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## Behavioural changes in slime moulds over time.

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## Behavioural changes in slime moulds over time

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### Summary

Changes in behaviour over the lifetime of single cell organisms has primarily been investigated in response to environmental stressors. However, growing evidence suggests that unicellular organisms undergo behavioural changes throughout their lifetime independently of the external environment. Here we studied how behavioural performances across different tasks vary with age in the acellular slime mould *Physarum polycephalum*. We tested slime moulds aged from 1 week to 100 weeks. First, we showed that migration speed decreases with age in favourable and adverse environments. Second, we showed that decision making and learning abilities do not deteriorate with age. Third, we revealed that old slime moulds can recover their behavioural performances if they go throughout a dormant stage or if they fuse with a young congener. Lastly, we observed the response of slime mould facing a choice between cues released by clone mates of different age. We found that both old and young slime moulds are attracted toward cues left by young slime moulds. Although many studies have studied behaviour in unicellular organisms, few have taken the step of looking for changes in behaviour over the lifetime of individuals. This study extends our knowledge of the behavioural plasticity of single-celled organisms and establish slime moulds as a promising model to investigate the effect of aging on behaviour at the cellular level.

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## Introduction

One aim of this special issue of *Philosophical Transactions* is to understand collective behaviour over the lifetime of individuals. In unicellular organisms, changes in collective behaviour through time has been studied almost exclusively in response to environmental stressors. For instance, it has been shown that bacteria [1,2] and cellular slime moulds [3–5] transit to new collective states such as coordinated cellular migration or aggregation when nutrients become scarce [6]. On the contrary, how collective behaviour changes with correlates of age in unicellular organisms independently of the external environment remains an open question. The main reason for this might be that, single cell organisms were mistakenly believed to be short lived and immune to ageing under optimum growth conditions [7–11]. It has now been demonstrated that, some unicellular organisms such as bacteria *Escherichia coli* and *Caulobacter crescentus*, ciliates *Paramecium* and *Tetrahymena*, yeast *Saccharomyces cerevisiae*, cellular and acellular slime moulds *Dictyostelium discoideum* and *Physarum polycephalum* undergo intrinsic changes over time that affect cell behaviour and physiology [11–13]. In this paper, our aim is to understand how cell behaviour varies over the lifetime of individuals in the acellular slime mould *P. polycephalum*. In contrast to the cellular slime mould *D. discoideum* in which the vegetative state consists of solitary amoebas that divide about every 4 h when food is plentiful, in *P. polycephalum*, the vegetative state is a unique large cell containing a large collection of nuclei that divide about every 8 h and share a common cytoplasm. Acellular slime moulds are particularly attractive organisms to study collective behaviour changes over time in unicellular organisms for several reasons.

First of all, although they are not collective *per se*, acellular slime moulds embrace most of the key principles of collective behaviour (reviewed in [14]): synchronization and coordination [15–18], positive and negative feedbacks [19], variability [20–22], symmetry breaking [20,23], phase transition [24], redundancy [14], information transfer [25,26] capacity to make complex decisions [27–30]. In addition, due to its internal dynamic, *P. polycephalum* is often regarded as a collection of nonlinear oscillators which are interconnected, often coupled and partially dependent on each other (reviewed in [16]). Furthermore, similarly to more traditional model systems in collective behaviour such as ant colonies or fish schools, its behaviour has successfully been simulated using a multi-agent approach [31,32]. In these models, *P. polycephalum* behaviour relies on particle like agents that follow simple rules but are capable collectively to generate self-organized amoeboid movement and to construct optimized networks [31,32].

Second, despite being unicellular, acellular slime moulds can produce seemingly complex behaviours. Indeed, since the seminal contribution of Toshiyuki Nakagaki twenty years ago [33], *P. polycephalum* has become an essential model organism for studying problem-solving in non-neural systems [32–37]. Past experiments have shown that acellular slime moulds can find the shortest path in a maze [33,38], build optimized networks to connect several food sources [34], anticipate events

1 [39], learn to ignore irrelevant stimuli [40,41], encode memory in their environment [42] or in their  
2 morphology [43], interact with their congeners [21,44], optimize nutrient intake [28,45], make optimal  
3 decisions [29,46,47], etc. *P. polycephalum*'s behaviour relies on its self-organized internal architecture  
4 which consists of a transport network of interconnected tubes [48]. The tubes contract and relax  
5 periodically, causing the cytoplasm to flow back and forth, a phenomenon termed 'shuttle streaming'  
6 [49]. *P. polycephalum* can migrate at a speed of up to few millimetres per hour through the interplay  
7 of intracellular flow, adhesion and rhythmic contractions of the tubes [49–51]. The frequency and the  
8 amplitude of the contractions depend on external stimuli and as a result, *P. polycephalum* is capable of  
9 altering its shape and motion as a function of a variety of external stimuli such as chemicals [52–54],  
10 light [55], temperature [56–58], humidity [44], gravity [59] or substrate distortion [46].

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Third, acellular slime moulds are easy to track through time and relatively long-lived. In unicellular organisms, lifetime can either be chronological if we consider the time a cell remains viable before dividing, or replicative if we consider the number of times a cell divide before it dies [9]. Tracking division is usually difficult as it requires to follow and distinguish individual cells. *P. polycephalum* offers the possibility to consider both chronological and replicative time simultaneously. During its development, the zygote undergoes synchronous mitotic divisions every 8 to 12 hours but without cytokinesis to form a single multinucleated cell, called a plasmodium [60,61]. Hence, the cell grows over time but there is no cell division. A plasmodium reared on solid medium by routine serial subculture under laboratory condition can be maintained from a few weeks to several months [62,63]. The life span of the plasmodium is in part genetically determined as different strains have different lifespans and subclones derived from a single plasmodium age in a coordinated manner, i.e death occurs at approximately the same time [62,64,65].

Fourth, *P. polycephalum* provides unique possibilities for experimental manipulations [66,67]. The plasmodium can extend up to hundreds of square centimetres. It can be severed into viable and structurally similar yet smaller plasmodia. Upon contact, genetically identical plasmodia can fuse with each other to form a single plasmodium. In response to environmental stressors, the plasmodium can enter a resting stage called sclerotium and remain dormant for years without deterioration. During the sclerotization process, the plasmodium lose 50% of their total protein content together with 40% of their DNA and 65% of their RNA [60]. After transfer to a humid and nutritive medium, a sclerotium can revert back to a mobile plasmodium within 24 h. Plasmodial cultures can be easily initiated from sclerotia after up to 3 years. Interestingly, numerous authors have reported that, a sclerotium issued from a seemingly ageing plasmodium may produce a vigorous plasmodium again, a form of "rejuvenescence". Following a period of growth this secondary plasmodium will undergo senescence again. This cycle of growth and senescence can be repeated several times and, thus, cultures can be maintained over many generations.

1 Lastly, *P. polycephalum* displays senescence, which is defined as a loss of fitness during ageing.  
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3 A plasmodium can be maintained for months during which no change is apparent until a reproducible  
4 decline in viability occurs [62]. Senescence in the plasmodial stage is characterized by morphological  
5 change [62], reduction of growth rate [62,68], reduced cytoplasmic flow [64], loss of yellow pigment  
6 [64], accumulation of polyploid nuclei [69], high level of mitochondrial DNA fragmentation [68],  
7 decreased replication of mitochondrial DNA [63], increase in nuclear size and DNA content [69,70].  
8 Senescence ends with the fragmentation of the plasmodium into several small spherical structures with  
9 a concomitant lysis of the plasmodium [64]. Longevity in slime moulds is not affected by most  
10 environmental factors with the exception of high temperature [71]. Senescence in slime moulds has  
11 been mainly studied at the cellular level. The potential effects of ageing on the plasmodium behaviour  
12 remained to be investigated.

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20 In this paper, we studied how slime mould behaviour changes over time. First, we explored the  
21 effect of age on migration speed in nutritive and adverse environments. Second, we investigated if  
22 decision making and learning performances changed with age. Third, we tested if older slime moulds  
23 were able to recover after a dormant period or after fusing with younger clones. Lastly, we examined  
24 if slime moulds were able to discriminate cues from congeners of different age.  
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## 30 **Methods**

### 31 **(a) Species studied and rearing conditions**

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35 *Physarum polycephalum*, also called the acellular slime mould, belongs to the supergroup Amoebozoa  
36 and the class Myxomycetes. In nature, slime moulds are found on organic substrates like tree bark or  
37 forest soil where they feed on microorganisms such as bacteria or fungi. *P. polycephalum* vegetative  
38 morph is a vast multinucleated cell named plasmodium. We used a strain of *P. polycephalum* : LU352  
39 kindly provided by Professor Dr Wolfgang Marwan (Max Planck Institute for Dynamics of Complex  
40 Technical Systems, Magdeburg, Germany). 80 large plasmodia were initiated from sclerotia at  
41 different time to obtain slime moulds of different age. In this paper, by age of the slime mould, we  
42 understand the time which elapses from the moment of the inoculation of a sclerotium onto a nutritive  
43 medium. The oldest plasmodia were kept after this study and are still being cultivated in our laboratory.  
44 They are currently more than 2 years old and none of them has died so far. In previous reports,  
45 plasmodium lifespan has been shown to vary from one month to one year, depending on the strain [62-  
46 64]. Hence, we considered as “old”, slime molds which were over one year old and “very old” slime  
47 molds which were almost two years old.

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60 Plasmodia were reared on a 1% w/v (weight/volume) agar medium with rolled oat flakes (Quaker Oats  
Company®) in Petri dishes (90mm Ø). They were kept in the dark in a thermoregulated chamber at a  
temperature of 20 degrees Celsius and a humidity of 80% from Monday to Friday. They were

1 transferred every day on a new agar medium on which was spread a single layer of rolled oat flakes.  
2 During the weekends, slime moulds were unfed and kept at 12°C. One day before each experiment, all  
3 plasmodia were transferred on a food medium consisting of finely ground oat flakes (5% w/v) mixed  
4 with 1% agar gel, hereafter referred as “oat gel”.  
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### 9 **(b) Migration speed on various substrates as a function of slime moulds age**

10 The aim of this experiment was to investigate how age affects the slime mould migration speed on  
11 various substrates (Figure 1a). Six groups of 10 large plasmodia of different ages (1, 17, 32, 54, 74  
12 and 94 weeks old) were used to conduct this experiment. Circular samples (10 mm Ø) were cut from  
13 each plasmodium and gently placed in contact with an oat gel bridge (35 mm long, 10 mm wide) in an  
14 experimental arena (Petri dish 12mm\*12mm). The bridge either contained an aversive substance: NaCl  
15 (100mM, 0.6% w/v) (Oat + NaCl bridge) or NaNO<sub>3</sub> (100mM, 0.8% w/v) (Oat + NaNO<sub>3</sub> bridge) or not  
16 (Oat). Experimental arenas housed 8 bridges and were kept in a thermoregulated chamber at a  
17 temperature of 25 degrees Celsius. After about 8 hours of incubation, the distance travelled by the  
18 slime moulds on each bridge was measured with a ruler. We computed the migration speed as the  
19 distance travelled divided by the time spent in contact with the bridge. A minimum of 48 samples were  
20 tested for each substrate and each age group, leading to total of 2396 assays.  
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### 31 **(c) Migration speed at different temperatures as a function of slime moulds** 32 **age**

33 Slime moulds have been shown to suffer both from low and high temperature which affect the shuttle  
34 streaming and therefore locomotion [72]. The aim of this experiment was to investigate how age affects  
35 the slime mould migration speed under different temperatures (Figure 1b). Five groups of 10 large  
36 plasmodia of different ages (8, 24, 61, 81 and 101 weeks old) were used to conduct this experiment.  
37 Circular samples (10 mm Ø) were cut from each plasmodium and gently placed in contact with an oat  
38 gel bridge (35 mm long, 10 mm wide) in an experimental arena (Petri dish 12mm\*12mm).  
39 Experimental arenas housed 8 bridges and were kept in thermoregulated chambers at a temperature of  
40 10, 25 or 38.5°C degrees Celsius. 25°C is a standard temperature to rear *Physarum polycephalum* while  
41 10°C and 38.5°C represents extremes conditions. After about 8 hours of incubation, the distance  
42 travelled by the slime moulds on each bridge was measured with a ruler. We computed the migration  
43 speed as the distance travelled divided by the time spent in contact with the bridge. Numerous slime  
44 moulds did not move at all during the experiments at extreme temperatures (10 or 38.5°C), they were  
45 not taken into account to compute speed but we calculated the proportion of motionless slime moulds  
46 for each age group and each temperature. A minimum of 80 samples were tested for each temperature  
47 and each age group, leading to total of 3533 assays.  
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#### **(d) Decision making ability as a function of slime moulds age**

Slime moulds have been shown to succeed in selecting the best option when offered multiple alternatives [22,27,45,73]. To investigate how age affects slime mould ability to make a decision, we studied how a slime mould distributed itself between two agar gel bridges containing or not an aversive substance (NaCl or NaNO<sub>3</sub>) (Figure 1c). Under non aversive condition, the slime moulds were offered a choice between a high-quality oat gel (5% w/v) and a low-quality one (2.5% w/v). Under aversive condition, the slime moulds were given a choice between a high-aversive option (0.6% w/v NaCl or NaNO<sub>3</sub>) and a low-aversive option (0.4% w/v NaCl or NaNO<sub>3</sub>). Five groups of 10 large plasmodia of different ages (6, 22, 59, 79 and 99 weeks old) were used to conduct this experiment. Circular samples (10 mm Ø) were cut from each plasmodium, placed between two bridges (35 mm long, 10 mm wide) in experimental arenas (Petri dish 12mm\*12mm). Experimental arenas housed 4 binary choices and were kept in a thermoregulated chamber at a temperature of 25 degrees Celsius. After about 8 hours of incubation, the distance travelled (in mm) on each bridge was measured with a ruler. We computed a decision-making performance as the distance covered on the best bridge (high quality or low aversive substrate) divided by the total distance. A minimum of 235 samples were tested for each age and each binary choice, leading to total of 4206 assays

#### **(e) Learning ability as a function of slime moulds age**

Slime moulds have been shown to be capable of habituation, a simple form of learning, when repeatedly exposed to an innocuous aversive substance [40,74]. To investigate how age affects slime moulds' ability to learn, we studied how slime moulds habituate to an aversive substance [25,40]. A bridge crossing experiment adapted from [40] was conducted to habituate the slime mould to an aversive substance (NaCl 100mM, 0.6% w/v) (Figure 1d). We used two groups of 10 large plasmodia of two different ages 12 and 74 weeks old. Before starting the habituation experiment, to accustom the slime moulds to the experimental set-up, 10 circular samples (Ø 10 mm) were cut from each plasmodium and introduced in an experimental arena (Petri dish 12mm\*12mm). The samples were then connected to an oat gel bridge (35 mm long, 10 mm wide). After a day, the slime moulds had covered the bridge, and a circular sample was cut from the bridge and transferred to new experimental arenas to start the habituation. On day 1 of the habituation, half of the samples were offered an oat gel bridge containing NaCl (substrate: Oat+NaCl, treatment: habituated, N=50 for each age group) while the remaining half had to cross a bridge without NaCl (substrate: Oat, treatment: control, N=50 for each age group). The following two days (day 2 and day 3), a circular sample was cut from each bridge daily and transferred to another arena where they were offered a new bridge. On day 4, once the habituation training was completed, we cut one or two circular samples from each bridge and transferred them to a new arena. Half of the habituated and control samples were offered a bridge containing NaCl (substrate: Oat+NaCl) while the remaining half had to cross a bridge without NaCl

(substrate: Oat). Thus, on day 4 the control group is either facing a bridge containing NaCl for the first time (N=30 for each age group) or a bridge without NaCl (N=30 for each age group) while the habituated group is either facing a bridge containing NaCl for the fourth time (N=30 for each age group) or a bridge without NaCl (N=30 for each age group). Every day, after about 8 hours of incubation, the distance travelled (in mm) on each bridge was measured with a ruler. Data collected on day 1 allowed us to confirm that slime moulds showed a clear aversive behaviour toward the NaCl while data collected on day 4 enable us to test if slime mould learn to ignore the aversive substance. On day 4 we synthesized the results with an aversion index HI and CI as in [41].

$$HI = \left| \frac{1}{N} \sum_{i=1}^N \frac{HON_i - \overline{HO}}{sd(\overline{HO})} \right| \quad \text{or} \quad CI = \left| \frac{1}{N} \sum_{i=1}^N \frac{CON_i - \overline{CO}}{sd(\overline{CO})} \right|$$

HON, CON, HO and CO are the habituated (H) and control slime moulds (C) on Oat+NaCl (ON) and Oat (O) respectively. Using those indexes allowed us to normalize each variable value corresponding to the treatments HON and CON by the mean and the standard deviation of their respective control HO and CO. Values clearly above 0 indicate an aversion towards the repellent, whereas values close to zero indicate habituation to the repellent *i.e.* slime moulds react the same way to Oat+NaCl and Oat.

#### (f) Recovery ability following dormancy as a function of slime moulds age

To investigate how age affects slime mould ability to recover, we studied how fast old slime moulds migrate after a dormancy period (Figure 1e). We used two groups of 10 large plasmodia of two different ages 60 and 100 weeks old, referred as “old” and “very old”. To initiate the transition from plasmodia to sclerotia, we cut 2 circular samples from each plasmodium ( $\varnothing = 20$  mm), placed each of them on a moist paper filter ( $\varnothing = 145$  mm) in a petri dish ( $\varnothing = 145$  mm) and kept them in a thermoregulated chamber at a temperature of 30 degrees Celsius for two days to dry. The sclerotia were then stored for a week before being re-initiated to obtain new plasmodia. A week later, we then had 40 large plasmodia, 20 were one week old and originated from old and very old plasmodia (treatment: dormancy) and 20 were old and very old (treatment: no dormancy). Circular samples ( $\varnothing 10$  mm) were cut from each plasmodium and gently placed in contact with an oat gel bridge (nutritive substrate) or an agar gel bridge (agar 1%w/v, non-nutritive substrate) in an experimental arena (Petri dish 12mm\*12mm). Experimental arenas housed 8 bridges and were kept in a thermoregulated chamber at a temperature of 25 degrees Celsius. After about 8 hours of incubation, the distance travelled by the slime moulds on each bridge was measured with a ruler. We computed the migration speed as the distance travelled divided by the time spent in contact with the bridge. A minimum of 80 samples were tested for each age group (old, very old) and each treatment (dormancy or not), leading to total of 640 assays.

### **(g) Fusion as a function of slime moulds age**

Fusion constitutes a defining feature of the lifestyle of slime moulds. To investigate how age affects slime mould ability to fuse, we studied how fast two slime moulds fuse as function of their respective age (Figure 1f). We used two groups of 10 large plasmodia of two different ages 18 and 75 weeks old, referred as “young” and “old”. Circular samples (10 mm Ø) were cut from each plasmodium and gently placed in contact with an agar gel bridge (15 mm long, 15 mm wide) in an experimental arena (Petri dish 12mm\*12mm). We placed a sample at each extremity of the bridge and tested the following pairs: young/young (N=220), young/old (N=330) and old/old (N=220). Experimental arenas housed 8 pairs of slime moulds. They were kept in a thermoregulated dark room at a temperature of 21 degrees Celsius. We conducted behavioural scan observations using a flash light to record the latency to membrane and vein fusion. Membranes were considered as fused when we could not see the delimitation between the two slime moulds while veins were considered as fused when we could discern a vein going from one slime mould to the other. The latency between two scans was less than 5 minutes. Pairs that did not fuse after 9h or that fused with their neighbouring slime moulds in the arenas were removed from the analysis (young/young: 26 out of 220, young/old: 25 out of 330 and old/old: 18 out of 220)

### **(h) Recovery ability following fusion as a function of slime moulds age**

Slime mould fusion could provide the potential for cell recovery through resource sharing. Recently, it was shown that a learned behaviour can be transferred during fusion [25]. In this experiment, we measure migration speed after fusion as a function of slime moulds age (Figure 1g). We used two groups of 10 large plasmodia of two different ages 5 and 54 weeks old, referred as “young” and “old”. Circular samples (10 mm Ø) were cut from each plasmodium and gently placed by pairs in an experimental arena (Petri dish 12mm\*12mm). Each experimental arenas housed 4 pairs. We tested the following pairs: young/young (N=100), young/old (N=100) and old/old (N=100). Fusion was allowed by bringing the slime moulds into contact for 3 hours (see Vogel and Dussutour 2016). After the fusion, the slime moulds were gently separated at the point of contact using a spatula. Then, all slime moulds were required to cross an oat gel bridge (35 mm long, 10 mm wide). Experimental arenas housed 8 bridges and were kept in a thermoregulated chamber at a temperature of 25 degrees Celsius. After about 8 hours of incubation, the distance travelled by the slime moulds on each bridge was measured with a ruler.

### **(i) Detection of conspecifics as a function of slime moulds age**

Slime moulds can sense and respond to chemical cues released in the environment by conspecifics in a foraging context [21,44]. They release calcium while foraging which is attractive to other slime moulds [21]. In this experiment we monitored the directional movement response evoked in slime

1 moulds in the presence of substrates previously explored by clone mates of different age (Figure 1*h*).  
2 We used two groups of 10 large plasmodia of two different ages 3 and 52 weeks old, referred as  
3 “young” and “old”. Circular Petri dishes (diameter,  $\text{\O} = 90$  mm) containing a layer of 1% w/v agar gel  
4 were used as experimental arenas. Once the agar in the Petri dish had set, three holes were punched ( $\text{\O}$   
5 = 10 mm, inter- distance = 20 mm). One hole was filled with a young and an old slime mould ( $\text{\O} = 10$   
6 mm) sitting on 5% w/v oat gel. The other two holes were filled with experimental substrates ( $\text{\O} = 10$   
7 mm) which consisted in agar gel explored for 24 h by well-fed young and old slime mould. To obtain  
8 the experimental substrates, a slime mould was allowed to cover completely an agar gel while being  
9 fed with oat flakes (making sure that the food was never in contact with the agar substrate, see [21] for  
10 details). Then we removed the slime mould, and rinsed the agar substrate with distilled water just before  
11 the experiment. The slime mould would typically explore its environment by expanding in all  
12 directions for a short distance to finally migrate in a specific direction, eventually contacting one  
13 experimental substrate. The experimental substrate that was reached first was taken to imply a positive  
14 response (*i.e.*, a relative preference for the cues enclosed in the experimental substrate over the  
15 alternative). For each assay, we recorded which substrate was contacted first and the latency to reach  
16 each substrate. We replicated each binary choice 300 times with old and young slime moulds.  
17 Experiments were recorded using digital cameras (EOS 70D, Canon) which took a picture every 5 min  
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## 31 (j) Statistical analyses

32 All statistics were performed with RStudio (Version 1.2.1335). To assess the difference in the various  
33 parameters measured between the treatments, we used linear mixed models (LMM) or generalised  
34 mixed models (GLMM) (function lmer or glmer). The package lme4 [75] was used for all mixed  
35 models. The lmerTest and Car packages were then used to run Type III analysis of variance on the  
36 calculated LMMs and GLMMs, extracting p-values for F-tests and Wald-tests. Assumptions for all  
37 LMMs were checked using standard procedure: diagnostic of quantile-quantile normal plots and  
38 Shapiro-Wilk test. The models were fitted by specifying the fixed effects (explanatory variables)  
39 depending on the experiment and a random effect: the plasmodium identity. The dependent variables  
40 that did not fit linear model requirements were transformed using the “bestNormalize” function  
41 (“bestNormalize” package). Continuous variables were centred and scaled if needed. The outcomes of  
42 all the models are presented in the supplementary information (Table S1-S13)  
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## 54 Results

### 55 (a) Migration speed on various substrates as a function of slime moulds age

56 In the first experiment, we investigated if slime moulds migration speed is affected by age on different  
57 substrates. Slime moulds migrated faster on plain oat gel than on oat gels containing an aversive  
58 substance (LMM, substrate  $F=67.01$ ,  $p<0.001$ , table S1, figure 2). For all substrates, migration speed  
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1 decreased as age increased (LMM, age  $F=80.52$ ,  $p<0.001$ , table S1, figure 2). On plain oat gel,  
2 migration speed declined drastically until the slime moulds were 30 weeks old then decreased gently.  
3 On oat gel containing  $\text{NaNO}_3$ , migration speed reached a plateau when the slime moulds were over 30  
4 weeks old. On oat gel containing  $\text{NaCl}$ , migration speed declined continuously with age (LMM age \*  
5 substrate  $F=28.08$ ,  $p<0.001$ , table S1, figure 2). Hence,  $\text{NaNO}_3$  was the most aversive substance for  
6 young slime moulds while for old slime moulds it was  $\text{NaCl}$ .  
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### 11 12 **(b) Migration speed at different temperatures as a function of slime moulds** 13 **age** 14

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16 In the second experiment, we examined if slime moulds' migration speed was affected by age at  
17 different temperatures. Slime moulds migrated faster at standard temperature than at cold or hot  
18 temperature (GLMM, temperature  $\chi^2=4724.85$   $p<0.001$ , table S2, figure 3). As previously, at standard  
19 temperature, migration speed declined drastically with age at the beginning then decreased gently when  
20 the slime moulds were over 25 weeks old. At both low and high temperatures, the speed was the lowest  
21 for young slime moulds (8 weeks old), then increased until the slime moulds reached an intermediate  
22 age between 24 and 61 weeks old and finally decreased slightly with age (GLMM, age  $\chi^2=5.67$   
23  $p=0.017$ , age \* temperature  $\chi^2=43.26$   $p<0.001$ , table S2, figure 3). The proportion of motionless slime  
24 moulds was also affected by age (GLMM, age  $\chi^2=10.11$   $p<0.001$ , Table S3, figure 3) and temperature  
25 (GLMM, temperature  $\chi^2=200.47$   $p<0.001$ , table S3, figure 3). The youngest (8 weeks old) and oldest  
26 slime moulds (101 weeks old) remained most often motionless at both extreme temperatures with the  
27 youngest being more vulnerable to cold and the oldest to heat (GLMM, age \* temperature  $\chi^2=39.58$   
28  $p<0.001$ , table S3, figure 3).  
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### 40 **(c) Decision making ability as a function of slime moulds age** 41

42 In the third experiment, we estimated slime moulds' ability to make a decision as a function of age and  
43 substrate. As previously, slime moulds migrated faster on plain oat gel than on oat gels containing an  
44 aversive substance (GLMM, substrate  $\chi^2=451.80$   $p<0.001$ , table S4) and for all substrates migration  
45 speed decreased as age increases (GLMM, age  $\chi^2=5.91$   $p<0.001$ , age \* substrate  $\chi^2=18.88$   $p<0.001$ ,  
46 table S4). On average, slime moulds had better performances under non aversive conditions (GLMM,  
47 substrate  $\chi^2=141.34$   $p<0.001$ , table S5, figure 4). However, under aversive conditions, decision making  
48 performance increased with age while it remained constant under non aversive conditions (GLMM,  
49 age  $\chi^2=4.22$   $p=0.040$ , age \* substrate  $\chi^2=111.82$   $p<0.001$ , table S5, figure 4). Hence, young slime mould  
50 had better performances under non aversive condition while it was the contrary for old slime moulds.  
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### 59 **(d) Learning ability as a function of slime moulds age** 60

In the fourth experiment, we investigated if slime moulds' ability to habituate to an aversive substance  
depended on age. On day 1, slime moulds migrated faster on plain oat gel than on oat gels containing

1 NaCl (LMM, substrate  $F=75.86$   $p<0.001$ , table S6, figure 5a) and migration speed was higher for  
2 young than for old slime moulds (LMM, age  $F=15.27$   $p=0.001$ , age \* substrate  $F=3.76$   $p=0.054$ , table  
3 S6, figure 5a). On day 4, habituated and control slime moulds were tested for habituation and were  
4 required to cross a bridge containing NaCl. Control slime moulds showed a strong aversive behaviour  
5 and a high aversion index. In contrast, habituated slime moulds, encountering NaCl for the fourth time,  
6 showed no aversive behaviour and an aversion index close to zero (LMM, treatment  $F=23.54$   $p<0.001$ ,  
7 table S7, figure 5b). Hence, they learned to ignore the aversive substance. Slime moulds age did not  
8 affect their learning performance (LMM, age  $F=2.15$   $p=0.145$ , age\*treatment  $F=1.92$   $p=0.169$ , table  
9 S7, figure 5b)

### 20 (e) Recovery ability as a function of slime moulds age

21 In the fifth experiment, we tested if slime moulds recovered their original migration speed after a  
22 dormancy period. Slime moulds migrated faster on oat gel than on agar gels (LMM, substrate  $F=30.10$   
23  $p<0.001$ , table S8, figure 6) regardless of age and treatment (LMM, substrate \* age  $F=0.11$   $p=0.741$ ,  
24 substrate \* treatment  $F=2.54$   $p=0.111$ , table S8, figure 6). Slime moulds that were turned into a dormant  
25 state and reinitiated were significantly faster than their counterparts that remained as plasmodia (LMM,  
26 treatment  $F=59.52$   $p<0.001$ , table S8, figure 6). Age of the dormant slime mould had no effect on speed  
27 following dormancy (treatment \* age,  $F=0.09$   $p=0.772$ , table S8, figure 6).

### 34 (f) Fusion as a function of slime moulds age

35 In the sixth experiment we examined if the fusion process was affected by age. The latency for the  
36 membrane to fuse was shorter for pairs of young slime moulds or mixed pair (young/old) than for pairs  
37 of old ones (LMM, age  $F=3.63$   $p=0.034$  table S9, figure 7a). Vein fusion, following membrane fusion,  
38 also occurred earlier in pair of young slime moulds than in the other pairs (LMM, age  $F=4.21$   $p=0.030$ ,  
39 table S10, figure 7b).

### 46 (g) Recovery ability following fusion as a function of slime moulds age

47 In the fifth experiment, we tested if old slime moulds recovered their original migration speed after  
48 fusing with young ones. Young slime moulds were faster than old slime mould when they were allowed  
49 to fuse with a slime mould of the same age (LMM, age  $F=19.68.52$   $p<0.001$ , table S11, figure 8).  
50 However, old slime moulds, that were allowed to fuse with a young one, were significantly faster than  
51 their counterparts that fused with an old one (LMM, age\*pair type  $F=33.84$   $p<0.001$ , table S11, figure  
52 8). On the contrary young ones that were allowed to fuse with an old slime mould were slower than  
53 their counterparts that fused with a young one.  
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## (h) Detection of conspecifics as a function of slime moulds age

In the last experiment, we investigated if slime moulds could perceive a difference in cues left by slime moulds of different ages. As previously shown, young slime moulds were faster than old ones (GLMM, age  $\chi^2=14.44$   $p<0.001$ , table S12, figure 9). Both young and old slime moulds contacted first the substrate explored by a young slime mould significantly more often than the substrate explored by an old one (GLMM, observed vs expected:  $\chi^2=5.67$   $p=0.017$ , table S13, figure 9).

## Discussion

In many organisms, ageing is accompanied by deficits in behavioural performance. In this study, we were able to maintain a culture of slime moulds for more than two years and track their behaviour throughout time. We revealed that old slime moulds move slower than young ones on neutral, nutritive and aversive substrates in all our experiments. We confirmed that aversive substrates slowed down slime moulds regardless of age [25,41,76,77]. Sodium is known to decrease migration rate in slime moulds via a depolarization of the membrane potential [78]. An interesting observation was that sodium chloride had a stronger effect than sodium nitrate on old slime moulds while it was the opposite for young slime moulds. In many eukaryotic cells, high extracellular NaCl increases reactive oxygen species (ROS), causes DNA damage and promotes senescence [79,80]. Given that old slime moulds already suffered from molecular damages due to senescence (effects listed in the introduction [62–64,68–70]), NaCl might have amplified those damages. Interestingly, old slime moulds were better at avoiding high concentration of NaCl or NaNO<sub>3</sub> than young slime moulds, suggesting that they were indeed more susceptible to chemical stressors and actively avoided them. As a matter of fact, we did not observe any significant differences in decision abilities when slime moulds were facing two food sources of different quality.

We then revealed that migration speed depended on age under extreme temperature. Temperature is known to be one of the main factors affecting the shuttle streaming in slime moulds. The contraction period decreases from 13min at 1°C to 1.26 min at 30°C and then increases past 32°C [57,58,81,82]. At 42°C, the shuttle streaming is totally arrested and the membrane exhibits alterations [81]. In our experiment, all slime moulds were slowed down by extreme temperatures and this effect increased with age when slime moulds age ranged from 25 to 100 weeks. Temperature is known to contribute to senescence in most living-organisms [83], including slime moulds [71]. Susceptibility to thermal stress increases as an organism ages due to more molecular damage being generated and accumulated over time [84]. Yet, surprisingly, the effect of temperature was the strongest for the youngest slime moulds which were 8 weeks old. As our culture alternated between 25°C during the week and 12°C during the weekend, it might be possible that a thermotolerance was indirectly induced over time. Signs of habituation to low temperature has already been observed in slime moulds [85]. In *P. polycephalum* as in most living organisms, expression of heat-shock protein (HSP) increases after

1 exposure to a moderate thermal stress [86]. HSPs function as molecular chaperones, they reduce  
2 molecular damage induced by extreme temperatures allowing the organism to cope with this stressor  
3 [87–90]. Usually, how much HSPs an organism produces correlates with the level of stress to which it  
4 is exposed. Therefore, in our experiment, we can presume that the level of expression of HSPs was  
5 higher in older slime moulds than in younger ones as they experienced more periodic changes in  
6 temperature over time. Thus, older slime moulds might have been more susceptible to thermal stress  
7 owing to their age but also more thermotolerant due to their past experiences than younger slime  
8 moulds.  
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10 Recently [74,91], it was revealed that slime moulds are capable of habituation, a simple form  
11 of learning, when they are repeatedly exposed to a chemical stimulus. In animals, learning performance  
12 displays a progressive decrease with age. However when it comes to habituation, it was observed that  
13 older organisms habituate more rapidly than young ones in *Aplysia californica* [92] and *Caenorhabditis*  
14 *elegans* [93] or as fast as young ones in *Drosophila melanogaster* [94], mice [95] and rats [96]. Here,  
15 we showed that, habituation performance is preserved in old slime moulds as well. In animals, it was  
16 shown that the response threshold to the stimulus was higher in old animals than in young ones which  
17 could explain their performance [92,95]. In contrast, in our experiment, we showed that the aversion  
18 level toward NaCl did not vary with age. On the contrary, in our first experiment, older individuals  
19 seemed more susceptible to NaCl than younger ones. Habituation to NaCl in slime moulds relies, in  
20 part, on NaCl uptake [41]. In plants, yeast and cellular slime moulds, sodium is driven into the cell  
21 passively through non selective cationic channels [97–99]. Therefore, it is possible that this passive  
22 transport of sodium throughout the membrane is not affected by age.  
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24 In slime moulds, sclerotia have been found to survive under adverse environmental conditions  
25 for long periods up to 3 years. In the slime mould *Didymimim iridis*, a related species, the aging process  
26 stops when the plasmodium differentiates into a sclerotium [100]. Once the sclerotium is revived, the  
27 plasmodium then lives the remainder of its characteristic life spans [100]. In contrast in this study, we  
28 observed a recovery phenomenon when aged slime moulds went throughout a dormant stage. After  
29 dormancy, old slime moulds migrated as fast as young slime moulds and faster than old slime moulds  
30 which had remained in a plasmodial stage. Past works have shown that oxidative stress defences are  
31 involved in the transition from plasmodium to sclerotium in *P. polycephalum* [101,102]. However,  
32 cell senescence is in part due to oxidative damage to DNA, RNA and proteins by mitochondrial reactive  
33 oxygen species (ROS) which accumulate with age [103]. Hence, one hypothesis might be that oxidative  
34 stress defences expressed during differentiation were responsible for the elimination of harmful ROS  
35 and, as a consequence, the behavioural recovery observed in old slime molds after dormancy.  
36 However, we only recorded the behaviour of the slime moulds one week after they were reinitiated  
37 from sclerotia and we cannot rule out that this rejuvenation phenomenon was only transient and did  
38 not affect the plasmodium lifespan.  
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1 In slime moulds, cell fusion is extremely common. In a related species *Didymium nigripes*, it  
2 was shown that slime moulds lose the ability to fuse over time [104]. In this study, in contrast, we  
3 showed that fusion occurred regardless of slime mould age. As old slime moulds were slower than their  
4 younger counterparts, their latency to fuse was simply longer. Vein fusion took also a longer time in  
5 old slime moulds than in young ones, but fusion was never aborted. We also revealed that an old slime  
6 mould could recover its migration speed performance after fusing with a young counterpart. Resource  
7 sharing between cells is often observed as a strategy to cope with environmental stress. For instance,  
8 cells can exchange learned information [25], mitochondria [105] or membrane material [106] via cell  
9 fusion. It is well known that cells accumulate DNA, protein and metabolite damage over time [107]. In  
10 young cells, most of the damages are cleared by maintenance processes [107]. However, as not all  
11 damages can be repaired, irreparable damage rises in the cell through time [107]. In aged cells, the  
12 maintenance systems can be damaged themselves, amplifying the burden of damages [107]. In our  
13 experiment, it might be possible that the damages accumulated over time in an old slime mould were  
14 cleaned or repaired by the maintenance system of its younger counterpart. Interestingly, in a related  
15 species, *Didymium iridis*, when age-hybrid slime moulds were formed by the fusion of young and old  
16 individuals, 58 % of the new slime moulds acquired the remaining life span of the older individuals,  
17 22 % died concurrently with the younger individuals and 20% died at an intermediate age [108]. The  
18 authors concluded that it is the oldest slime mould that determines the lifespan of the age-hybrid slime  
19 mould [100,109]. In our experiment, we did not follow the age-hybrid slime moulds behaviour over  
20 time. Hence, even if we observed a form of recovery process in old slime moulds after fusion, we  
21 cannot rule out that they would have died concurrently with slime moulds of the same age in absence of  
22 fusion.

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39 As *P. polycephalum* migrates on the substrate, it leaves behind an extracellular slime [42,110].  
40 Previous works have shown that previously explored substrate might be repulsive or attractive  
41 depending on the physiological status of the slime mould that explored the substrate [21,44]. A  
42 substrate explored by a starved, irradiated or poisoned slime mould is actively avoided [44] while a  
43 substrate explored by a well-fed slime mould is attractive [21]. Calcium has been identified as the main  
44 chemical that mediates attraction [21] whereas the substances responsible for the aversion remain to  
45 be identified. In this study, we showed that slime moulds were able to use age-related cues released by  
46 conspecifics and make a decision accordingly. We revealed that slime moulds, regardless of their age,  
47 were more attracted by cues released by younger slime moulds than by older ones. As we did not notice  
48 any fusion delay when a young slime mould was facing an old one in the fusion experiment, we believe  
49 that the substrate explored by an old slime mould was not aversive but only less attractive. The  
50 information supplied by these excretions would appear to be crucial for both young and old slime  
51 mould. A young slime mould would benefit from avoiding fusion with an aged slime mould as it could  
52 inherit its damages. Indeed, we noticed that after fusing with an old counterpart, a young slime mould

1 was somewhat slower. In contrast, an old slime mould would gain from fusing with a young slime  
2 mould, as it would allow it to recover. In many animals from flies to humans [111–115], as an organism  
3 ages, the effect of senescence on both the perception and processing of information might affect their  
4 response to the presence of others. Here, we did not observe any effect of age on social interactions.  
5 since older individuals were still able to fuse with others (although in a slower fashion) and detect  
6 conspecifics.  
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11 Although many studies have studied behaviour in single cell organisms, few have taken the  
12 step of looking for changes in behaviour over the lifetime of individuals. This study extends our  
13 knowledge of the behavioural plasticity of unicellular organisms and establish acellular slime molds as  
14 a promising model to investigate the effect of aging on behaviour at the cellular level. Their ability to  
15 fuse with congeners and their peculiar lifecycle, might help us to understand how cellular metabolic  
16 activity and cellular defences are regulated and coordinated, and how a cell can counteract the effects  
17 of ageing.  
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## **Data Accessibility**

The datasets supporting this article are provided as supplementary material.

## **Authors' Contributions**

AD conceived the study. AD designed the study. AR, EP, PM, CC and MD performed the experiments. AR, EP, PM and CC carried out data acquisition. AD analysed the data. AD wrote the manuscript. AD secured funding. All authors gave final approval for publication.

## **Competing Interests**

We have no competing interests.

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3 **Figure 1:** Experimental design. (a) Effect of various substrate (nutritive substrate: 5% oat gel, aversive  
4 substrates: 0.6% NaCl + oat gel, 0.8% NaNO<sub>3</sub> + oat gel) on migration speed experiment as a function  
5 of slime mould age (6 groups of different age were tested, from 1 to 94 weeks old). (b) Effect of  
6 temperature (standard temperature : 25°C, extreme temperature : 10°C and 38.5°C) on migration speed  
7 as a function of slime mould age (5 groups of different age were tested from 8 to 101 weeks old). (c)  
8 Decision making under nutritive (2.5% oat gel vs 5% oat gel) and aversive conditions (0.4% NaCl +  
9 5% oat gel vs 0.6% NaCl + 5% oat gel, 0.4% NaNO<sub>3</sub> + 5% oat gel vs 0.6% NaNO<sub>3</sub> + 5% oat gel) as a  
10 function of slime mould age (5 groups of different age were tested, from 6 to 99 weeks old). (d)  
11 Habituation to an aversive substance (0.6% NaCl) as a function of slime mould age (2 groups of  
12 different age were tested : young slime moulds of 12 weeks and old ones of 74 weeks). (e) Effect of  
13 dormancy on migration speed on a neutral (agar gel) and nutritive substrate (oat gel) as a function of  
14 slime mould age (2 groups of different age were tested : old slime mould of 60 weeks and very old  
15 ones of 100 weeks). (f) Fusion ability as a function of slime mould age (2 groups of different age were  
16 tested: : relatively young slime moulds of 18 weeks and old ones of 75 weeks old). (g) Effect of fusion  
17 on migration speed on a nutritive substrate (oat gel) as a function of slime mould age (2 groups of  
18 different age were tested: young slime mould of 5 weeks and old ones of 54 weeks). (h) Detection of  
19 chemical cues left by conspecifics (young and old) as a function of slime mould age (2 groups of  
20 different ages were tested: young slime moulds of 3 weeks and old ones of 52 weeks).

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37 **Figure 2:** Mean migration speed as a function of age and substrate. Six groups of 10 large plasmodia  
38 of different ages (1, 17, 32, 54, 74 and 94 weeks old) were used to conduct this experiment. The  
39 substrates were made of oat gel (5%w/v) containing one of two aversive substances (Oat + NaCl (0.6%  
40 w/v), Oat + NaNO<sub>3</sub> (0.8% w/v)) or not (Oat). N > 48 for each age and each substrate. Total numbers  
41 of assays= 2396. Error bars indicate CI95.

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50 **Figure 3:** Mean migration speed and mean proportion of motionless slime moulds as a function of age  
51 and temperature. Five groups of 10 large plasmodia of different ages (8, 24, 61, 81 and 101 weeks old)  
52 were used to conduct this experiment. Three temperatures were tested: a standard one (25°C) and two  
53 extremes (10°C and 38.5°C). The substrates were made of oat gel (5% w/v). All the slime moulds at  
54 25°C migrated on the bridge so the proportion of motionless slime mould was equal to zero for all age  
55 groups. N > 80 for each age and each temperature. Total numbers of assays= 3533. Error bars indicate  
56 CI95.  
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3 **Figure 4:** Decision making as a function of age and substrates. Five groups of 10 large plasmodia of  
4 different ages (6, 22, 59, 79 and 99 weeks old) were used to conduct this experiment. Under non  
5 aversive conditions (oat), the slime moulds were offered a choice between a high-quality oat gel (5%  
6 w/v) and a low-quality one (2.5% w/v). Under aversive conditions (Oat+ NaCl or Oat+NaNO<sub>3</sub>), the  
7 slime moulds were given a choice between a high-aversive option (0.6% w/v) and a low-aversive  
8 option (0.4% w/v). Decision making performance was computed as the distance covered on the best  
9 bridge divided by the total distance. A value equal to 0.5 means that the slime mould distribution is  
10 symmetric between the two bridges, a value equal to 1 or 0 means that the slime moulds migrated only  
11 one the best or the worse bridge respectively. N > 235 for each age and each condition. Total numbers  
12 of assays= 4206. Error bars indicate CI95.  
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22 **Figure 5:** Learning as a function of age. (a) Migration speed on day 1. Two groups of 10 large  
23 plasmodia of two different ages 12 and 74 weeks old were used to conduct this experiment. Slime  
24 moulds were required to migrate on an oat gel bridge with an aversive substance NaCl (Oat+ NaCl)  
25 (0.6% w/v, habituated treatment) or a plain oat gel bridge (Oat) without the repellent (control  
26 treatment). Low values of speed indicate an aversive response. (b) Aversion index on day 4 computed  
27 after the habituation phase. On day 4, all slime moulds (habituated and control) had to migrate either  
28 on a bridge with the aversive substance or without. An aversion index closes to 0 indicates habituation  
29 while values clearly above 0 indicate an aversion to the repellent. N = 50 for each age (young and old)  
30 and each treatment (habituated and control). Total numbers of assays= 200. Error bars indicate 95%  
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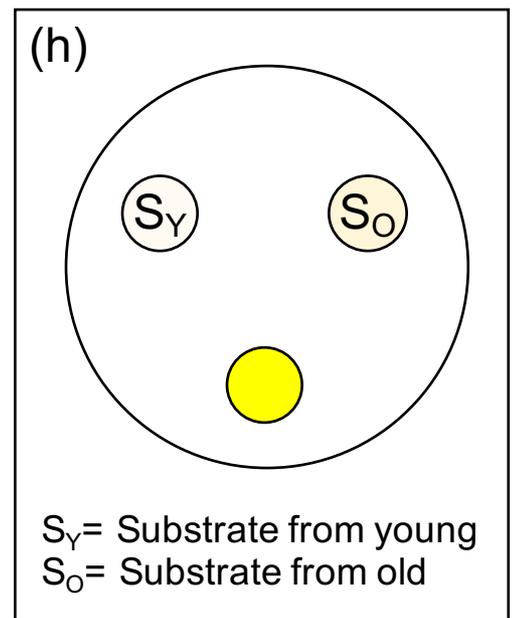
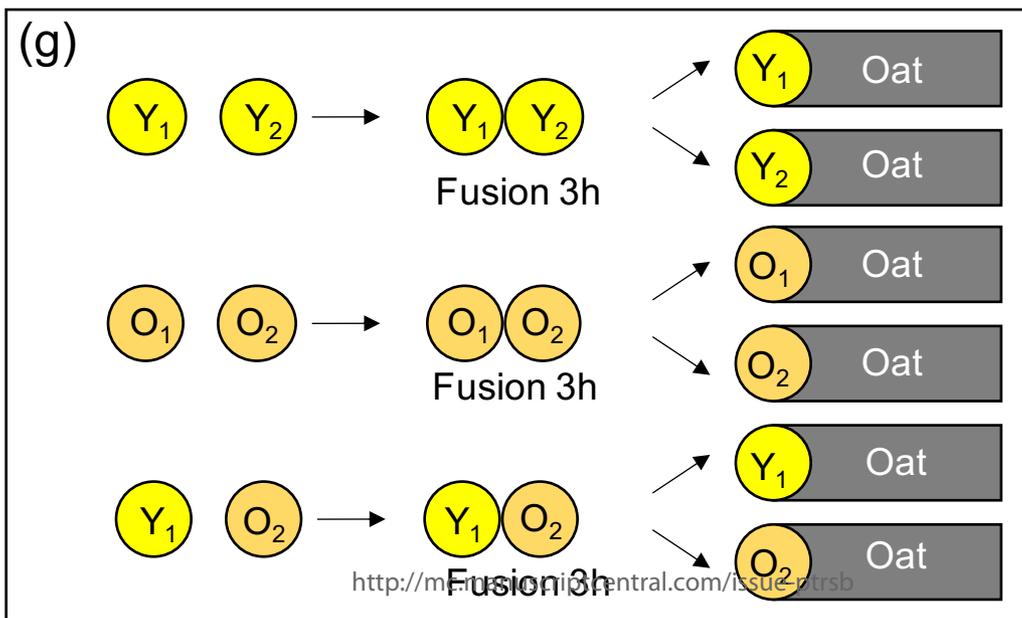
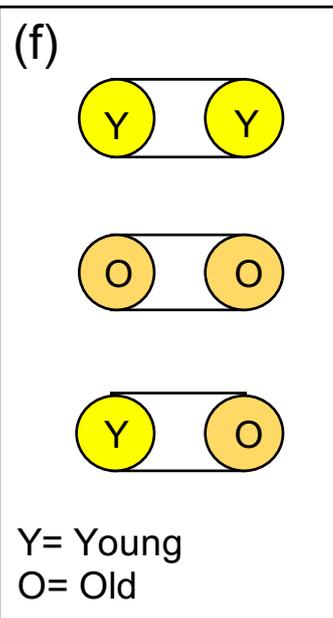
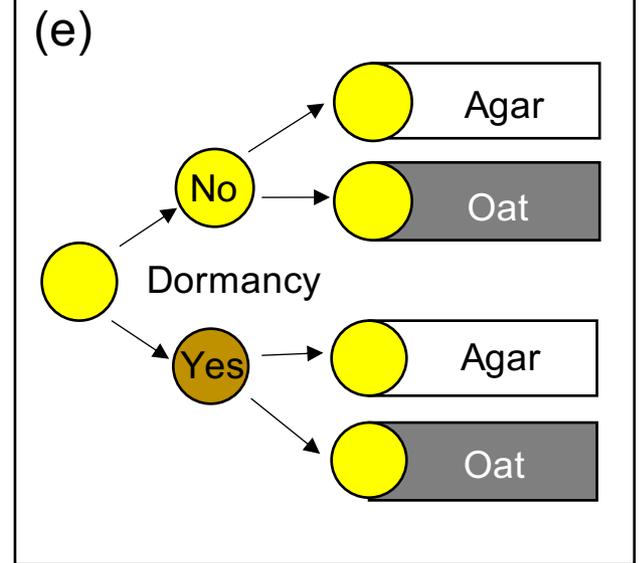
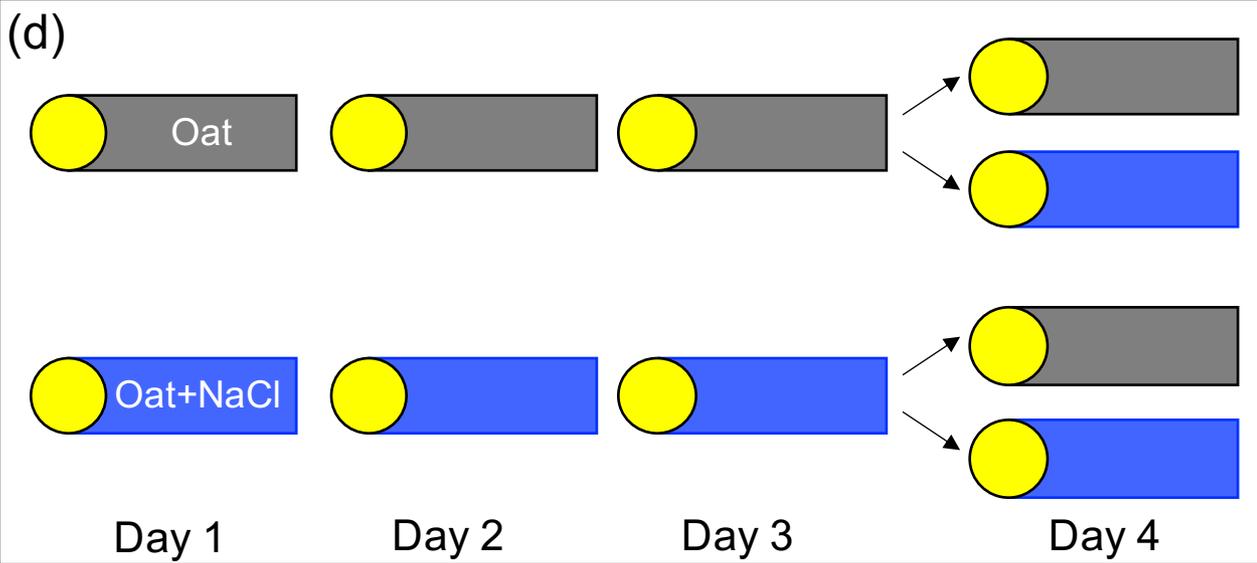
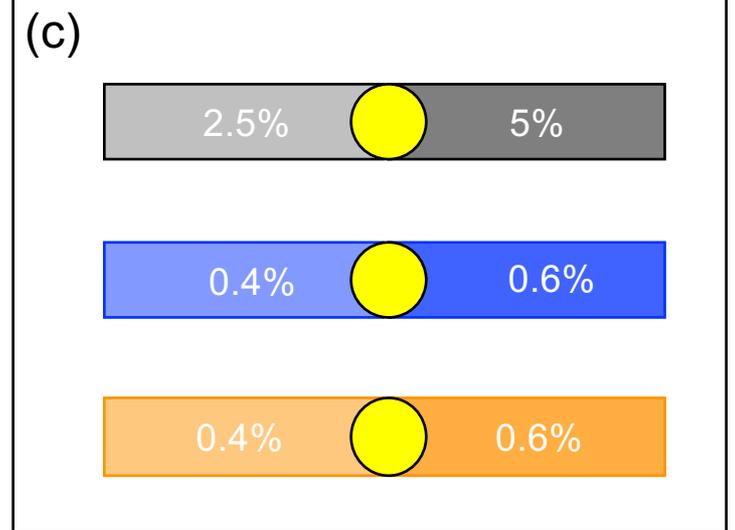
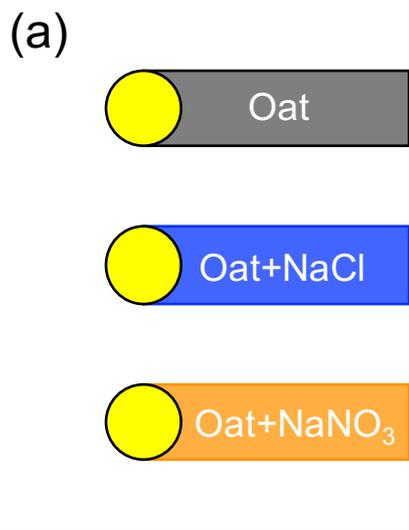
43 **Figure 6:** Mean migration speed following a dormancy period. Two groups of 10 large plasmodia of  
44 two different ages 60 (old) and 100 (very old) weeks old were used to conduct this experiment. Before  
45 the migration speed assays, slime moulds were either turn into sclerotia for a week and reinitiated  
46 (dormancy: yes) or kept as plasmodia (dormancy: no). The substrates were made of oat gel 5%w/v  
47 (Oat) or plain agar gel 1%w/v (Agar). N =80 for each age (old and very old) and each treatment  
48 (dormancy yes or no). Total numbers of assays= 640. Error bars indicate CI95.  
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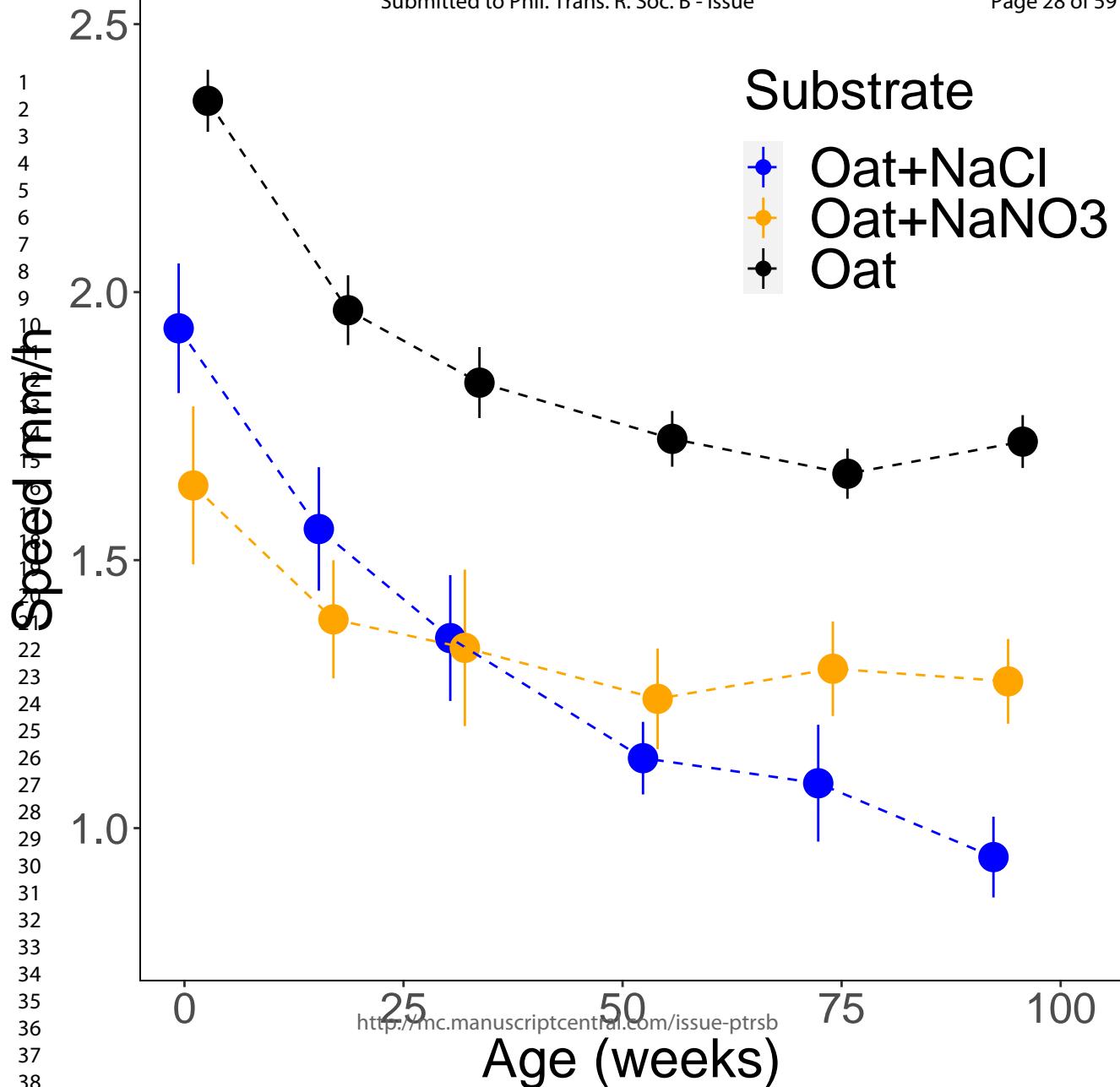
56 **Figure 7:** Fusion as a function of age. Two groups of 10 large plasmodia of two different ages 18  
57 (young) and 75 (old) weeks old were used to conduct this experiment. Young (old) slime moulds were  
58 paired with an individual of the same age or older (younger). The bridge separating the two slime  
59 moulds within a pair was made of plain agar gel 1% w/v. (a) Latency to membrane fusion as a function  
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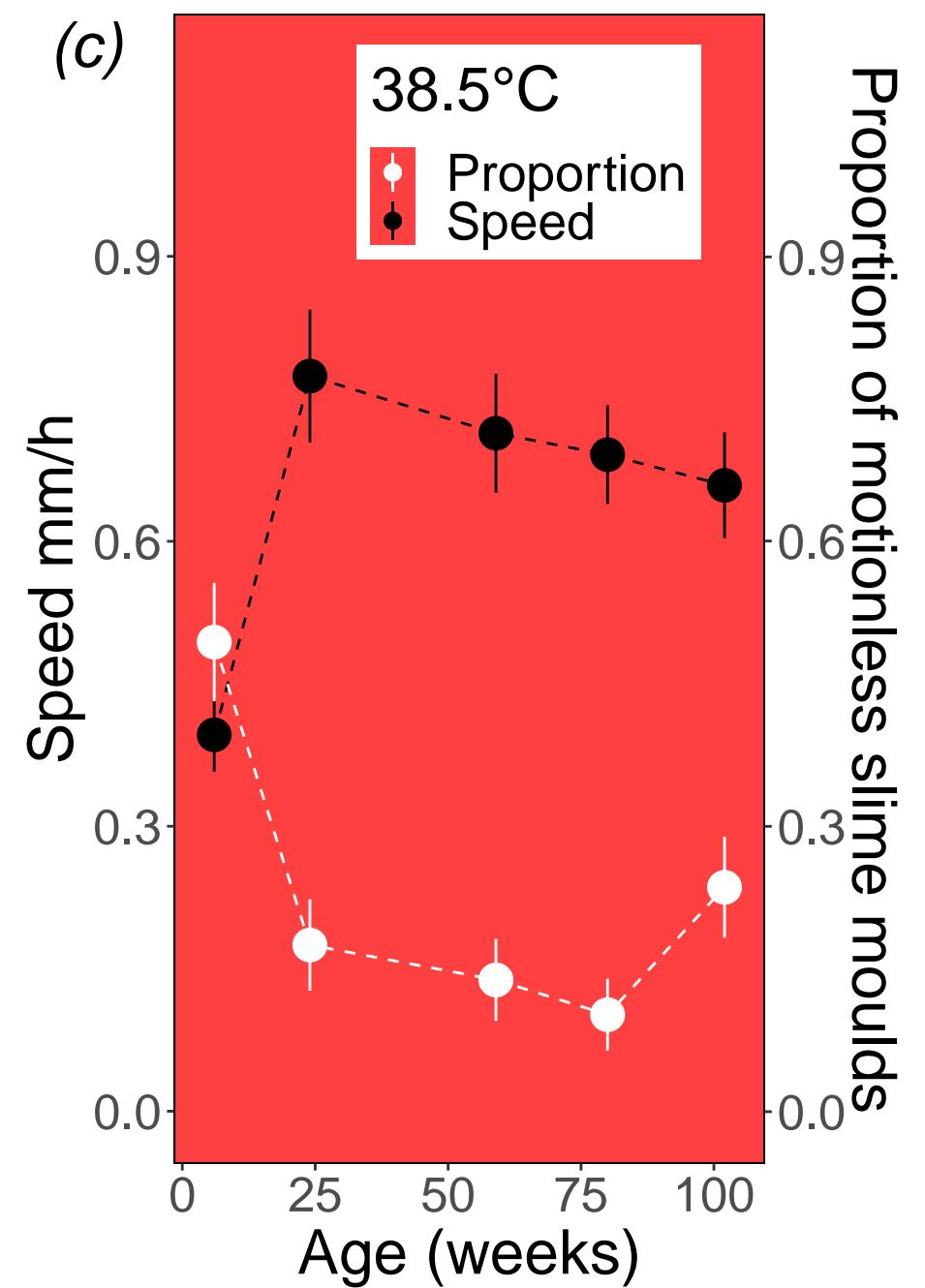
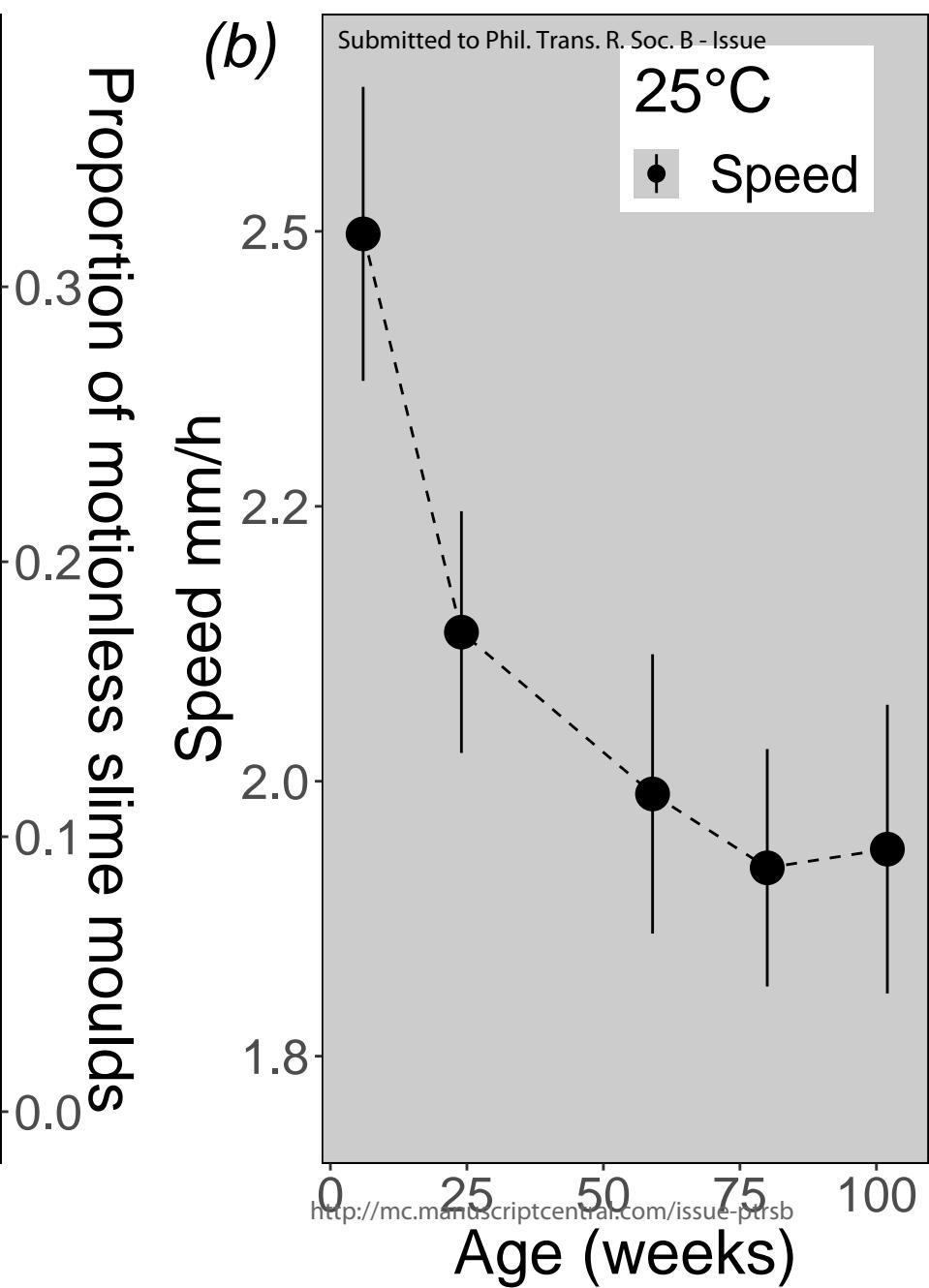
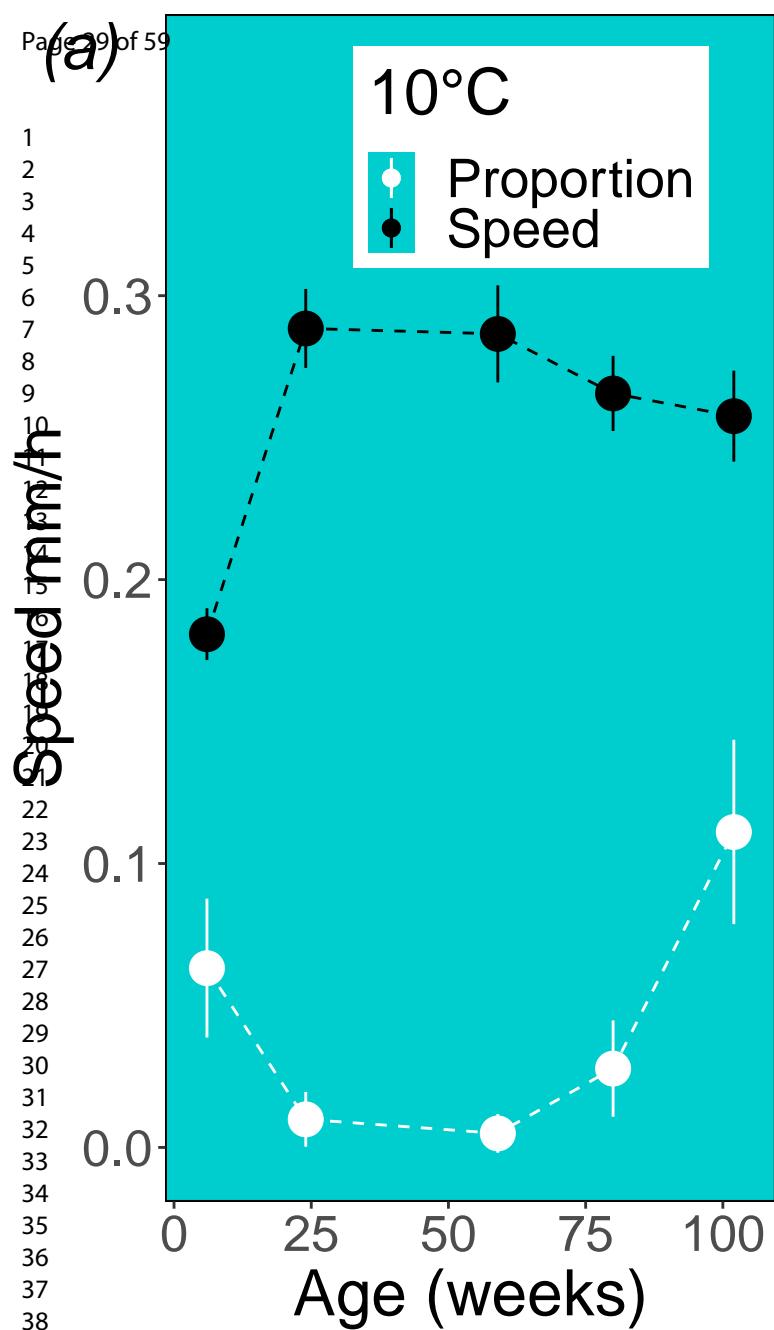
1 of age. The time of membrane fusion was recorded for each pair. (b) Latency to vein fusion as a  
2 function of age. The time of vein connection following membrane fusion was recorded for each pair.  
3 N=198, N=305 and N=202 for young/ young, young/old and old/old, respectively. Error bars indicate  
4 CI95.  
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11 **Figure 8:** Mean migration speed following fusion. Two groups of 10 large plasmodia of two different  
12 ages 5 (young) and 54 (very old) weeks old were used to conduct this experiment. Before the migration  
13 speed assays, slime moulds were allowed to fuse either with a slime mould of the same age or with a  
14 slime mould of a different age. The substrates were made of oat gel 5%w/v (Oat). N =100 for each par  
15 type (young/young, young/old, old/old). Total numbers of assays= 600. Error bars indicate CI95.  
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23 **Figure 9:** Detection of conspecifics as a function of age. Two groups of 10 large plasmodia of two  
24 different ages 3 (young) and 52 (old) weeks old were used to conduct this experiment. Slime moulds  
25 were offered a choice between two substrates previously explored by individuals of different ages. We  
26 computed the proportion of slime moulds that reached each substrate first. A value equal to 0.5 means  
27 that the slime moulds contacted the substrate explored by a young individual as often as the substrate  
28 explored by a young individual (random choice). N = 300 for each age. The CI of the random choice  
29 are delineated by shaded areas. Error bars indicate CI95.  
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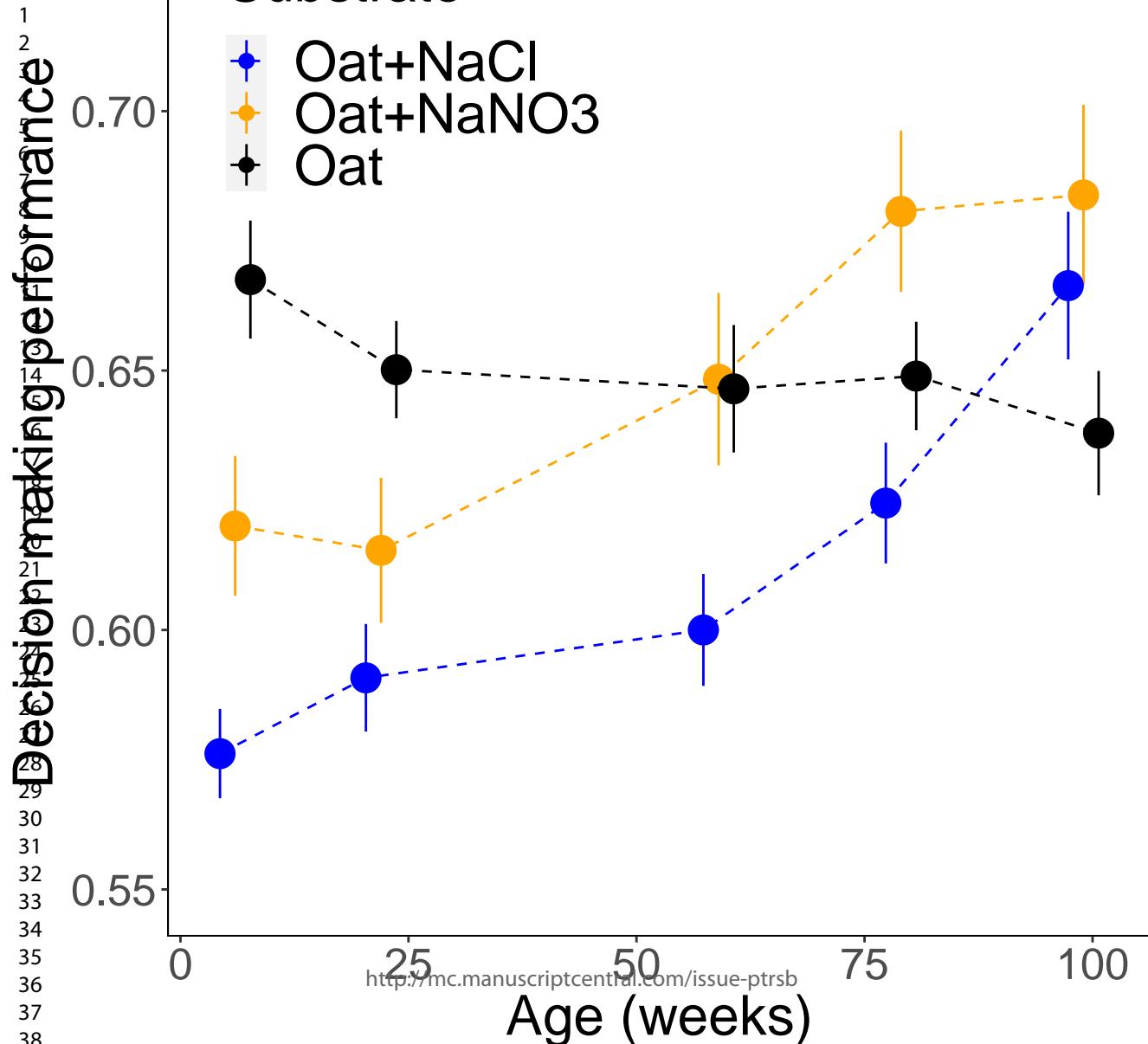


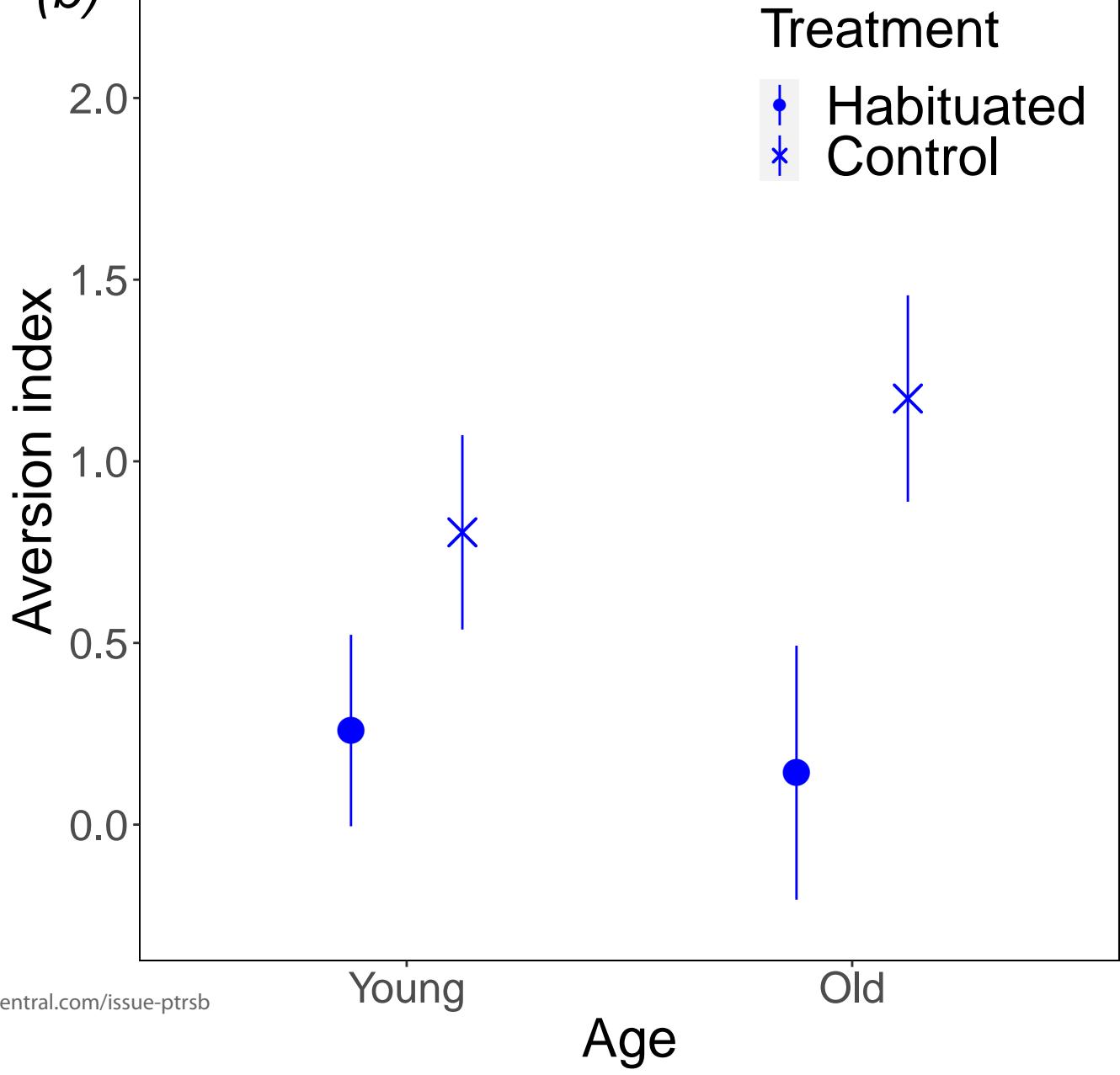
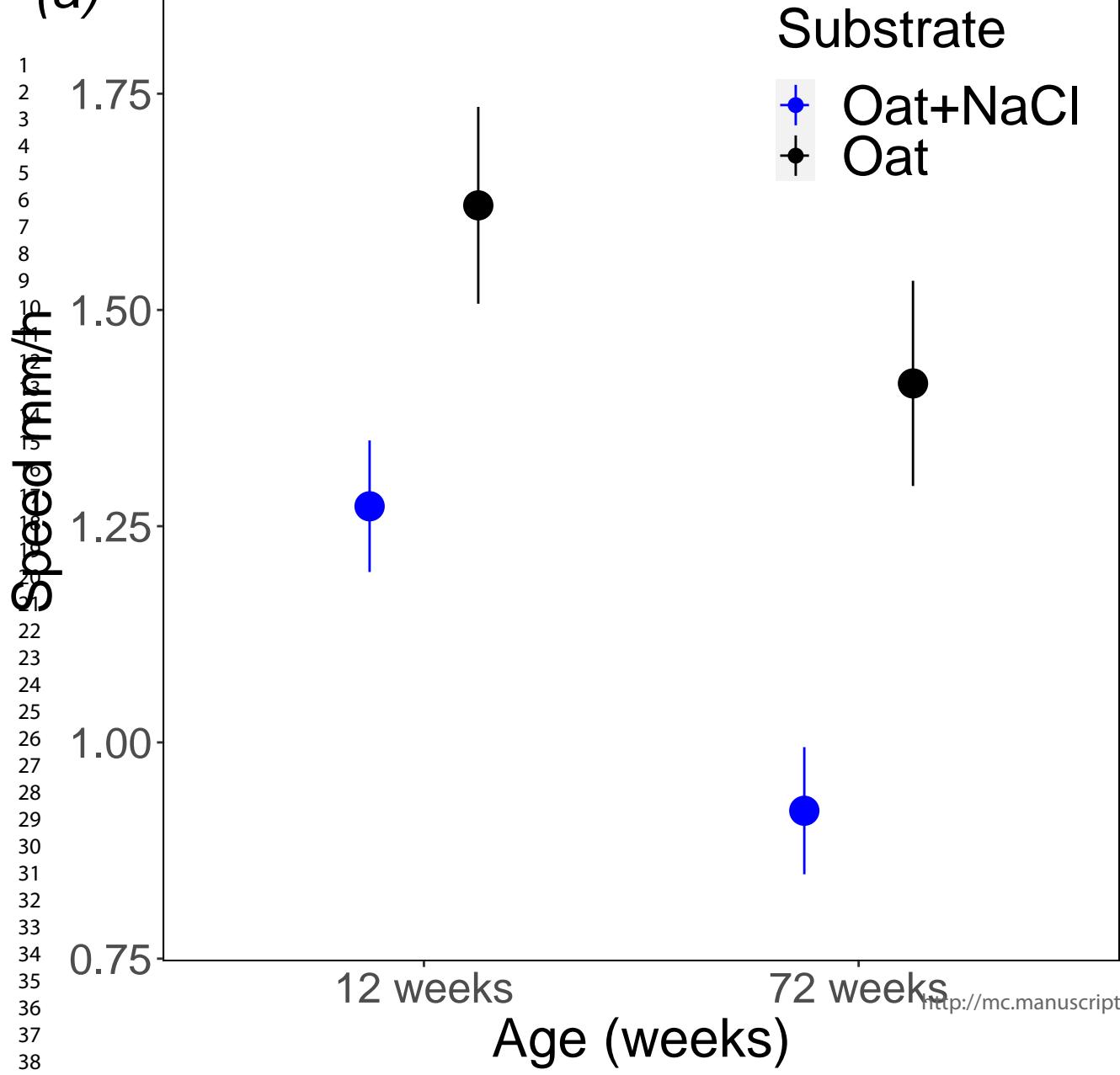




## Substrate

- Oat+NaCl
- Oat+NaNO<sub>3</sub>
- Oat





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Old

Substrate

○ Agar  
● Oat

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No

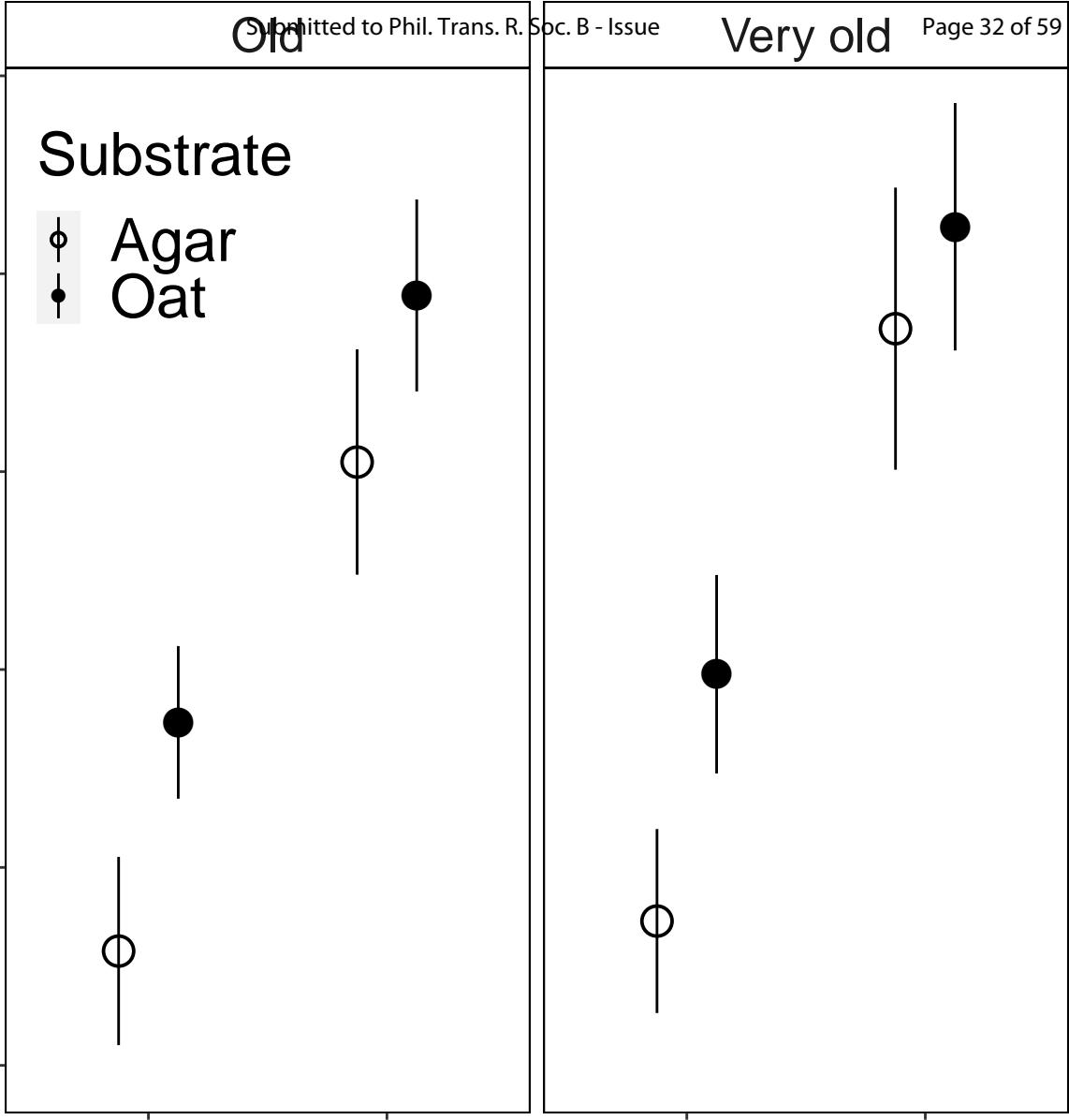
Yes

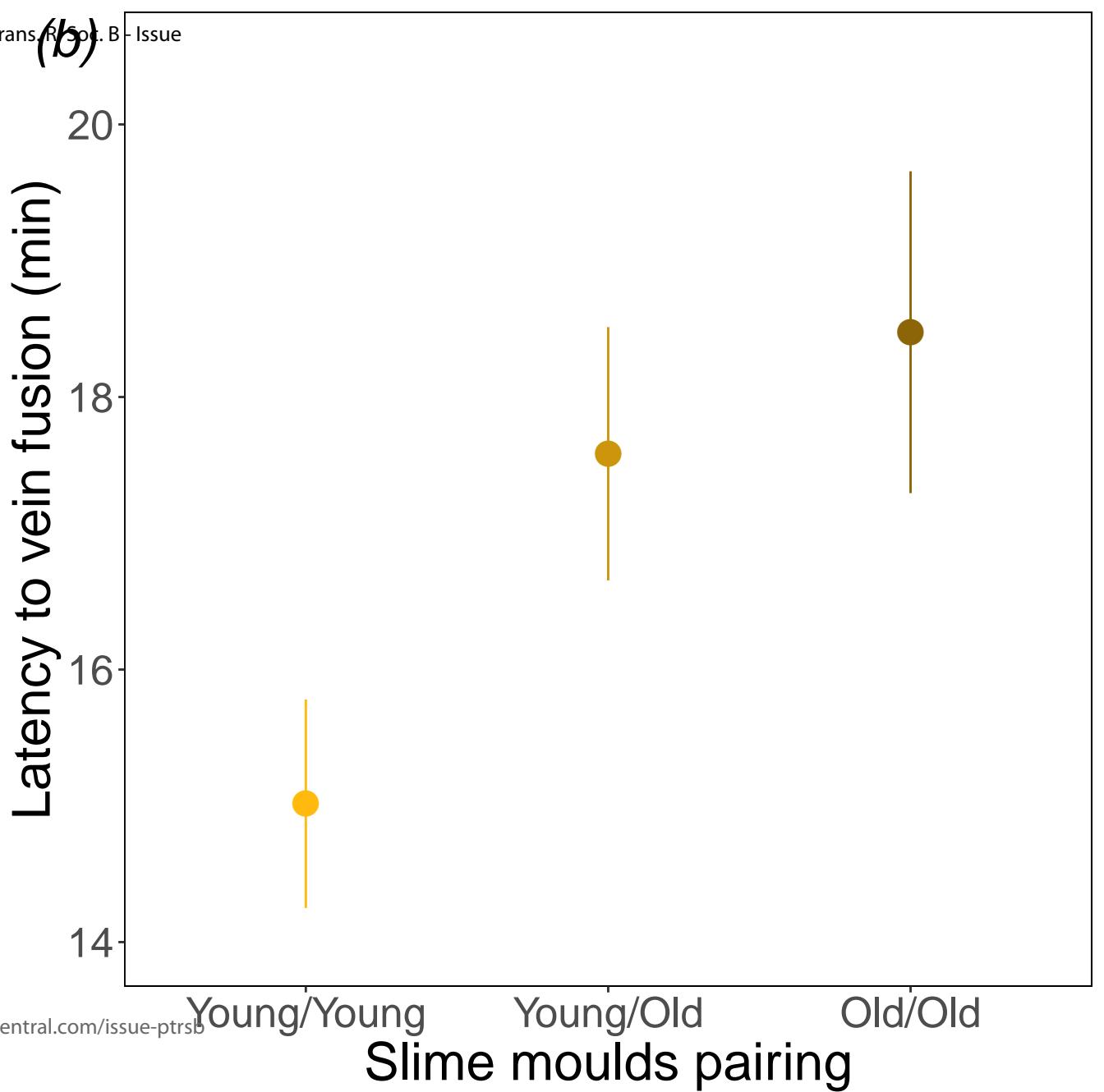
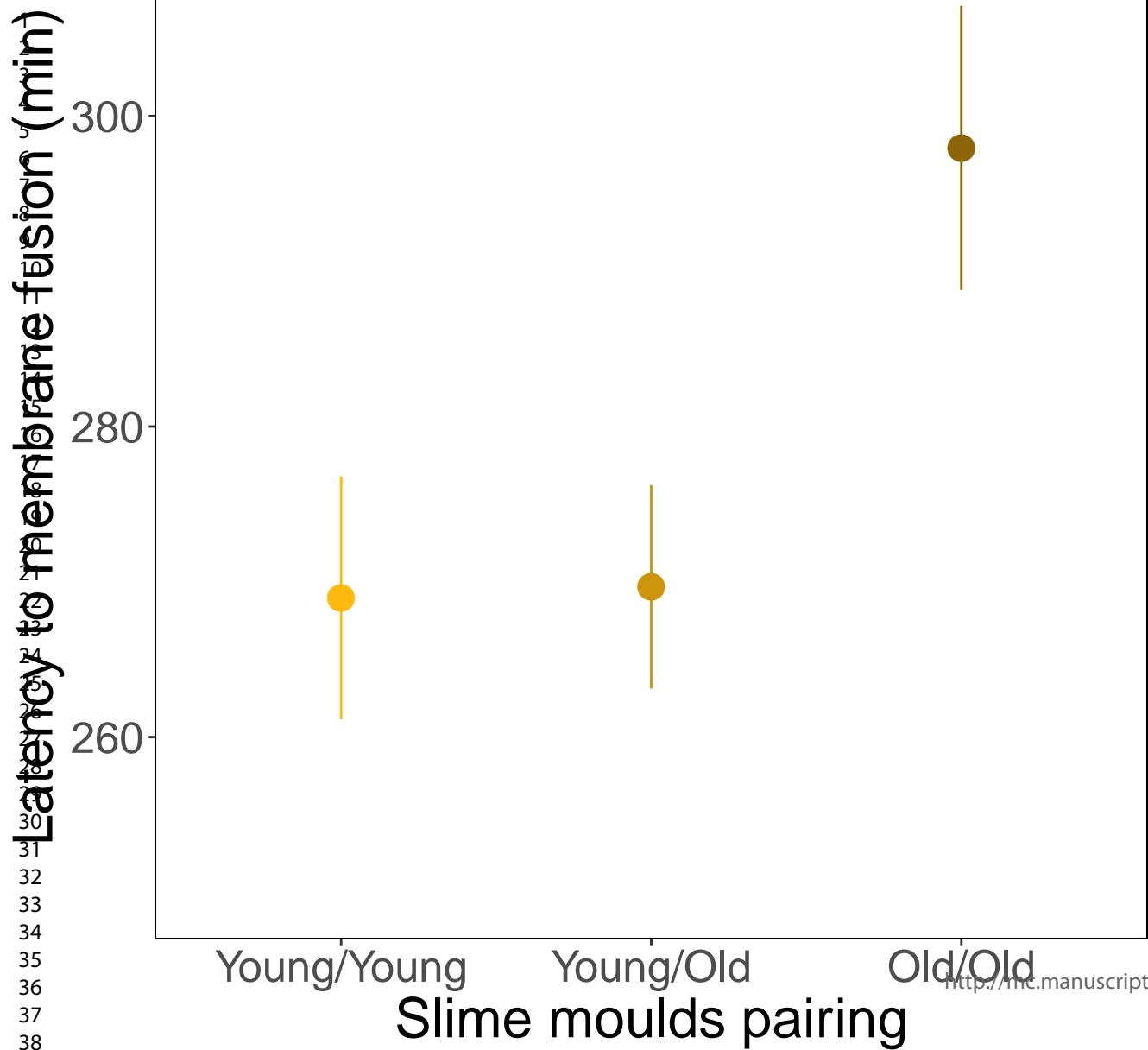
No

Yes

Dormancy

<http://mc.manuscriptcentral.com/issue-ptrsb>





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# Fused with

-  A young slime mould
-  An old slime mould

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2.75  
2.50  
2.25  
2.00  
1.75  
1.50

Speed mm/h

Young

Old

<http://mc.manuscriptcentral.com/issue-ptrsb>

Age

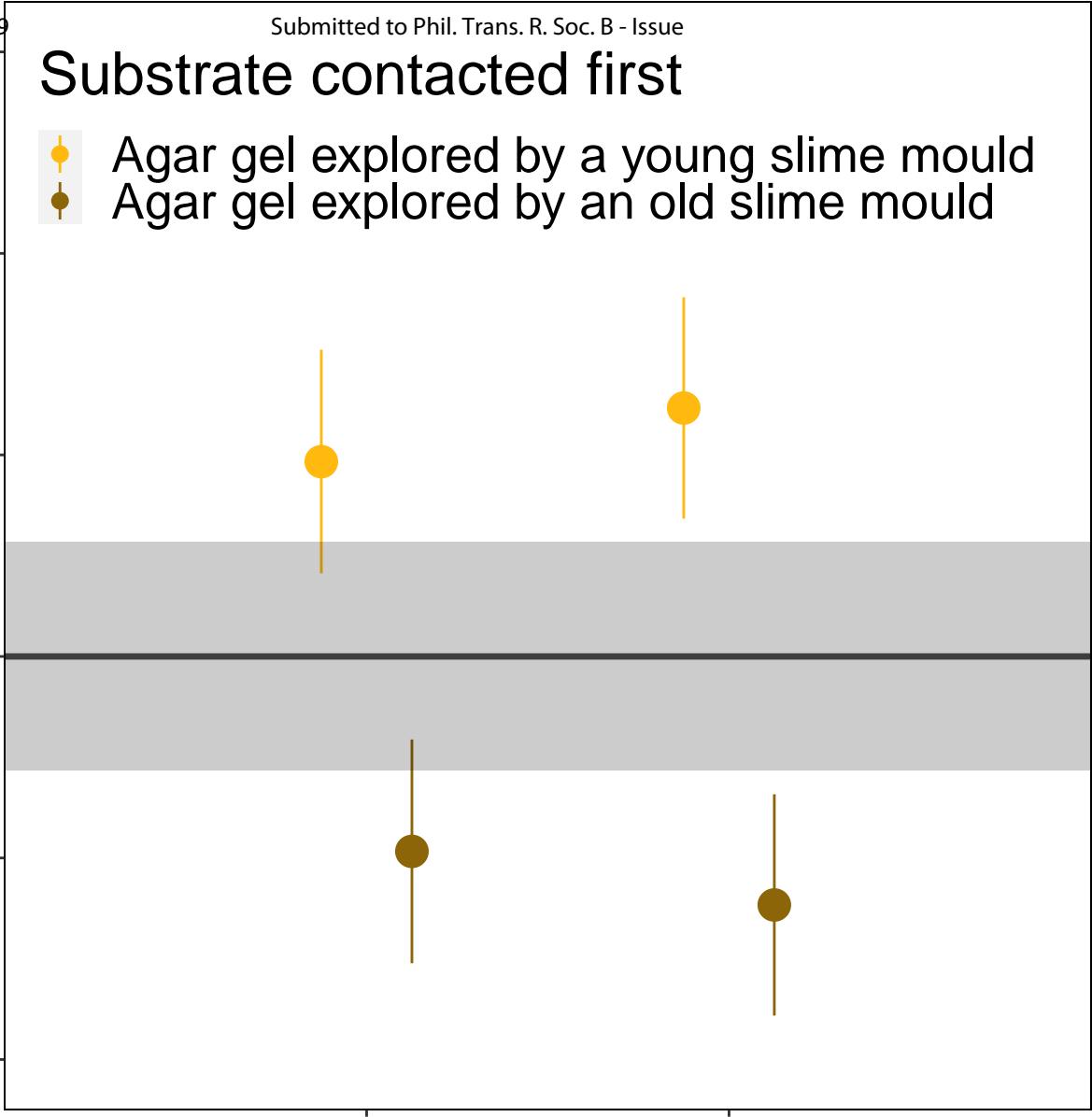
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# Substrate contacted first

Agar gel explored by a young slime mould  
Agar gel explored by an old slime mould

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Age

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## Behavioural changes in slime moulds over time

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<sup>1</sup>Contributed equally to the work

**Keywords:** Ageing, learning, decision making, *Physarum polycephalum*, slime moulds

### 4 Summary

5 Changes in behaviour over the lifetime of single cell organisms has primarily been investigated in  
6 response to environmental stressors. However, growing evidence suggests that unicellular organisms  
7 undergo behavioural changes throughout their lifetime independently of the external environment.  
8 Here we studied how behavioural performances across different tasks vary with age in the acellular  
9 slime mould *Physarum polycephalum*. We tested slime moulds aged from 1 week to 100 weeks. First,  
10 we showed that migration speed decreases with age in favourable and adverse environments. Second,  
11 we showed that decision making and learning abilities do not deteriorate with age. Third, we revealed  
12 that old slime moulds can recover their behavioural performances if they go throughout a dormant  
13 stage or if they fuse with a young congener. Lastly, we observed the response of slime mould facing a  
14 choice between cues released by clone mates of different age. We found that both old and young slime  
15 moulds are attracted toward cues left by young slime moulds. Although many studies have studied  
16 behaviour in unicellular organisms, few have taken the step of looking for changes in behaviour  
17 over the lifetime of individuals. This study extends our knowledge of the behavioural plasticity of single-  
18 celled organisms and establish slime moulds as a promising model to investigate the effect of aging on  
19 behaviour at the cellular level.

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## 22 Introduction

23 One aim of this special issue of *Philosophical Transactions* is to understand collective  
24 behaviour over the lifetime of individuals. In unicellular organisms, changes in collective behaviour  
25 through time has been studied almost exclusively in response to environmental stressors. For instance,  
26 it has been shown that bacteria [1,2] and cellular slime moulds [3–5] transit to new collective states  
27 such as coordinated cellular migration or aggregation when nutrients become scarce [6]. On the  
28 contrary, how collective behaviour changes with correlates of age in unicellular organisms  
29 independently of the external environment remains an open question. The main reason for this might  
30 be that, single cell organisms were mistakenly believed to be short lived and immune to ageing under  
31 optimum growth conditions [7–11]. It has now been demonstrated that, some unicellular organisms  
32 such as bacteria *Escherichia coli* and *Caulobacter crescentus*, ciliates *Paramecium* and *Tetrahymena*,  
33 yeast *Saccharomyces cerevisiae*, cellular and acellular slime moulds *Dictyostelium discoideum* and  
34 *Physarum polycephalum* undergo intrinsic changes over time that affect cell behaviour and physiology  
35 [11–13]. In this paper, our aim is to understand how cell behaviour varies over the lifetime of  
36 individuals in the acellular slime mould *P. polycephalum*. In contrast to the cellular slime mould *D.*  
37 *discoideum* in which the vegetative state consists of solitary amoebas that divide about every 4 h when  
38 food is plentiful, in *P. polycephalum*, the vegetative state is a unique large cell containing a large  
39 collection of nuclei that divide about every 8 h and share a common cytoplasm. Acellular slime moulds  
40 are particularly attractive organisms to study collective behaviour changes over time in unicellular  
41 organisms for several reasons.

42 First of all, although they are not collective *per se*, acellular slime moulds embrace most of the  
43 key principles of collective behaviour (reviewed in [14]): synchronization and coordination [15–18],  
44 positive and negative feedbacks [19], variability [20–22], symmetry breaking [20,23], phase transition  
45 [24], redundancy [14], information transfer [25,26] capacity to make complex decisions [27–30]. In  
46 addition, due to its internal dynamic, *P. polycephalum* is often regarded as a collection of nonlinear  
47 oscillators which are interconnected, often coupled and partially dependent on each other (reviewed in  
48 [16]). Furthermore, similarly to more traditional model systems in collective behaviour such as ant  
49 colonies or fish schools, its behaviour has successfully been simulated using a multi-agent approach  
50 [31,32]. In these models, *P. polycephalum* behaviour relies on particle like agents that follow simple  
51 rules but are capable collectively to generate self-organized amoeboid movement and to construct  
52 optimized networks [31,32].

53 Second, despite being unicellular, acellular slime moulds can produce seemingly complex  
54 behaviours. Indeed, since the seminal contribution of Toshiyuki Nakagaki twenty years ago [33], *P.*  
55 *polycephalum* has become an essential model organism for studying problem-solving in non-neural  
56 systems [32–37]. Past experiments have shown that acellular slime moulds can find the shortest path  
57 in a maze [33,38], build optimized networks to connect several food sources [34], anticipate events

1 58 [39], learn to ignore irrelevant stimuli [40,41], encode memory in their environment [42] or in their  
2 morphology [43], interact with their congeners [21,44], optimize nutrient intake [28,45], make optimal  
3 decisions [29,46,47], etc. *P. polycephalum*'s behaviour relies on its self-organized internal architecture  
4 which consists of a transport network of interconnected tubes [48]. The tubes contract and relax  
5 periodically, causing the cytoplasm to flow back and forth, a phenomenon termed 'shuttle streaming'  
6 [49]. *P. polycephalum* can migrate at a speed of up to few millimetres per hour through the interplay  
7 of intracellular flow, adhesion and rhythmic contractions of the tubes [49–51]. The frequency and the  
8 amplitude of the contractions depend on external stimuli and as a result, *P. polycephalum* is capable of  
9 altering its shape and motion as a function of a variety of external stimuli such as chemicals [52–54],  
10 light [55], temperature [56–58], humidity [44], gravity [59] or substrate distortion [46].

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Third, acellular slime moulds are easy to track through time and relatively long-lived. In unicellular organisms, lifetime can either be chronological if we consider the time a cell remains viable before dividing, or replicative if we consider the number of times a cell divide before it dies [9]. Tracking division is usually difficult as it requires to follow and distinguish individual cells. *P. polycephalum* offers the possibility to consider both chronological and replicative time simultaneously. During its development, the zygote undergoes synchronous mitotic divisions every 8 to 12 hours but without cytokinesis to form a single multinucleated cell, called a plasmodium [60,61]. Hence, the cell grows over time but there is no cell division. A plasmodium reared on solid medium by routine serial subculture under laboratory condition can be maintained from a few weeks to several months [62,63]. The life span of the plasmodium is in part genetically determined as different strains have different lifespans and subclones derived from a single plasmodium age in a coordinated manner, i.e death occurs at approximately the same time [62,64,65].

Fourth, *P. polycephalum* provides unique possibilities for experimental manipulations [66,67]. The plasmodium can extend up to hundreds of square centimetres. It can be severed into viable and structurally similar yet smaller plasmodia. Upon contact, genetically identical plasmodia can fuse with each other to form a single plasmodium. In response to environmental stressors, the plasmodium can enter a resting stage called sclerotium and remain dormant for years without deterioration. During the sclerotization process, the plasmodium lose 50% of their total protein content together with 40% of their DNA and 65% of their RNA [60]. After transfer to a humid and nutritive medium, a sclerotium can revert back to a mobile plasmodium within 24 h. Plasmodial cultures can be easily initiated from sclerotia after up to 3 years. Interestingly, numerous authors have reported that, a sclerotium issued from a seemingly ageing plasmodium may produce a vigorous plasmodium again, a form of "rejuvenescence". Following a period of growth this secondary plasmodium will undergo senescence again. This cycle of growth and senescence can be repeated several times and, thus, cultures can be maintained over many generations.

1 93 Lastly, *P. polycephalum* displays senescence, which is defined as a loss of fitness during ageing.  
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3 94 A plasmodium can be maintained for months during which no change is apparent until a reproducible  
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5 95 decline in viability occurs [62]. Senescence in the plasmodial stage is characterized by morphological  
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7 96 change [62], reduction of growth rate [62,68], reduced cytoplasmic flow [64], loss of yellow pigment  
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9 97 [64], accumulation of polyploid nuclei [69], high level of mitochondrial DNA fragmentation [68],  
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11 98 decreased replication of mitochondrial DNA [63], increase in nuclear size and DNA content [69,70].  
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13 99 Senescence ends with the fragmentation of the plasmodium into several small spherical structures with  
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15 100 a concomitant lysis of the plasmodium [64]. Longevity in slime moulds is not affected by most  
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17 101 environmental factors with the exception of high temperature [71]. Senescence in slime moulds has  
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19 102 been mainly studied at the cellular level. The potential effects of ageing on the plasmodium behaviour  
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21 103 remained to be investigated.

22 104 In this paper, we studied how slime mould behaviour changes over time. First, we explored the  
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24 105 effect of age on migration speed in nutritive and adverse environments. Second, we investigated if  
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26 106 decision making and learning performances changed with age. Third, we tested if older slime moulds  
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28 107 were able to recover after a dormant period or after fusing with younger clones. Lastly, we examined  
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30 108 if slime moulds were able to discriminate cues from congeners of different age.

## 31 109 **Methods**

### 32 33 110 **(a) Species studied and rearing conditions**

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35 111 *Physarum polycephalum*, also called the acellular slime mould, belongs to the supergroup Amoebozoa  
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37 112 and the class Myxomycetes. In nature, slime moulds are found on organic substrates like tree bark or  
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39 113 forest soil where they feed on microorganisms such as bacteria or fungi. *P. polycephalum* vegetative  
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41 114 morph is a vast multinucleated cell named plasmodium. We used a strain of *P. polycephalum* : LU352  
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43 115 kindly provided by Professor Dr Wolfgang Marwan (Max Planck Institute for Dynamics of Complex  
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45 116 Technical Systems, Magdeburg, Germany). 80 large plasmodia were initiated from sclerotia at  
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47 117 different time to obtain slime moulds of different age. In this paper, by age of the slime mould, we  
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49 118 understand the time which elapses from the moment of the inoculation of a sclerotium onto a nutritive  
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51 119 medium. The oldest plasmodia were kept after this study and are still being cultivated in our laboratory.  
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53 120 They are currently more than 2 years old and none of them has died so far. In previous reports,  
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55 121 plasmodium lifespan has been shown to vary from one month to one year, depending on the strain [62-  
56  
57 122 64]. Hence, we considered as “old”, slime molds which were over one year old and “very old” slime  
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59 123 molds which were almost two years old.

60  
61 124 Plasmodia were reared on a 1% w/v (weight/volume) agar medium with rolled oat flakes (Quaker Oats  
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63 125 Company®) in Petri dishes (90mm Ø). They were kept in the dark in a thermoregulated chamber at a  
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65 126 temperature of 20 degrees Celsius and a humidity of 80% from Monday to Friday. They were

1 127 transferred every day on a new agar medium on which was spread a single layer of rolled oat flakes.  
2  
3 128 During the weekends, slime moulds were unfed and kept at 12°C. One day before each experiment, all  
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5 129 plasmodia were transferred on a food medium consisting of finely ground oat flakes (5% w/v) mixed  
6  
7 130 with 1% agar gel, hereafter referred as “oat gel”.

### 9 131 **(b) Migration speed on various substrates as a function of slime moulds age**

11 132 The aim of this experiment was to investigate how age affects the slime mould migration speed on  
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13 133 various substrates (Figure 1a). Six groups of 10 large plasmodia of different ages (1, 17, 32, 54, 74  
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15 134 and 94 weeks old) were used to conduct this experiment. Circular samples (10 mm Ø) were cut from  
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17 135 each plasmodium and gently placed in contact with an oat gel bridge (35 mm long, 10 mm wide) in an  
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19 136 experimental arena (Petri dish 12mm\*12mm). The bridge either contained an aversive substance: NaCl  
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21 137 (100mM, 0.6% w/v) (Oat + NaCl bridge) or NaNO<sub>3</sub> (100mM, 0.8% w/v) (Oat + NaNO<sub>3</sub> bridge) or not  
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23 138 (Oat). Experimental arenas housed 8 bridges and were kept in a thermoregulated chamber at a  
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25 139 temperature of 25 degrees Celsius. After about 8 hours of incubation, the distance travelled by the  
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27 140 slime moulds on each bridge was measured with a ruler. We computed the migration speed as the  
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29 141 distance travelled divided by the time spent in contact with the bridge. A minimum of 48 samples were  
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31 142 tested for each substrate and each age group, leading to total of 2396 assays.

### 31 143 **(c) Migration speed at different temperatures as a function of slime moulds** 32 33 144 **age**

35 145 Slime moulds have been shown to suffer both from low and high temperature which affect the shuttle  
36  
37 146 streaming and therefore locomotion [72]. The aim of this experiment was to investigate how age affects  
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39 147 the slime mould migration speed under different temperatures (Figure 1b). Five groups of 10 large  
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41 148 plasmodia of different ages (8, 24, 61, 81 and 101 weeks old) were used to conduct this experiment.  
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43 149 Circular samples (10 mm Ø) were cut from each plasmodium and gently placed in contact with an oat  
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45 150 gel bridge (35 mm long, 10 mm wide) in an experimental arena (Petri dish 12mm\*12mm).  
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47 151 Experimental arenas housed 8 bridges and were kept in thermoregulated chambers at a temperature of  
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49 152 10, 25 or 38.5°C degrees Celsius. 25°C is a standard temperature to rear *Physarum polycephalum* while  
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51 153 10°C and 38.5°C represents extremes conditions. After about 8 hours of incubation, the distance  
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53 154 travelled by the slime moulds on each bridge was measured with a ruler. We computed the migration  
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55 155 speed as the distance travelled divided by the time spent in contact with the bridge. Numerous slime  
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57 156 moulds did not move at all during the experiments at extreme temperatures (10 or 38.5°C), they were  
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59 157 not taken into account to compute speed but we calculated the proportion of motionless slime moulds  
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158 for each age group and each temperature. A minimum of 80 samples were tested for each temperature  
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and each age group, leading to total of 3533 assays.

#### **(d) Decision making ability as a function of slime moulds age**

Slime moulds have been shown to succeed in selecting the best option when offered multiple alternatives [22,27,45,73]. To investigate how age affects slime mould ability to make a decision, we studied how a slime mould distributed itself between two agar gel bridges containing or not an aversive substance (NaCl or NaNO<sub>3</sub>) (Figure 1c). Under non aversive condition, the slime moulds were offered a choice between a high-quality oat gel (5% w/v) and a low-quality one (2.5% w/v). Under aversive condition, the slime moulds were given a choice between a high-aversive option (0.6% w/v NaCl or NaNO<sub>3</sub>) and a low-aversive option (0.4% w/v NaCl or NaNO<sub>3</sub>). Five groups of 10 large plasmodia of different ages (6, 22, 59, 79 and 99 weeks old) were used to conduct this experiment. Circular samples (10 mm Ø) were cut from each plasmodium, placed between two bridges (35 mm long, 10 mm wide) in experimental arenas (Petri dish 12mm\*12mm). Experimental arenas housed 4 binary choices and were kept in a thermoregulated chamber at a temperature of 25 degrees Celsius. After about 8 hours of incubation, the distance travelled (in mm) on each bridge was measured with a ruler. We computed a decision-making performance as the distance covered on the best bridge (high quality or low aversive substrate) divided by the total distance. A minimum of 235 samples were tested for each age and each binary choice, leading to total of 4206 assays

#### **(e) Learning ability as a function of slime moulds age**

Slime moulds have been shown to be capable of habituation, a simple form of learning, when repeatedly exposed to an innocuous aversive substance [40,74]. To investigate how age affects slime moulds' ability to learn, we studied how slime moulds habituate to an aversive substance [25,40]. A bridge crossing experiment adapted from [40] was conducted to habituate the slime mould to an aversive substance (NaCl 100mM, 0.6% w/v) (Figure 1d). We used two groups of 10 large plasmodia of two different ages 12 and 74 weeks old. Before starting the habituation experiment, to accustom the slime moulds to the experimental set-up, 10 circular samples (Ø 10 mm) were cut from each plasmodium and introduced in an experimental arena (Petri dish 12mm\*12mm). The samples were then connected to an oat gel bridge (35 mm long, 10 mm wide). After a day, the slime moulds had covered the bridge, and a circular sample was cut from the bridge and transferred to new experimental arenas to start the habituation. On day 1 of the habituation, half of the samples were offered an oat gel bridge containing NaCl (substrate: Oat+NaCl, treatment: habituated, N=50 for each age group) while the remaining half had to cross a bridge without NaCl (substrate: Oat, treatment: control, N=50 for each age group). The following two days (day 2 and day 3), a circular sample was cut from each bridge daily and transferred to another arena where they were offered a new bridge. On day 4, once the habituation training was completed, we cut one or two circular samples from each bridge and transferred them to a new arena. Half of the habituated and control samples were offered a bridge containing NaCl (substrate: Oat+NaCl) while the remaining half had to cross a bridge without NaCl

(substrate: Oat). Thus, on day 4 the control group is either facing a bridge containing NaCl for the first time (N=30 for each age group) or a bridge without NaCl (N=30 for each age group) while the habituated group is either facing a bridge containing NaCl for the fourth time (N=30 for each age group) or a bridge without NaCl (N=30 for each age group). Every day, after about 8 hours of incubation, the distance travelled (in mm) on each bridge was measured with a ruler. Data collected on day 1 allowed us to confirm that slime moulds showed a clear aversive behaviour toward the NaCl while data collected on day 4 enable us to test if slime mould learn to ignore the aversive substance. On day 4 we synthesized the results with an aversion index HI and CI as in [41].

$$HI = \left| \frac{1}{N} \sum_{i=1}^N \frac{HON_i - \overline{HO}}{sd(\overline{HO})} \right| \quad \text{or} \quad CI = \left| \frac{1}{N} \sum_{i=1}^N \frac{CON_i - \overline{CO}}{sd(\overline{CO})} \right|$$

HON, CON, HO and CO are the habituated (H) and control slime moulds (C) on Oat+NaCl (ON) and Oat (O) respectively. Using those indexes allowed us to normalize each variable value corresponding to the treatments HON and CON by the mean and the standard deviation of their respective control HO and CO. Values clearly above 0 indicate an aversion towards the repellent, whereas values close to zero indicate habituation to the repellent *i.e.* slime moulds react the same way to Oat+NaCl and Oat.

#### (f) Recovery ability following dormancy as a function of slime moulds age

To investigate how age affects slime mould ability to recover, we studied how fast old slime moulds migrate after a dormancy period (Figure 1e). We used two groups of 10 large plasmodia of two different ages 60 and 100 weeks old, referred as “old” and “very old”. To initiate the transition from plasmodia to sclerotia, we cut 2 circular samples from each plasmodium ( $\varnothing = 20$  mm), placed each of them on a moist paper filter ( $\varnothing = 145$  mm) in a petri dish ( $\varnothing = 145$  mm) and kept them in a thermoregulated chamber at a temperature of 30 degrees Celsius for two days to dry. The sclerotia were then stored for a week before being re-initiated to obtain new plasmodia. A week later, we then had 40 large plasmodia, 20 were one week old and originated from old and very old plasmodia (treatment: dormancy) and 20 were old and very old (treatment: no dormancy). Circular samples ( $\varnothing 10$  mm) were cut from each plasmodium and gently placed in contact with an oat gel bridge (nutritive substrate) or an agar gel bridge (agar 1%w/v, non-nutritive substrate) in an experimental arena (Petri dish 12mm\*12mm). Experimental arenas housed 8 bridges and were kept in a thermoregulated chamber at a temperature of 25 degrees Celsius. After about 8 hours of incubation, the distance travelled by the slime moulds on each bridge was measured with a ruler. We computed the migration speed as the distance travelled divided by the time spent in contact with the bridge. A minimum of 80 samples were tested for each age group (old, very old) and each treatment (dormancy or not), leading to total of 640 assays.

### **(g) Fusion as a function of slime moulds age**

Fusion constitutes a defining feature of the lifestyle of slime moulds. To investigate how age affects slime mould ability to fuse, we studied how fast two slime moulds fuse as function of their respective age (Figure 1f). We used two groups of 10 large plasmodia of two different ages 18 and 75 weeks old, referred as “young” and “old”. Circular samples (10 mm Ø) were cut from each plasmodium and gently placed in contact with an agar gel bridge (15 mm long, 15 mm wide) in an experimental arena (Petri dish 12mm\*12mm). We placed a sample at each extremity of the bridge and tested the following pairs: young/young (N=220), young/old (N=330) and old/old (N=220). Experimental arenas housed 8 pairs of slime moulds. They were kept in a thermoregulated dark room at a temperature of 21 degrees Celsius. We conducted behavioural scan observations using a flash light to record the latency to membrane and vein fusion. Membranes were considered as fused when we could not see the delimitation between the two slime moulds while veins were considered as fused when we could discern a vein going from one slime mould to the other. The latency between two scans was less than 5 minutes. Pairs that did not fuse after 9h or that fused with their neighbouring slime moulds in the arenas were removed from the analysis (young/young: 26 out of 220, young/old: 25 out of 330 and old/old: 18 out of 220)

### **(h) Recovery ability following fusion as a function of slime moulds age**

Slime mould fusion could provide the potential for cell recovery through resource sharing. Recently, it was shown that a **learned** behaviour can be transferred during fusion [25]. In this experiment, we measure migration speed after fusion as a function of slime moulds age (Figure 1g). We used two groups of 10 large plasmodia of two different ages 5 and 54 weeks old, referred as “young” and “old”. Circular samples (10 mm Ø) were cut from each plasmodium and gently placed by pairs in an experimental arena (Petri dish 12mm\*12mm). Each experimental arenas housed 4 pairs. We tested the following pairs: young/young (N=100), young/old (N=100) and old/old (N=100). Fusion was allowed by bringing the slime moulds into contact for 3 hours (see Vogel and Dussutour 2016). After the fusion, the slime moulds were gently separated at the point of contact using a spatula. Then, all slime moulds were required to cross an oat gel bridge (35 mm long, 10 mm wide). Experimental arenas housed 8 bridges and were kept in a thermoregulated chamber at a temperature of 25 degrees Celsius. After about 8 hours **of** incubation, the distance travelled by the slime moulds on each bridge was measured with a ruler.

### **(i) Detection of conspecifics as a function of slime moulds age**

Slime moulds can sense and respond to chemical cues released in the environment by conspecifics in a foraging context [21,44]. They release calcium while foraging which is attractive to other slime moulds [21]. In this experiment we monitored the directional movement response evoked in slime

1 263 moulds in the presence of substrates previously explored by clone mates of different age (Figure 1*h*).  
2  
3 264 We used two groups of 10 large plasmodia of two different ages 3 and 52 weeks old, referred as  
4  
5 265 “young” and “old”. Circular Petri dishes (diameter,  $\text{\O} = 90$  mm) containing a layer of 1% w/v agar gel  
6  
7 266 were used as experimental arenas. Once the agar in the Petri dish had set, three holes were punched ( $\text{\O}$   
8  
9 267 = 10 mm, inter- distance = 20 mm). One hole was filled with a young and an old slime mould ( $\text{\O} = 10$   
10  
11 268 mm) sitting on 5% w/v oat gel. The other two holes were filled with experimental substrates ( $\text{\O} = 10$   
12  
13 269 mm) which consisted in agar gel explored for 24 h by well-fed young and old slime mould. To obtain  
14  
15 270 the experimental substrates, a slime mould was allowed to cover completely an agar gel while being  
16  
17 271 fed with oat flakes (making sure that the food was never in contact with the agar substrate, see [21] for  
18  
19 272 details). Then we removed the slime mould, and rinsed the agar substrate with distilled water just before  
20  
21 273 the experiment. The slime mould would typically explore its environment by expanding in all  
22  
23 274 directions for a short distance to finally migrate in a specific direction, eventually contacting one  
24  
25 275 experimental substrate. The experimental substrate that was reached first was taken to imply a positive  
26  
27 276 response (*i.e.*, a relative preference for the cues enclosed in the experimental substrate over the  
28  
29 277 alternative). For each assay, we recorded which substrate was contacted first and the latency to reach  
30  
31 278 each substrate. We replicated each binary choice 300 times with old and young slime moulds.  
32  
33 279 Experiments were recorded using digital cameras (EOS 70D, Canon) which took a picture every 5 min

## 31 280 (j) Statistical analyses

33 281 All statistics were performed with RStudio (Version 1.2.1335). To assess the difference in the various  
34  
35 282 parameters measured between the treatments, we used linear mixed models (LMM) or generalised  
36  
37 283 mixed models (GLMM) (function lmer or glmer). The package lme4 [75] was used for all mixed  
38  
39 284 models. The lmerTest and Car packages were then used to run Type III analysis of variance on the  
40  
41 285 calculated LMMs and GLMMs, extracting p-values for F-tests and Wald-tests. Assumptions for all  
42  
43 286 LMMs were checked using standard procedure: diagnostic of quantile-quantile normal plots and  
44  
45 287 Shapiro-Wilk test. The models were fitted by specifying the fixed effects (explanatory variables)  
46  
47 288 depending on the experiment and a random effect: the plasmodium identity. The dependent variables  
48  
49 289 that did not fit linear model requirements were transformed using the “bestNormalize” function  
50  
51 290 (“bestNormalize” package). Continuous variables were centred and scaled if needed. The outcomes of  
52  
53 291 all the models are presented in the supplementary information (Table S1-S13)

## 54 292 Results

### 57 293 (a) Migration speed on various substrates as a function of slime moulds age

58  
59 294 In the first experiment, we investigated if slime moulds migration speed is affected by age on different  
60  
295 substrates. Slime moulds migrated faster on plain oat gel than on oat gels containing an aversive  
296 substance (LMM, substrate  $F=67.01$ ,  $p<0.001$ , table S1, figure 2). For all substrates, migration speed

297 decreased as age increased (LMM, age  $F=80.52$ ,  $p<0.001$ , table S1, figure 2). On plain oat gel,  
298 migration speed declined drastically until the slime moulds were 30 weeks old then decreased gently.  
299 On oat gel containing  $\text{NaNO}_3$ , migration speed reached a plateau when the slime moulds were over 30  
300 weeks old. On oat gel containing  $\text{NaCl}$ , migration speed declined continuously with age (LMM age \*  
301 substrate  $F=28.08$ ,  $p<0.001$ , table S1, figure 2). Hence,  $\text{NaNO}_3$  was the most aversive substance for  
302 young slime moulds while for old slime moulds it was  $\text{NaCl}$ .

### 303 **(b) Migration speed at different temperatures as a function of slime moulds** 304 **age**

305 In the second experiment, we examined if slime moulds' migration speed was affected by age at  
306 different temperatures. Slime moulds migrated faster at standard temperature than at cold or hot  
307 temperature (GLMM, temperature  $\chi^2=4724.85$   $p<0.001$ , table S2, figure 3). As previously, at standard  
308 temperature, migration speed declined drastically with age at the beginning then decreased gently when  
309 the slime moulds were over 25 weeks old. At both low and high temperatures, the speed was the lowest  
310 for young slime moulds (8 weeks old), then increased until the slime moulds reached an intermediate  
311 age between 24 and 61 weeks old and finally decreased slightly with age (GLMM, age  $\chi^2=5.67$   
312  $p=0.017$ , age \* temperature  $\chi^2=43.26$   $p<0.001$ , table S2, figure 3). The proportion of motionless slime  
313 moulds was also affected by age (GLMM, age  $\chi^2=10.11$   $p<0.001$ , Table S3, figure 3) and temperature  
314 (GLMM, temperature  $\chi^2=200.47$   $p<0.001$ , table S3, figure 3). The youngest (8 weeks old) and oldest  
315 slime moulds (101 weeks old) remained most often motionless at both extreme temperatures with the  
316 youngest being more vulnerable to cold and the oldest to heat (GLMM, age \* temperature  $\chi^2=39.58$   
317  $p<0.001$ , table S3, figure 3).

### 318 **(c) Decision making ability as a function of slime moulds age**

319 In the third experiment, we estimated slime moulds' ability to make a decision as a function of age and  
320 substrate. As previously, slime moulds migrated faster on plain oat gel than on oat gels containing an  
321 aversive substance (GLMM, substrate  $\chi^2=451.80$   $p<0.001$ , table S4) and for all substrates migration  
322 speed decreased as age increases (GLMM, age  $\chi^2=5.91$   $p<0.001$ , age \* substrate  $\chi^2=18.88$   $p<0.001$ ,  
323 table S4). On average, slime moulds had better performances under non aversive conditions (GLMM,  
324 substrate  $\chi^2=141.34$   $p<0.001$ , table S5, figure 4). However, under aversive conditions, decision making  
325 performance increased with age while it remained constant under non aversive conditions (GLMM,  
326 age  $\chi^2=4.22$   $p=0.040$ , age \* substrate  $\chi^2=111.82$   $p<0.001$ , table S5, figure 4). Hence, young slime mould  
327 had better performances under non aversive condition while it was the contrary for old slime moulds.

### 328 **(d) Learning ability as a function of slime moulds age**

329 In the fourth experiment, we investigated if slime moulds' ability to habituate to an aversive substance  
330 depended on age. On day 1, slime moulds migrated faster on plain oat gel than on oat gels containing

NaCl (LMM, substrate  $F=75.86$   $p<0.001$ , table S6, figure 5a) and migration speed was higher for young than for old slime moulds (LMM, age  $F=15.27$   $p=0.001$ , age \* substrate  $F=3.76$   $p=0.054$ , table S6, figure 5a). On day 4, habituated and control slime moulds were tested for habituation and were required to cross a bridge containing NaCl. Control slime moulds showed a strong aversive behaviour and a high aversion index. In contrast, habituated slime moulds, encountering NaCl for the fourth time, showed no aversive behaviour and an aversion index close to zero (LMM, treatment  $F=23.54$   $p<0.001$ , table S7, figure 5b). Hence, they learned to ignore the aversive substance. Slime moulds age did not affect their learning performance (LMM, age  $F=2.15$   $p=0.145$ , age\*treatment  $F=1.92$   $p=0.169$ , table S7, figure 5b)

### (e) Recovery ability as a function of slime moulds age

In the fifth experiment, we tested if slime moulds recovered their original migration speed after a dormancy period. Slime moulds migrated faster on oat gel than on agar gels (LMM, substrate  $F=30.10$   $p<0.001$ , table S8, figure 6) regardless of age and treatment (LMM, substrate \* age  $F=0.11$   $p=0.741$ , substrate \* treatment  $F=2.54$   $p=0.111$ , table S8, figure 6). Slime moulds that were turned into a dormant state and reinitiated were significantly faster than their counterparts that remained as plasmodia (LMM, treatment  $F=59.52$   $p<0.001$ , table S8, figure 6). **Age of the dormant slime mould had no effect on speed following dormancy** (treatment \* age,  $F=0.09$   $p=0.772$ , table S8, figure 6).

### (f) Fusion as a function of slime moulds age

In the sixth experiment we examined if the fusion process was affected by age. The latency for the membrane to fuse was shorter for pairs of young slime moulds or mixed pair (young/old) than for pairs of old ones (LMM, age  $F=3.63$   $p=0.034$  table S9, figure 7a). Vein fusion, following membrane fusion, also occurred earlier in pair of young slime moulds than in the other pairs (LMM, age  $F=4.21$   $p=0.030$ , table S10, figure 7b).

### (g) Recovery ability following fusion as a function of slime moulds age

In the fifth experiment, we tested if old slime moulds recovered their original migration speed after fusing with young ones. **Young slime moulds were faster** than old slime mould when they were allowed to fuse with a slime mould of the same age (LMM, age  $F=19.68.52$   $p<0.001$ , table S11, figure 8). However, old slime moulds, that were allowed to fuse with a young one, were significantly faster than their counterparts that fused with an old one (LMM, age\*pair type  $F=33.84$   $p<0.001$ , table S11, figure 8). On the contrary young ones that were allowed to fuse with an old slime mould were slower than their counterparts that fused with a young one.

## (h) Detection of conspecifics as a function of slime moulds age

In the last experiment, we investigated if slime moulds could perceive a difference in cues left by slime moulds of different ages. As previously shown, young slime moulds were faster than old ones (GLMM, age  $\chi^2=14.44$   $p<0.001$ , table S12, figure 9). Both young and old slime moulds contacted first the substrate explored by a young slime mould significantly more often than the substrate explored by an old one (GLMM, observed vs expected:  $\chi^2=5.67$   $p=0.017$ , table S13, figure 9).

## Discussion

In many organisms, ageing is accompanied by deficits in behavioural performance. In this study, we were able to maintain a culture of slime moulds for more than two years and track their behaviour throughout time. We revealed that old slime moulds move slower than young ones on neutral, nutritive and aversive substrates in all our experiments. We confirmed that aversive substrates slowed down slime moulds regardless of age [25,41,76,77]. Sodium is known to decrease migration rate in slime moulds via a depolarization of the membrane potential [78]. An interesting observation was that sodium chloride had a stronger effect than sodium nitrate on old slime moulds while it was the opposite for young slime moulds. In many eukaryotic cells, high extracellular NaCl increases reactive oxygen species (ROS), causes DNA damage and promotes senescence [79,80]. Given that old slime moulds already suffered from molecular damages due to senescence (effects listed in the introduction [62–64,68–70]), NaCl might have amplified those damages. Interestingly, old slime moulds were better at avoiding high concentration of NaCl or NaNO<sub>3</sub> than young slime moulds, suggesting that they were indeed more susceptible to chemical stressors and actively avoided them. As a matter of fact, we did not observe any significant differences in decision abilities when slime moulds were facing two food sources of different quality.

We then revealed that migration speed depended on age under extreme temperature. Temperature is known to be one of the main factors affecting the shuttle streaming in slime moulds. The contraction period decreases from 13min at 1°C to 1.26 min at 30°C and then increases past 32°C [57,58,81,82]. At 42°C, the shuttle streaming is totally arrested and the membrane exhibits alterations [81]. In our experiment, all slime moulds were slowed down by extreme temperatures and this effect increased with age when slime moulds age ranged from 25 to 100 weeks. Temperature is known to contribute to senescence in most living-organisms [83], including slime moulds [71]. Susceptibility to thermal stress increases as an organism ages due to more molecular damage being generated and accumulated over time [84]. Yet, surprisingly, the effect of temperature was the strongest for the youngest slime moulds which were 8 weeks old. As our culture alternated between 25°C during the week and 12°C during the weekend, it might be possible that a thermotolerance was indirectly induced over time. Signs of habituation to low temperature has already been observed in slime moulds [85]. In *P. polycephalum* as in most living organisms, expression of heat-shock protein (HSP) increases after

1 398 exposure to a moderate thermal stress [86]. HSPs function as molecular chaperones, they reduce  
2 399 molecular damage induced by extreme temperatures allowing the organism to cope with this stressor  
3 400 [87–90]. Usually, how much HSPs an organism produces correlates with the level of stress to which it  
4 401 is exposed. Therefore, in our experiment, we can presume that the level of expression of HSPs was  
5 402 higher in older slime moulds than in younger ones as they experienced more periodic changes in  
6 403 temperature over time. Thus, older slime moulds might have been more susceptible to thermal stress  
7 404 owing to their age but also more thermotolerant due to their past experiences than younger slime  
8 405 moulds.

15 406 Recently [74,91], it was revealed that slime moulds are capable of habituation, a simple form  
16 407 of learning, when they are repeatedly exposed to a chemical stimulus. In animals, learning performance  
17 408 displays a progressive decrease with age. However when it comes to habituation, it was observed that  
18 409 older organisms habituate more rapidly than young ones in *Aplysia californica* [92] and *Caenorhabditis*  
19 410 *elegans* [93] or as fast as young ones in *Drosophila melanogaster* [94], mice [95] and rats [96]. Here,  
20 411 we showed that, habituation performance is preserved in old slime moulds as well. In animals, it was  
21 412 shown that the response threshold to the stimulus was higher in old animals than in young ones which  
22 413 could explain their performance [92,95]. In contrast, in our experiment, we showed that the aversion  
23 414 level toward NaCl did not vary with age. On the contrary, in our first experiment, older individuals  
24 415 seemed more susceptible to NaCl than younger ones. Habituation to NaCl in slime moulds relies, in  
25 416 part, on NaCl uptake [41]. In plants, yeast and cellular slime moulds, sodium is driven into the cell  
26 417 passively through non selective cationic channels [97–99]. Therefore, it is possible that this passive  
27 418 transport of sodium throughout the membrane is not affected by age.

37 419 In slime moulds, sclerotia have been found to survive under adverse environmental conditions  
38 420 for long periods up to 3 years. In the slime mould *Didymimim iridis*, a related species, the aging process  
39 421 stops when the plasmodium differentiates into a sclerotium [100]. Once the sclerotium is revived, the  
40 422 plasmodium then lives the remainder of its characteristic life spans [100]. In contrast in this study, we  
41 423 observed a recovery phenomenon when aged slime moulds went throughout a dormant stage. After  
42 424 dormancy, old slime moulds migrated as fast as young slime moulds and faster than old slime moulds  
43 425 which had remained in a plasmodial stage. Past works have shown that oxidative stress defences are  
44 426 involved in the transition from plasmodium to sclerotium in *P. polycephalum* [101,102]. However,  
45 427 cell senescence is in part due to oxidative damage to DNA, RNA and proteins by mitochondrial reactive  
46 428 oxygen species (ROS) which accumulate with age [103]. Hence, one hypothesis might be that oxidative  
47 429 stress defences expressed during differentiation were responsible for the elimination of harmful ROS  
48 430 and, as a consequence, the behavioural recovery observed in old slime molds after dormancy.  
49 431 However, we only recorded the behaviour of the slime moulds one week after they were reinitiated  
50 432 from sclerotia and we cannot rule out that this rejuvenation phenomenon was only transient and did  
51 433 not affect the plasmodium lifespan.

1 434 In slime moulds, cell fusion is extremely common. In a related species *Didymium nigripes*, it  
2 435 was shown that slime moulds lose the ability to fuse over time [104]. In this study, in contrast, we  
3 436 showed that fusion occurred regardless of slime mould age. As old slime moulds were slower than their  
4 437 younger counterparts, their latency to fuse was simply longer. Vein fusion took also a longer time in  
5 438 old slime moulds than in young ones, but fusion was never aborted. We also revealed that an old slime  
6 439 mould could recover its migration speed performance after fusing with a young counterpart. Resource  
7 440 sharing between cells is often observed as a strategy to cope with environmental stress. For instance,  
8 441 cells can exchange learned information [25], mitochondria [105] or membrane material [106] via cell  
9 442 fusion. It is well known that cells accumulate DNA, protein and metabolite damage over time [107]. In  
10 443 young cells, most of the damages are cleared by maintenance processes [107]. However, as not all  
11 444 damages can be repaired, irreparable damage rises in the cell through time [107]. In aged cells, the  
12 445 maintenance systems can be damaged themselves, amplifying the burden of damages [107]. In our  
13 446 experiment, it might be possible that the damages accumulated over time in an old slime mould were  
14 447 cleaned or repaired by the maintenance system of its younger counterpart. Interestingly, in a related  
15 448 species, *Didymium iridis*, when age-hybrid slime moulds were formed by the fusion of young and old  
16 449 individuals, 58 % of the new slime moulds acquired the remaining life span of the older individuals,  
17 450 22 % died concurrently with the younger individuals and 20% died at an intermediate age [108]. The  
18 451 authors concluded that it is the oldest slime mould that determines the lifespan of the age-hybrid slime  
19 452 mould [100,109]. In our experiment, we did not follow the age-hybrid slime moulds behaviour over  
20 453 time. Hence, even if we observed a form of recovery process in old slime moulds after fusion, we  
21 454 cannot rule out that they would have died concurrently with slime moulds of the same age in absence of  
22 455 fusion.

23 456 As *P. polycephalum* migrates on the substrate, it leaves behind an extracellular slime [42,110].  
24 457 Previous works have shown that previously explored substrate might be repulsive or attractive  
25 458 depending on the physiological status of the slime mould that explored the substrate [21,44]. A  
26 459 substrate explored by a starved, irradiated or poisoned slime mould is actively avoided [44] while a  
27 460 substrate explored by a well-fed slime mould is attractive [21]. Calcium has been identified as the main  
28 461 chemical that mediates attraction [21] whereas the substances responsible for the aversion remain to  
29 462 be identified. In this study, we showed that slime moulds were able to use age-related cues released by  
30 463 conspecifics and make a decision accordingly. We revealed that slime moulds, regardless of their age,  
31 464 were more attracted by cues released by younger slime moulds than by older ones. As we did not notice  
32 465 any fusion delay when a young slime mould was facing an old one in the fusion experiment, we believe  
33 466 that the substrate explored by an old slime mould was not aversive but only less attractive. The  
34 467 information supplied by these excretions would appear to be crucial for both young and old slime  
35 468 mould. A young slime mould would benefit from avoiding fusion with an aged slime mould as it could  
36 469 inherit its damages. Indeed, we noticed that after fusing with an old counterpart, a young slime mould

was somewhat slower. In contrast, an old slime mould would gain from fusing with a young slime mould, as it would allow it to recover. **In many animals from flies to humans [111–115], as an organism ages, the effect of senescence on both the perception and processing of information might affect their response to the presence of others. Here, we did not observe any effect of age on social interactions. since older individuals were still able to fuse with others (although in a slower fashion) and detect conspecifics.**

Although many studies have studied behaviour in single cell organisms, few have taken the step of looking for changes in behaviour over the lifetime of individuals. This study extends our knowledge of the behavioural plasticity of unicellular organisms and establish acellular slime molds as a promising model to investigate the effect of aging on behaviour at the cellular level. Their ability to fuse with congeners and their peculiar lifecycle, might help us to understand how cellular metabolic activity and cellular defences are regulated and coordinated, and how a cell can counteract the effects of ageing.

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8 730 **Data Accessibility**

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10 732 The datasets supporting this article are provided as supplementary material.

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16 735 **Authors' Contributions**

17 736

18 737 AD conceived the study. AD designed the study. AR, EP, PM, CC and MD performed the experiments. AR, EP, PM and CC carried out

19 738 data acquisition. AD analysed the data. AD wrote the manuscript. AD secured funding. All authors gave final approval for publication.

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24 740 **Competing Interests**

25 741

26 742 We have no competing interests.

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1 748  
2  
3 749 **Figure 1:** Experimental design. (a) Effect of various substrate (nutritive substrate: 5% oat gel, aversive  
4 substrates: 0.6% NaCl + oat gel, 0.8% NaNO<sub>3</sub> + oat gel) on migration speed experiment as a function  
5 750 of slime mould age (6 groups of different age were tested, from 1 to 94 weeks old). (b) Effect of  
6 751 temperature (standard temperature : 25°C, extreme temperature : 10°C and 38.5°C) on migration speed  
7 752 as a function of slime mould age (5 groups of different age were tested from 8 to 101 weeks old). (c)  
8 753 Decision making under nutritive (2.5% oat gel vs 5% oat gel) and aversive conditions (0.4% NaCl +  
9 754 5% oat gel vs 0.6% NaCl + 5% oat gel, 0.4% NaNO<sub>3</sub> + 5% oat gel vs 0.6% NaNO<sub>3</sub> + 5% oat gel) as a  
10 755 function of slime mould age (5 groups of different age were tested, from 6 to 99 weeks old). (d)  
11 756 Habituation to an aversive substance (0.6% NaCl) as a function of slime mould age (2 groups of  
12 757 different age were tested : young slime moulds of 12 weeks and old ones of 74 weeks). (e) Effect of  
13 758 dormancy on migration speed on a neutral (agar gel) and nutritive substrate (oat gel) as a function of  
14 759 slime mould age (2 groups of different age were tested : old slime mould of 60 weeks and very old  
15 760 ones of 100 weeks). (f) Fusion ability as a function of slime mould age (2 groups of different age were  
16 761 tested: : relatively young slime moulds of 18 weeks and old ones of 75 weeks old). (g) Effect of fusion  
17 762 on migration speed on a nutritive substrate (oat gel) as a function of slime mould age (2 groups of  
18 763 different age were tested: young slime mould of 5 weeks and old ones of 54 weeks). (h) Detection of  
19 764 chemical cues left by conspecifics (young and old) as a function of slime mould age (2 groups of  
20 765 different ages were tested: young slime moulds of 3 weeks and old ones of 52 weeks).

21 766  
22 767  
23 768  
24 769 **Figure 2:** Mean migration speed as a function of age and substrate. Six groups of 10 large plasmodia  
25 770 of different ages (1, 17, 32, 54, 74 and 94 weeks old) were used to conduct this experiment. The  
26 771 substrates were made of oat gel (5%w/v) containing one of two aversive substances (Oat + NaCl (0.6%  
27 772 w/v), Oat + NaNO<sub>3</sub> (0.8% w/v)) or not (Oat). N > 48 for each age and each substrate. Total numbers  
28 773 of assays= 2396. Error bars indicate CI95.

29 774  
30 775  
31 776 **Figure 3:** Mean migration speed and mean proportion of motionless slime moulds as a function of age  
32 777 and temperature. Five groups of 10 large plasmodia of different ages (8, 24, 61, 81 and 101 weeks old)  
33 778 were used to conduct this experiment. Three temperatures were tested: a standard one (25°C) and two  
34 779 extremes (10°C and 38.5°C). The substrates were made of oat gel (5% w/v). All the slime moulds at  
35 780 25°C migrated on the bridge so the proportion of motionless slime mould was equal to zero for all age  
36 781 groups. N > 80 for each age and each temperature. Total numbers of assays= 3533. Error bars indicate  
37 782 CI95.

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3 785 **Figure 4:** Decision making as a function of age and substrates. Five groups of 10 large plasmodia of  
4 786 different ages (6, 22, 59, 79 and 99 weeks old) were used to conduct this experiment. Under non  
5 787 aversive conditions (oat), the slime moulds were offered a choice between a high-quality oat gel (5%  
6 788 w/v) and a low-quality one (2.5% w/v). Under aversive conditions (Oat+ NaCl or Oat+NaNO<sub>3</sub>), the  
7 789 slime moulds were given a choice between a high-aversive option (0.6% w/v) and a low-aversive  
8 790 option (0.4% w/v). Decision making performance was computed as the distance covered on the best  
9 791 bridge divided by the total distance. A value equal to 0.5 means that the slime mould distribution is  
10 792 symmetric between the two bridges, a value equal to 1 or 0 means that the slime moulds migrated only  
11 793 one the best or the worse bridge respectively. N > 235 for each age and each condition. Total numbers  
12 794 of assays= 4206. Error bars indicate CI95.

20 795

21  
22 796 **Figure 5:** Learning as a function of age. (a) Migration speed on day 1. Two groups of 10 large  
23 797 plasmodia of two different ages 12 and 74 weeks old were used to conduct this experiment. Slime  
24 798 moulds were required to migrate on an oat gel bridge with an aversive substance NaCl (Oat+ NaCl)  
25 799 (0.6% w/v, habituated treatment) or a plain oat gel bridge (Oat) without the repellent (control  
26 800 treatment). Low values of speed indicate an aversive response. (b) Aversion index on day 4 computed  
27 801 after the habituation phase. On day 4, all slime moulds (habituated and control) had to migrate either  
28 802 on a bridge with the aversive substance or without. An aversion index closes to 0 indicates habituation  
29 803 while values clearly above 0 indicate an aversion to the repellent. N = 50 for each age (young and old)  
30 804 and each treatment (habituated and control). Total numbers of assays= 200. Error bars indicate 95%  
31 805 CI.

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42 808 **Figure 6:** Mean migration speed following a dormancy period. Two groups of 10 large plasmodia of  
43 809 two different ages 60 (old) and 100 (very old) weeks old were used to conduct this experiment. Before  
44 810 the migration speed assays, slime moulds were either turn into sclerotia for a week and reinitiated  
45 811 (dormancy: yes) or kept as plasmodia (dormancy: no). The substrates were made of oat gel 5%w/v  
46 812 (Oat) or plain agar gel 1%w/v (Agar). N =80 for each age (old and very old) and each treatment  
47 813 (dormancy yes or no). Total numbers of assays= 640. Error bars indicate CI95.

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56 816 **Figure 7:** Fusion as a function of age. Two groups of 10 large plasmodia of two different ages 18  
57 817 (young) and 75 (old) weeks old were used to conduct this experiment. Young (old) slime moulds were  
58 818 paired with an individual of the same age or older (younger). The bridge separating the two slime  
59 819 moulds within a pair was made of plain agar gel 1% w/v. (a) Latency to membrane fusion as a function

1 820 of age. The time of membrane fusion was recorded for each pair. (b) Latency to vein fusion as a  
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3 821 function of age. The time of vein connection following membrane fusion was recorded for each pair.  
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5 822 N=198, N=305 and N=202 for young/ young, young/old and old/old, respectively. Error bars indicate  
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7 823 CI95.

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11 826 **Figure 8:** Mean migration speed following fusion. Two groups of 10 large plasmodia of two different  
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13 827 ages 5 (young) and 54 (very old) weeks old were used to conduct this experiment. Before the migration  
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15 828 speed assays, slime moulds were allowed to fuse either with a slime mould of the same age or with a  
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17 829 slime mould of a different age. The substrates were made of oat gel 5%w/v (Oat). N =100 for each par  
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19 830 type (young/young, young/old, old/old). Total numbers of assays= 600. Error bars indicate CI95.

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24 833 **Figure 9:** Detection of conspecifics as a function of age. Two groups of 10 large plasmodia of two  
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26 834 different ages 3 (young) and 52 (old) weeks old were used to conduct this experiment. Slime moulds  
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28 835 were offered a choice between two substrates previously explored by individuals of different ages. We  
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30 836 computed the proportion of slime moulds that reached each substrate first. A value equal to 0.5 means  
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32 837 that the slime moulds contacted the substrate explored by a young individual as often as the substrate  
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34 838 explored by a young individual (random choice). N = 300 for each age. The CI of the random choice  
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36 839 are delineated by shaded areas. Error bars indicate CI95.

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