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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

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

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.

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

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

Methods

(a) Species studied and rearing conditions

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

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

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

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

The aim of this experiment was to investigate how age affects the slime mould migration speed on various substrates (Figure 1a). Six groups of 10 large plasmodia of different ages (1, 17, 32, 54, 74 and 94 weeks old) were used to conduct this experiment. Circular samples (10 mm Ø) were cut from each plasmodium and gently placed in contact with an oat gel bridge (35 mm long, 10 mm wide) in an experimental arena (Petri dish 12mm*12mm). The bridge either contained an aversive substance: NaCl (100mM, 0.6% w/v) (Oat + NaCl bridge) or NaNO₃ (100mM, 0.8% w/v) (Oat + NaNO₃ bridge) or not (Oat). 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 48 samples were tested for each substrate and each age group, leading to total of 2396 assays.

(c) Migration speed at different temperatures as a function of slime moulds age

Slime moulds have been shown to suffer both from low and high temperature which affect the shuttle streaming and therefore locomotion [72]. The aim of this experiment was to investigate how age affects the slime mould migration speed under different temperatures (Figure 1b). Five groups of 10 large plasmodia of different ages (8, 24, 61, 81 and 101 weeks old) were used to conduct this experiment. Circular samples (10 mm Ø) were cut from each plasmodium and gently placed in contact with an oat gel bridge (35 mm long, 10 mm wide) in an experimental arena (Petri dish 12mm*12mm). Experimental arenas housed 8 bridges and were kept in thermoregulated chambers at a temperature of 10, 25 or 38.5°C degrees Celsius. 25°C is a standard temperature to rear *Physarum polycephalum* while 10°C and 38.5°C represents extremes conditions. 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. Numerous slime moulds did not move at all during the experiments at extreme temperatures (10 or 38.5°C), they were not taken into account to compute speed but we calculated the proportion of motionless slime moulds for each age group and each temperature. A minimum of 80 samples were tested for each temperature 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 NaNO₃) (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 NaNO₃) and a low-aversive option (0.4% w/v NaCl or NaNO₃). 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

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

(j) Statistical analyses

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

Results

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

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

decreased as age increased (LMM, age $F=80.52$, $p<0.001$, table S1, figure 2). On plain oat gel, migration speed declined drastically until the slime moulds were 30 weeks old then decreased gently. On oat gel containing NaNO_3 , migration speed reached a plateau when the slime moulds were over 30 weeks old. On oat gel containing NaCl , migration speed declined continuously with age (LMM age * substrate $F=28.08$, $p<0.001$, table S1, figure 2). Hence, NaNO_3 was the most aversive substance for young slime moulds while for old slime moulds it was NaCl .

(b) Migration speed at different temperatures as a function of slime moulds age

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

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

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

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

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

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 NaNO₃ 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

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

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

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

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

As *P. polycephalum* migrates on the substrate, it leaves behind an extracellular slime [42,110]. Previous works have shown that previously explored substrate might be repulsive or attractive depending on the physiological status of the slime mould that explored the substrate [21,44]. A substrate explored by a starved, irradiated or poisoned slime mould is actively avoided [44] while a substrate explored by a well-fed slime mould is attractive [21]. Calcium has been identified as the main chemical that mediates attraction [21] whereas the substances responsible for the aversion remain to be identified. In this study, we showed that slime moulds were able to use age-related cues released by conspecifics and make a decision accordingly. We revealed that slime moulds, regardless of their age, were more attracted by cues released by younger slime moulds than by older ones. As we did not notice any fusion delay when a young slime mould was facing an old one in the fusion experiment, we believe that the substrate explored by an old slime mould was not aversive but only less attractive. The information supplied by these excretions would appear to be crucial for both young and old slime mould. A young slime mould would benefit from avoiding fusion with an aged slime mould as it could 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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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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Figure 1: Experimental design. (a) Effect of various substrate (nutritive substrate: 5% oat gel, aversive substrates: 0.6% NaCl + oat gel, 0.8% NaNO₃ + oat gel) on migration speed experiment as a function of slime mould age (6 groups of different age were tested, from 1 to 94 weeks old). (b) Effect of temperature (standard temperature : 25°C, extreme temperature : 10°C and 38.5°C) on migration speed as a function of slime mould age (5 groups of different age were tested from 8 to 101 weeks old). (c) Decision making under nutritive (2.5% oat gel vs 5% oat gel) and aversive conditions (0.4% NaCl + 5% oat gel vs 0.6% NaCl + 5% oat gel, 0.4% NaNO₃ + 5% oat gel vs 0.6% NaNO₃ + 5% oat gel) as a function of slime mould age (5 groups of different age were tested, from 6 to 99 weeks old). (d) Habituation to an aversive substance (0.6% NaCl) as a function of slime mould age (2 groups of different age were tested : young slime moulds of 12 weeks and old ones of 74 weeks). (e) Effect of dormancy on migration speed on a neutral (agar gel) and nutritive substrate (oat gel) as a function of slime mould age (2 groups of different age were tested : old slime mould of 60 weeks and very old ones of 100 weeks). (f) Fusion ability as a function of slime mould age (2 groups of different age were tested: : relatively young slime moulds of 18 weeks and old ones of 75 weeks old). (g) Effect of fusion on migration speed on a nutritive substrate (oat gel) as a function of slime mould age (2 groups of different age were tested: young slime mould of 5 weeks and old ones of 54 weeks). (h) Detection of chemical cues left by conspecifics (young and old) as a function of slime mould age (2 groups of different ages were tested: young slime moulds of 3 weeks and old ones of 52 weeks).

Figure 2: Mean migration speed as a function of age and substrate. Six groups of 10 large plasmodia of different ages (1, 17, 32, 54, 74 and 94 weeks old) were used to conduct this experiment. The substrates were made of oat gel (5%w/v) containing one of two aversive substances (Oat + NaCl (0.6% w/v), Oat + NaNO₃ (0.8% w/v)) or not (Oat). N > 48 for each age and each substrate. Total numbers of assays= 2396. Error bars indicate CI95.

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

Figure 4: Decision making as a function of age and substrates. Five groups of 10 large plasmodia of different ages (6, 22, 59, 79 and 99 weeks old) were used to conduct this experiment. Under non aversive conditions (oat), 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 conditions (Oat+ NaCl or Oat+NaNO₃), the slime moulds were given a choice between a high-aversive option (0.6% w/v) and a low-aversive option (0.4% w/v). Decision making performance was computed as the distance covered on the best bridge divided by the total distance. A value equal to 0.5 means that the slime mould distribution is symmetric between the two bridges, a value equal to 1 or 0 means that the slime moulds migrated only one the best or the worse bridge respectively. N > 235 for each age and each condition. Total numbers of assays= 4206. Error bars indicate CI95.

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

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

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

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

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

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













