days of age. Currently, the age of plasmodia in most published re-
2268 search is unknown. It is possible that the variations found in current experiments are caused
2269 by differences in the age of plasmodia. In addition, if plasmodia of different ages are not spread
2270 equally throughout treatment groups, differences caused by age may be incorrectly attributed
2271 to differences in treatments.

2272 Some patterns of individual variations in *P. polycephalum* were linear. Plasmodia of both
2273 strains had faster movement speed and produced fewer branches with increasing age. This
2274 showed that older plasmodia were more likely to allocate their biomass into fewer branches
2275 that searched around the arena faster. I also found nucleus variation with increasing age,
2276 where there was a decrease in nucleus density with increasing age, suggesting that plasmodia
2277 were not able to duplicate nuclei as efficiently in older age. In my research, I found a wide
2278 range of behavioural, physiological and cellular changes in *P. polycephalum* over age. These
2279 findings highlights the need for researchers to be mindful of the age of plasmodia used in their
2280 experiments. In addition, my work demonstrates non-linear changes behaviour, physiology and
2281 within the cell of *P. polycephalum* over their life time.

2282 **Variations in individuals in different foraging environments**

2283 I also found behavioural variation in individuals in different foraging environments. In Chapter
2284 3, I observed acellular slime moulds in homogeneous and heterogeneous foraging environments.
2285 I found behavioural variation in all three species. Plasmodia of *P. polycephalum* were able to
2286 cover more food sources at a time in heterogeneous environments, despite finding its first food
2287 slower than in the homogeneous environment. *Didymium iridis* had a similar search strategy

to *P. polycephalum* but was able to create a larger network to cover more food sources at once. Plasmodia of *D. iridis* were able to forage with a similar effectiveness in both heterogeneous and homogeneous environments. Perhaps *P. polycephalum* plasmodia foraged less effectively in homogeneous environments due to their smaller network size. Unlike *D. iridis* and *P. polycephalum*, plasmodia of *P. melleum* did not create networks between food sources, instead plasmodia moved from one food patch to the next. The behaviour of *P. melleum* plasmodia were highly effective in heterogeneous environments, where they contacted significantly more food sources compared to the homogeneous environment. The slower and more directional foraging behaviour of *P. melleum* was better suited to the heterogeneous environment. Higher foraging success in heterogeneous environments in *P. polycephalum* and *P. melleum* may be related to the microhabitats that these species are found in. Heterogeneous environments are common in soil and leaf litter as organic matter is decomposed at different rates. It is likely that the foraging behaviour of *P. polycephalum* and *P. melleum* was more effective in the heterogeneous environment as the nutrient distribution mimicked their natural leaf litter microhabitat most closely. Nutrient heterogeneity in foraging environments can drive behavioural variation in individuals. For example, when foraging in fine-grained heterogeneous environments, grass species produce higher root density in high concentration food patches compared to in coarse-grained heterogeneous environments (Fransen et al., 2001; Mommer et al., 2012). In marine bacteria, motile bacteria are able to forage more effectively in turbulent heterogeneous environments compared to stagnant homogeneous environments (Taylor and Stocker, 2012). In contrast, *D. iridis* plasmodia did not change their foraging behaviour in the two foraging environments, despite also being found in leaf litter. In plants, dominant species that develop extensive leaf canopy and root systems were found to be less flexible and did not change their foraging behaviour in different environments (Campbell et al., 1991). As *D. iridis* plasmodia were able to contact a large amount of food, it is possible that *D. iridis* is a dominant species in the leaf litter environment.

Future investigations on behaviour of a variety of acellular slime mould species in different environments may further our knowledge on how variations in foraging behaviour help acellular

slime mould species adapt and forage effectively in different environments. The three species in my experiment are primarily found in leaf litter but acellular slime mould species can be found in a variety of different microhabitats, including more homogeneous environments such as dung (Bezerra et al., 2008; Calaça et al., 2020; Eliasson, 2013) or inflorescences (Black et al., 2004; Schnittler and Stephenson, 2002; Stephenson et al., 2004). Further investigation of foraging behaviour of acellular slime moulds found in microhabitats with more homogeneous nutrient distribution may help elucidate whether foraging behaviour relates to the nutrient distribution that species occur in, or whether the foraging behaviour of acellular slime moulds are generally more effective in heterogeneous environments.

Concluding remarks

The research in my thesis has expanded our knowledge on behavioural variation at the species, strain and individual level of acellular slime moulds. In addition, I observed the first example of facilitative interactions between species of acellular slime moulds in Chapter 3. Study of interspecific interactions between acellular slime mould species would further our understanding of how different species can co-exist in the same microhabitat and would help to develop our understanding of species interactions within decomposition systems. While investigating the effect of age on the acellular slime mould species *P. polycephalum* in Chapter 1 and 2, I found that plasmodia of this species can survive for significantly longer than previously thought, with both strains reaching a lifespan of over 600 days. The lifespan of my strains of *P. polycephalum* was over three times longer than previously recorded (Hu et al., 1985; Poulter, 1969) and highlights the longevity of plasmodia in this species. Further research on the cellular drivers of changes in behaviour and physiology of acellular slime moulds would help to develop our understand of the mechanism behind behaviour in brainless organisms. Additionally, research focused on the behaviour of a diverse range of acellular slime moulds from different climates and microhabitats would further our understanding of how different foraging strategies can develop, and how this behaviour can drive interactions between species of acellular slime moulds.

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