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# Stress signalling in acellular slime moulds and its detection by conspecifics

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Unicellular organisms live in unpredictable environments. Therefore, they need to continuously assess environmental conditions and respond appropriately to survive and thrive. When subjected to rapid changes in their environment or to cellular damages, unicellular organisms such as bacteria exhibit strong physiological reactions called stress responses that can be sensed by conspecifics. The ability to detect and use stress-related cues released by conspecifics to acquire information about the environment constitutes an adaptive survival response by prompting the organism to avoid potential dangers. Here, we investigate stress signalling and its detection by conspecifics in a unicellular organism, *Physarum polycephalum*. Slime moulds were subjected to either biotic (i.e. nutritional) or abiotic (i.e. chemical and light) stressors or left undisturbed while they were exploring a homogeneous environment. Then, we observed the responses of slime moulds facing a choice between cues released by stressed clone mates and cues released by undisturbed ones. We found that slime moulds actively avoided environments previously explored by stressed clone mates. These results suggest that slime moulds, like bacteria or social amoeba, exhibit physiological responses to biotic and abiotic stresses that can be sensed by conspecifics. Our results establish slime moulds as a promising new model to investigate the use of social information in unicellular organisms.

This article is part of the theme issue 'Signal detection theory in recognition systems: from evolving models to experimental tests'.

## 1. Introduction

Unicellular organisms live in unpredictable environments. They need to continuously monitor environmental parameters and adjust their behaviour in response to potentially life-threatening changes [1,2]. Unicellular organisms are capable of responding to a large range of stressors including extreme pH, drought, heat, osmotic challenge, oxidative stress, nutrient limitation and the presence of toxic molecules originating from their abiotic and biotic environment [3]. The specific mechanisms used to sense stressors and trigger adequate cellular responses have been extensively reviewed in bacteria [3]. These stress responses can be specific and specialized for a particular kind of stress or general and adapted to multiple stresses. Stress responses in single-cell organisms include the development of spores and competence, activation of motility, synthesis of antibiotics and enzymes, and changes in energy production systems (e.g. improving acquisition and use of growth-limiting nutrients).

To respond appropriately to stressful changes in their immediate environment and/or seek optimal living conditions, single-celled organisms must be able to process many external cues simultaneously and integrate them to adjust their response [1,4]. These cues might be environmental, such as temperature change or nutrient scarcity, or can be derived from conspecifics in the form of secreted molecules [3,5,6]. Unicellular organisms are capable of sensing and processing the metabolic activities of their conspecifics and use them to learn about the current status of the population [7]. For instance, many unicellular organisms, including prokaryotes and eukaryotes, can obtain information about their

surroundings using a process called quorum sensing [8–10]. Quorum sensing involves the production, release and perception of signalling molecules often referred as autoinducers and is implicated in many group activities, including aggregation, swarming, bioluminescence, biofilm formation, secretion of virulence factors, sporulation, antibiotic production and extracellular digestion [9,11,12]. Quorum sensing is considered to be a means of assessing local population density and its physiological state [12–14]. It facilitates adaptation to environmental stress as it improves access to nutrients, promotes defence against competitors and enhances cell differentiation to survive harsh environmental conditions [15]. For instance, in the social bacterium *Myxococcus xanthus*, the production of a mixture of amino acids termed A-factor, an early extracellular cell-density signal, increases under nutrient stress [3,16]. This way the bacteria can assess the physiological status of each other to properly coordinate their motion, aggregate and sporulate to resist starvation [17]. Another similar example can be found in the social amoeba *Dictyostelium discoideum* also known as cellular slime moulds. These single-celled amoebae feed on bacteria and other microbes in the soil. When food becomes scarce, the starving amoebae secrete and respond to cAMP, an extracellular signalling molecule, causing them to aggregate, form a multicellular assemblage called 'slug', and sporulate [18,19].

Yet, we know little about how chemical cues produced by conspecifics in response to stress influence the decision-making process in single-cell organisms. Can cells glean information from extracellular metabolites released by conspecifics to anticipate potential danger and move towards safer environments? In other words, are unicellular organisms able to extract information about the environment directly from the chemical cues released by stressed conspecifics to respond adaptively in the absence of the eliciting stressor? In the present study, we attempt to answer this question using the acellular slime mould *Physarum polycephalum*.

*Physarum polycephalum* is an amoebozoa belonging to the class of Myxomycetes also called the acellular slime mould [20–22]. It is notably characterized by a unicellular, multinucleated and vegetative state known as the plasmodium that can move around and extend up to hundreds of square centimetres. Migrating plasmodium extends two-dimensionally to adopt a fan-like shape with an intricate network of veins towards the rear [23]. The cytoplasm within the cell streams rhythmically back and forth through this network of tubular veins, circulating nutrients and chemical signals and forming pseudopods that allow the organism to navigate in its environment [24]. *Physarum polycephalum* is capable of moving at speeds of up to few cm $h^{-1}$. As it explores its environment, *P. polycephalum* continuously secretes a thick extracellular slime called 'mucus' [25]. The glycoprotein nature of this extracellular slime coat endows *P. polycephalum* with unique protective and structural properties that favour survival [26] including an externalized spatial memory that helps navigation in unknown environments [27,28].

*Physarum polycephalum* is well known for its ability to solve a large variety of problems [4,29–32]. It can find its way in a maze [33], avoid obstacles [27] and risky environments [34], build efficient networks [35], optimize its nutrient intake [34,36], learn to ignore repellents [37] and transfer learned information to clone mates [38]. All these abilities rely on the capacities of slime moulds to sense and respond to a wide range of biotic and abiotic environmental cues [22]. They sense nutritive cues such as amino acids [39–41], sugars [42,43], minerals [44], salts [45,46] and so on. They also respond to changes in oxygen, pH, osmolarity, temperature and light [22]. *Physarum polycephalum* is capable of directing its movement towards cues that are favourable for growth and survival such as nutrients [36] and away from cues that threaten survival such as toxins [47] or light [48]. Vogel *et al.* [44] demonstrated that slime moulds can sense and respond to chemical cues released in the environment by conspecifics in a foraging context. In their experiment, they identified that slime moulds release calcium while foraging and that calcium is attractive to other slime moulds.

In the present study, we tested the hypotheses that: (i) slime moulds release chemical cues in the environment when facing various stressors, and (ii) these cues can be sensed and used adaptively by other slime moulds. To test our hypotheses, we manipulated the physiological conditions of slime moulds exploring an environment using biotic or abiotic stresses. We then recorded the behavioural response of slime moulds facing substrates explored by stressed or undisturbed clone mates. To date, to our knowledge, no studies have demonstrated that slime moulds convey information regarding stressful situations that can be sensed by conspecifics. Sensing clone mates' stress would allow slime moulds to rapidly escape conditions that limit their growth by orienting their movement towards a more favourable environment.

## 2. Material and methods

### (a) Species and rearing conditions

Acellular slime moulds live in shady, cool and humid organic substrates where they feed on bacteria, yeasts and fungi [49]. Under adverse conditions (i.e. decreased humidity and food availability), slime moulds can turn into an encysted resting stage made of desiccated spherules called sclerotium. Slime mould cultures can be easily reinitiated from sclerotia for up to 3 years [50]. A single slime mould can be divided into many parts, each of which forms a new slime mould. Conversely, clone slime moulds can fuse to form a single slime mould. We used a strain of *P. polycephalum* provided by Southern Biological, Victoria, Australia. Experiments were initiated with a total of 50 sclerotia. We cultivated slime moulds on 10% (wt/vol) oatmeal–1% (wt/vol) agar mixture hereinafter referred to as 'oat gel' (Quaker Oats Company). Oat gel was renewed every day. Rearing and experiments were done in dark temperature-controlled chambers at 26°C and 80% relative humidity. Experiments were run for 24 h, and pictures were taken every 5 min with a digital Canon 70D camera. An LED-light panel was used to temporarily illuminate (approx. 4 s) the experimental set-up from below when taking a picture.

### (b) Experimental set-ups

We monitored the directional movement response evoked in slime moulds in the presence of substrates previously explored by clone mates, subjected to various stressors. We placed a semicircular slime mould in 145 mm Petri dishes filled with either agar gel (non-nutritive substrate) or oat gel (nutritive substrate), hereinafter both referred to as 'experimental substrates' (figure 1*a*). Slime moulds taken from the rearing culture always sit on a layer of oat gel which cannot be removed without damaging the cell. Thus, we placed a plastic sheet between the experimental substrate and the oat gel to prevent diffusion of any food substance in the experimental substrate (figure 1*a*). Once the slime mould had travelled a distance of 2 cm on the experimental substrate, it was either stressed or left undisturbed. After 24 h, the slime mould

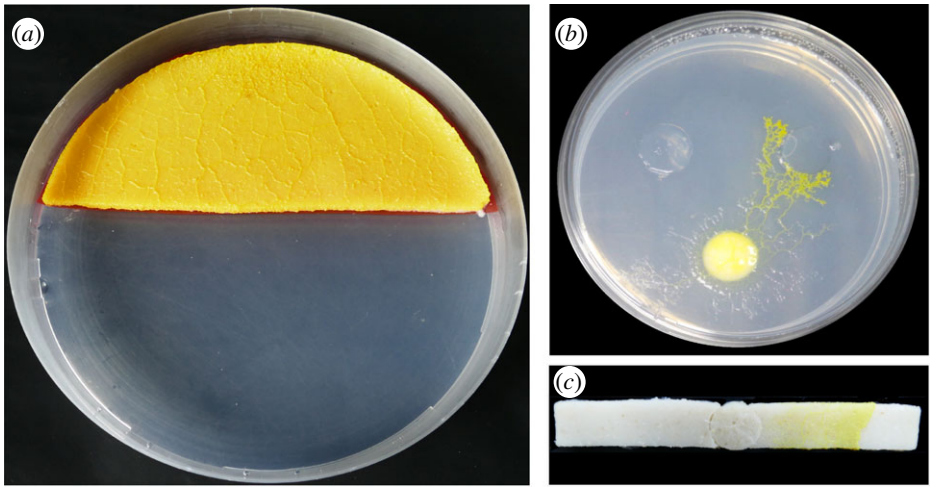


**Figure 1.** Experimental set-ups. (*a*) Stress set-up: a semicircular slime mould sitting on an oat gel placed in a 145 mm Petri dish containing a layer of agar gel. The underneath red semicircular plastic sheet prevents potential contact between the oat gel supporting the slime mould and the experimental substrate. A similar set-up was used for abiotic stresses, but the Petri dish contained a layer of oat gel instead of plain agar gel (*b*) Patch test: a circular cell sitting on oat gel was given a choice between two circular patches of previously explored substrates (experimental substrates). In this picture, the cell migrated towards the patch on the right. (*c*) Bridge test: a circular slime mould on a layer of oat gel was given a choice between two bridges made of previously explored substrates (experimental substrates). In this picture, the slime mould chose to migrate on the experimental substrate on the right. (Online version in colour.)

was removed, and the experimental substrate was lightly rinsed with distilled water to remove any slime residue. The experimental substrate was then cut up and used in binary choice experiments to evaluate stress-sensing abilities of focal slime moulds. The electronic supplementary material, figure S1 shows a schematic of the experimental protocol.

Binary choice experiments were conducted using two different experimental set-ups. The first experimental set-up ('patch set-up'; figure 1*b*) was identical to the one used in [44]. We used circular Petri dishes (diameter, Ø = 90 mm) containing a layer of 1% agar gel (height, $H = 5$ mm) as experimental arenas. Once the agar in the Petri dish had set, we punched three holes (Ø = 13 mm, inter-distance = 20 mm) and filled one with a focal slime mould (Ø = 13 mm) sitting on 10% oat gel, and the other two with experimental substrates cut up using a template (Ø = 13 mm, $H = 5$ mm). The focal 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 of the two experimental substrates (figure 1*b*). In this experimental set-up, the active migration of the slime mould towards one of the two experimental substrates relies on diffusion processes [44,51]. However, the substances released in the experimental substrate by the stressed slime moulds might differ depending on the stressors and their diffusion rate in the arena might vary.

Hence, we proposed a second experimental set-up ('bridge set-up', figure 1*c*) that does not bring diffusion into play. We placed a focal circular slime mould (Ø = 10 mm) sitting on a 10% oat gel ($H = 5$ mm) between two experimental substrates cut up using a template ($H = 5$ mm, length = 40 mm, width = 10 mm; figure 1*c*). Here, the experimental substrates were directly in contact with the focal slime mould, which could make an informed decision.

## (c) Detection of nutritional stress

In the first series of experiments, we recorded the choice of focal slime moulds facing experimental substrates explored by clone mates subjected to nutritional stress.

The experimental substrates were as follows:

— WF: agar gel explored by a well-fed slime mould. We allowed a slime mould to cover an agar gel and fed it with oat flakes for 24 h, making sure that the food was never in contact with the agar gel (see [44]);
— ST: agar gel explored by a slime mould starved for 24 h. We allowed a slime mould sitting on oat gel to migrate on an agar gel until it covered at least 2 cm. We then removed the oat gel, disconnecting the slime mould from any food source. The slime mould explored the agar gel for 24 h without being fed;
— $ST_{10}$, $ST_5$ and $ST_{2.5}$: agar gels explored by slime moulds differing in their nutritional status. Before being placed on the experimental substrate, the slime moulds were fed with one of three nutritional treatments: a standard oat gel (10% oat), a diluted oat gel (5%) or a highly diluted oat gel (2.5%) for 48 h. We then allowed the slime moulds sitting on oat gel to migrate on an agar gel and to explore it for 24 h without being fed. By contrast to the ST substrate, here the slime moulds were still connected to the oat gel supporting them while exploring the agar gel; and
— AG: agar gel stored 24 h in the same temperature-controlled chamber as the other experimental substrates. Agar gel was used as a control non-nutritive substrate.

The experimental substrates were combined pairwise to offer plasmodia the binary choices listed below:

(i) AG versus WF: to test whether a focal slime mould would be attracted by cues left by a well-fed clone mate;
(ii) AG versus ST: to test whether a focal slime mould would avoid cues left by a starved clone mate;
(iii) WF versus ST: to test whether a focal slime mould would prefer cues left by a well-fed clone mate;
(iv) AG versus $ST_{10}$, $ST_5$ or $ST_{2.5}$: to test whether a focal slime mould would avoid cues left by a starved clone mate depending on its nutritional status; and
(v) $ST_{10}$ versus $ST_5$, $ST_{10}$ versus $ST_{2.5}$, $ST_5$ versus $ST_{2.5}$: to test whether a focal slime mould would be able to discriminate cues left by clone mates with different nutritional status.

## (d) Detection of abiotic stress

In the second series of experiments, we recorded the choice of focal slime moulds facing experimental substrates explored by clone mates subjected to abiotic stress.

*Physarum polycephalum* respond to abiotic stress such as toxins present in the substrate and light. We used caffeine as a chemical stressor and a combination of blue and white light as

a physical stressor. Caffeine is a known repellent for slime moulds [37] and when applied topically cause characteristic cell surface blebbing and budding [52]. White and blue lights are harmful and trigger an avoidance response in slime moulds [53–55]. To prevent any interaction between nutritional stress and abiotic stress, this series of experiments were performed using oat gel as an experimental substrate instead of agar gel.

Here, the experimental substrates were as follows:

— CAF: oat gel explored for 24 h by a slime mould that was exposed to caffeine. We deposited five 50 µl droplets of 200 mM caffeine solution on the external curved edge of a semicircular slime mould (electronic supplementary material, figure S2) while it was migrating on an oat gel;
— $UN_{CAF}$: oat gel explored for 24 h by a slime mould that was exposed to tap water. We deposited five 50 µl droplets of tap water on the external curved edge of a semicircular slime mould (electronic supplementary material, figure S2) while it was migrating on an oat gel. This slime mould was kept in the same incubator as the slime mould exposed to caffeine;
— LUX: oat gel explored for 24 h by a slime mould exposed to light. We used blue and white LED lights (ref. SK6812RGBW-NW) to expose the slime mould to light for 10 s (10 010 Lux) every 30 s while it was migrating on an oat gel;
— $UN_{LUX}$: oat gel explored for 24 h by a slime mould kept in the dark. This slime mould was kept in the same incubator as the irradiated one and was sheltered from the light using an opaque plastic cover (00.2 Lux);
— UN: oat gel explored for 24 h by a slime mould. We allowed a slime mould sitting on the oat gel to migrate and feed on an oat gel for 24 h; and
— FD: oat gel stored 24 h in the same temperature-controlled chamber as the UN substrates, without any contact with a slime mould.

The experimental substrates were combined pairwise to offer plasmodia the binary choices listed below:

(vi) FD versus UN: to verify that an oat gel previously explored by a slime mould was not less attractive than an unexplored oat gel. In other words, we wanted to make sure that that a slime mould exploring an oat gel was not releasing starvation-related cues;
(vii) CAF versus $UN_{CAF}$: to test whether a focal slime mould would avoid cues left by a clone mate exposed to chemical stress; and
(viii) LUX versus $UN_{LUX}$: to test whether a focal slime mould would avoid cues left by a clone mate exposed to physical stress.

## (e) Measures

We replicated each binary choice (15 in total) at least 40 times (713 choice assays in total). When we used a patch set-up, focal slime moulds explored the agar gel by expanding a network of tubules in all directions for a short distance and then building one or few search fronts (figure 1*b*). The experimental substrate that 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.

When we used the bridge set-up, we measured the distance travelled over each experimental substrate after 24 h by focal slime moulds. For each assay, the difference between the distances travelled on each experimental substrate was used to determine which experimental substrate was the most attractive. A greater distance on one experimental substrate relative to the other indicated a stronger attraction. For each treatment, we counted how many times each substrate was preferred over the other. Graphs representing the differences in distances are made available in the electronic supplementary material, figure S3.

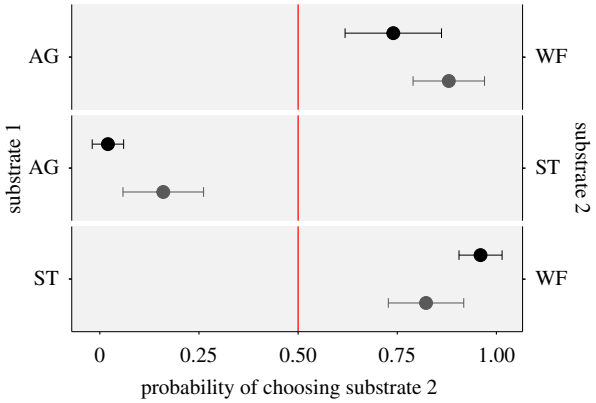


**Figure 2.** Detection of nutritional stress. Probability of choosing an experimental substrate over another when offered a choice between a neutral substrate (AG) and a substrate explored by a well-fed slime mould (WF), or a neutral substrate (AG) and a substrate explored by a starved slime mould (ST), or between the two explored substrates (ST versus WF). Grey dots and black dots represent the results obtained with the patch and the bridge set-ups, respectively. The red line ($x = 0.5$) indicates the expected probability if the slime mould migration had occurred indiscriminately between the two experimental substrates. From top to bottom, for the patch set-up, $n = 50$, $n = 62$, $n = 50$. From top to bottom, for the bridge set-up, $n = 50$, $n = 50$ and $n = 49$. Error bars indicate ±95% confidence interval (CI). (Online version in colour.)

## (f) Statistical analysis

For all experiments, we compared the proportion of focal slime moulds that preferred one substrate over the other with that expected from a binomial distribution using generalized linear models (GLMs). The models were fitted by specifying the fixed effects (experimental substrates) and the error family (binomial). The focal slime moulds used in the experiment originated from multiple plasmodia of the Australian strain. However, plasmodia belonging to the same strain may sometimes behave differently [56]. Thus, to take into account this potential source of variability, we compared each GLM with a generalized linear mixed model (GLMM) including the plasmodium identity as a random factor. The GLM were a better fit in every case (ANOVAs, $p > 0.05$, Akaike information criteria (AIC) GLM < AIC GLMM). All statistical analyses were performed using R 3.5.1 software (The R Foundation for Statistical Computing 2018) and the significance threshold was $\alpha = 0.05$.

# 3. Results

## (a) Detection of nutritional state

When given a choice between a substrate explored by a well-fed clone mate (WF) and a non-nutritive substrate (AG), 88% and 74% of focal slime moulds migrated towards the WF substrate first when using the patch set-up and the bridge set-up, respectively (figure 2; binomial GLM, $p < 0.001$; electronic supplementary material, tables S4 and S5). On the other hand, when given a choice between a substrate explored by a starved clone mate (ST) and a non-nutritive substrate (AG), only 16% (patch set-up) and 0.02% (bridge set-up) of the focal slime moulds chose the ST substrate (figure 2; binomial GLMs, $p < 0.001$; electronic supplementary material, tables S4 and S5). As expected from the previous results, when focal slime moulds had to choose between a WF and a ST substrate, 82% (patch set-up) and 96% (bridge set-up) of them migrated towards the WF substrate (figure 2; binomial GLMs, $p < 0.001$; electronic supplementary material, tables S4 and S5). In comparison to the patch set-up, the bridge set-up

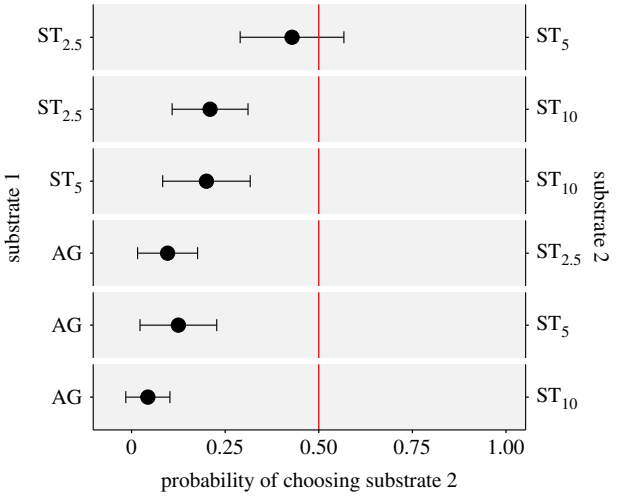


**Figure 3.** Detection of nutritional status. Probability of choosing between two experimental substrates explored by slime moulds varying in their nutritional status ($ST_{2.5}$, $ST_5$ and $ST_{10}$), or versus a neutral stimulus (AG). The red line ($x = 0.5$) indicates the expected probability if the slime mould migration had occurred indiscriminately between the two experimental substrates. From top to bottom $n = 49$, $n = 62$, $n = 45$, $n = 52$, $n = 40$, $n = 46$. Error bars indicate ±95% CI. (Online version in colour.)

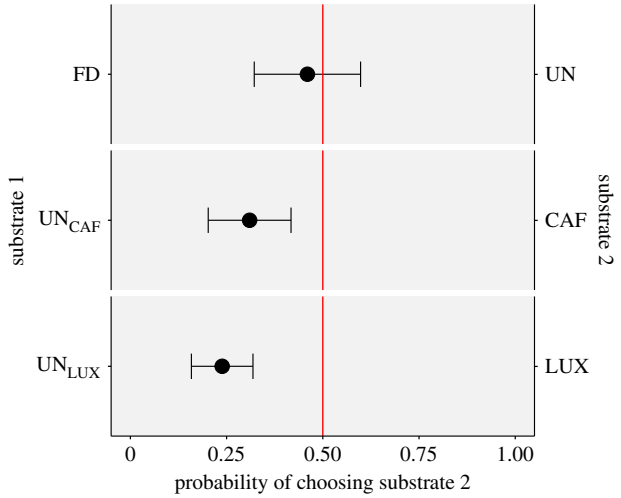


**Figure 4.** Detection of abiotic stress. Probability of choosing: an oat gel (FD) versus an oat gel explored by an undisturbed slime mould (UN); a substrate explored by an undisturbed slime mould ($UN_{CAF}$) versus a substrate explored by a slime mould exposed to caffeine (CAF); and a substrate explored by an undisturbed slime mould ($UN_{LUX}$) versus a substrate explored by an irradiated slime mould (LUX). The red line ($x = 0.5$) indicates the expected probability if the slime mould migration had occurred indiscriminately between the two experimental substrates. From top to bottom, $n = 50$, $n = 71$ and $n = 109$. Error bars indicate ±95% CI. (Online version in colour.)

led to more clear-cut decisions. Hence, we decided to only use the bridge set-up for the rest of the experiments.

When given a choice between a non-nutritive substrate (AG) and any substrate explored by clone mates varying in nutritional status ($ST_{2.5}$, $ST_5$ or $ST_{10}$), focal slime moulds preferred to migrate on the AG substrate (binomial GLMs, $p < 0.01$; electronic supplementary material, table S6). This confirmed that non-focal slime moulds sitting on food and constrained to migrate on a non-nutritive substrate for 24 h (figure 1*a*), excreted cues that were repulsive to focal slime moulds. The degree of repulsion was inversely proportional to the oat concentration offered to the non-focal slime moulds for 2 days (10%, 5% or 2.5% oat gel; figure 3). When given a choice between two $ST_x$ substrates, focal slime moulds migrate preferentially towards the experimental substrates explored by a clone mate fed on the most diluted oat gel (binomial GLMs, $p < 0.001$; electronic supplementary material, table S6) except when facing a choice between $ST_{5.0}$ and $ST_{2.5}$, in which case they showed no preference (binomial GLM, $p > 0.05$; electronic supplementary material, table S6). These results confirm that slime moulds that migrated from a standard oat gel to a non-nutritive agar gel released cues that were more repulsive than the ones released by slime moulds that migrated from a diluted oat gel to a non-nutritive agar gel (figure 3).

### (b) Detection of abiotic stress

When given a choice between an unexplored oat gel (FD) and an oat gel explored by an undisturbed clone mate (UN), slime moulds did not show a significant preference for one of the substrates (binomial GLM, $p > 0.05$; figure 4; electronic supplementary material, table S7). We, therefore, used the UN substrate as a control for the abiotic stress experiments.

When given a choice between an oat gel explored by a clone mate exposed to caffeine for 24 h (CAF) and an oat gel explored by an undisturbed clone mate ($UN_{CAF}$), slime moulds preferred to explore the $UN_{CAF}$ substrate (binomial GLMs, $p < 0.001$; figure 4; electronic supplementary material, table S7). Similarly, when given a choice between an oat gel explored by a clone mate exposed to light for 24 h (LUX) and an oat gel explored by an undisturbed clone mate ($UN_{LUX}$), slime moulds preferred to grow on the $UN_{LUX}$ substrate (binomial GLMs, $p < 0.001$; figure 4; electronic supplementary material, table S7).

## 4. Discussion

Our study supported our hypotheses that: (i) in response to biotic and abiotic stress slime moulds released substances in their environment, and (ii) other slime moulds can detect these substances and adapt their behaviour accordingly. Our results provide new insights into the complex social life of unicellular organisms.

As *P. polycephalum* explores its environment, it leaves behind a trail of non-living extracellular slime (mucus). Slime moulds are less likely to explore a substrate that contains this extracellular slime than an unexplored substrate [27,36]. Here, we demonstrated that a previously explored substrate might be repulsive or attractive depending on the nutritional status of the slime mould that explored the substrate. A substrate explored by a starved clone mate was actively avoided by slime moulds, which preferred to migrate on an unexplored substrate. By contrast, a substrate explored by a well-fed individual was more attractive than an unexplored substrate. These first results suggest that slime moulds might release different chemical substances depending on their nutritional status. Vogel *et al.* [44] has already demonstrated that well-fed slime moulds release calcium in the environment, a substance that triggers positive chemotaxis in slime moulds. The substances released under starvation contexts remain to be identified.

Results were consistent between experimental set-ups; yet, the least preferred experimental substrate (ST) was less often preferred when we used the bridge set-up. This set-up provided immediate access to information as the slime moulds were directly in contact with both experimental substrates.

Slime moulds could, therefore, gather enough information to make an accurate decision. On the contrary, in the experiments with the patch set-up, the slime moulds were placed away from the experimental substrates. Their decisions then relied on their ability to sense and track the chemical substances diffusing from experimental substrates using chemotaxis. Thus, in this setting, decisions were probably based on noisier and more partial evidence, and therefore more prone to error. In addition, the potential difference between the diffusion coefficients of the substances secreted by well-fed and starved slime moulds could also have affected decision accuracy. For these reasons, we decided to use the bridge set-up for the remaining experiments.

We found that slime moulds were also able to detect the magnitude of a nutritional stress. Clone mates that experienced the greatest gap in nutrient concentration when migrating from their food substrate to a non-nutritive substrate, i.e. from 10% oat gel to 1% plain agar gel, elicited the strongest repellent effect on focal slime moulds. Three hypotheses could be proposed to account for such differences in degree of repellency. First, this difference might be explained by differences in slime moulds' biomass. *Physarum polycephalum* has been shown to respond to nutrient dilution by growing across a greater area and in a sparser manner [36]. Therefore, in our experiments, if substances secretion was correlated to slime mould mass, thinner and lighter slime moulds would have secreted a lesser quantity of substances in 24 h than thicker and heavier ones. To test this hypothesis, we sampled and weighed nine different slime moulds per nutritional treatment (48 h on 10%, 5% or 2.5% oat gel). We did not find any significant differences in terms of biomass (see the electronic supplementary material, figure S8 and table S8). Thus, we can safely dismiss this hypothesis. Second, the difference in degree of repellency might be related to differences in the stress response dynamic. Contrary to slime moulds reared on a poorly nutritive substrate (2.5% oat gel), slime moulds reared on a highly nutritive substrate (10% oat gel) and transferred on a non-nutritive substrate (plain 1% agar gel) might have experienced a more sudden nutritional stress. Therefore, they might have reacted earlier to nutrient limitation and/or exhibited a faster build-up of their stress response, leading to a higher concentration of stress-related substances in the experimental substrate after 24 h. Finally, the secretion of stress-related substances was proportional to the intensity of the stress, i.e. the difference in food availability, experienced by the slime mould when migrating between the nutritional substrate towards the non-nutritive ones. In our experiments, the change in nutrient concentration was more severe when a slime mould migrated from a highly nutritive substrate to a non-nutritive one than when a slime mould migrated from a poorly nutritive substrate to a non-nutritive one. An acute change might have elicited a stronger response than a mild change, leading to an experimental substrate. Such variability in the stress response intensity has also been observed in cellular slime moulds (*D. discoideum*) and bacteria [57–59]. In these unicellular organisms, mild changes in stimulus intensity are often ignored and generate no change in behaviour while abrupt changes elicit strong responses [57–59].

Nutrient limitation is not the only factor that triggers a stress response in unicellular organisms [1,3,5,14,60,61] and *P. polycephalum*'s ability to detect stress-related cues from clone mates extended beyond starvation levels. Slime moulds can also avoid an environment explored by clone mates exposed to chemical stress. Caffeine is a repellent that slime moulds can learn to ignore at low concentration (less than 2 mM, [37]) but can be harmful at higher concentrations because it causes extrusion of cytoplasm (greater than 5 mM, [52]) and delayed mitosis [62]. In our experiments, slime moulds significantly avoided substrates explored by clone mates exposed to caffeine by growing preferentially on substrates explored by undisturbed clone mates. Slime moulds were also able to detect a stress response resulting from irradiation. *Physarum polycephalum* is photophobic and naturally lives in shady areas [49,54,55]. Latty & Beekman [34] have shown that when selecting food patches, slime moulds trade-off the nutritional content of food sources with the risk of light exposure. In addition, in a follow up study [48], they demonstrated that direct light exposure of slime moulds hinders the speed-accuracy trade-off that underlies their foraging decisions. Exposure to white and blue lights has been shown to interfere with cellular processes such as glucose metabolism, migration, growth, respiration and sporulation [53,63]. In our study, after a 24 h exposure to intermittent irradiation with blue and white lights, we noted that slime moulds exhibited a significant bleaching of their yellow-coloured plasmodium, which confirms a previous observation [64]. We showed that slime moulds presented with a substrate explored by an irradiated clone mate significantly preferred to migrate on a substrate explored by an undisturbed clone mate.

Our experiments also showed that regardless of the type of stress, slime moulds avoided the substrate explored by a stressed clone mate. However, some stressors elicited stronger responses than others. When presented against a substrate explored by undisturbed slime moulds, the experimental substrates explored by starved slime moulds were more aversive than the experimental substrates explored by slime moulds exposed to chemical or light stress. Therefore, starvation might have elicited a stronger response from the slime moulds than the two other stressors. Although this study is the first to our knowledge to present experimental evidence that slime moulds are able to detect stress-related substances from clone mates, the type of substances slime moulds release in their environment remains to be identified but we can propose two alternative hypotheses.

A first hypothesis is stressed slime moulds release a single chemical substance but in varying concentrations depending on the stressors. For instance, in *Escherichia coli*, the levels and activity of RNA polymerase σ-factor $\sigma^S$ can be induced not only by low levels of nutrients but also by DNA damage, a decrease in oxygen levels, and an increase in temperature or in osmolarity. For this reason, $\sigma^S$ is considered to be a general stress regulator [14]. Cyclic 3′,5′ adenosine monophosphate (cAMP) could be a potential candidate to signal general stress in acellular slime moulds. cAMP is a second messenger that plays a vital role in cell signalling and is implicated in nutrient metabolism in most organisms. In the cellular slime mould *D. discoideum*, cAMP is released extracellularly in response to nutritional stress and acts as a chemoattractant promoting aggregation [18,19]. Interestingly, in *P. polycephalum*, Kincaid & Mansour [65] found out that cAMP is repulsive at high concentration while being an effective chemoattractant at lower concentration. In both cellular and acellular slime moulds, caffeine affects the activation of cAMP synthesis and alters the distribution of intracellular calcium (it enhances calcium flux within the cell) [66,67]. Illumination with ultraviolet (UV) or blue light also increased cAMP levels [68] and causes drastic

changes in the cytoplasmic calcium concentration, as observed with caffeine [69]. Based on this information, cAMP, if secreted extracellularly in high enough concentration by slime moulds stressed with either nutrient deprivation, light or toxin, could be used by their clone mates as a cue to avoid risky environments.

Alternatively, stressed slime moulds could release specific chemical substances depending on the type of stressors. In the case of chemical stress, the chemical substance used to stress slime moulds could itself be the cue used by focal slime moulds to avoid potential danger. Boussard *et al.* [70] have shown in *P. polycephalum* that when exploring a substrate containing salt (NaCl), slime moulds absorb and store the salt intracellularly. Then, when introduced in salt-free environment, these slime moulds release the salt extracellularly. In our study, the set-up prevented any direct diffusion of caffeine in the experimental substrate. Therefore, any caffeine that might be found in the experimental substrate would have to be absorbed and released by the slime mould itself. In plants, alkaloids such as quinine and caffeine are easily taken up, accumulated, stored and released through alkaloid transporters [71]. Such transporters are part of a large, ubiquitous superfamily of proteins found in many organisms including slime moulds [72]. Visible light and UVs affect the slime mould by damaging the cell and DNA integrity. Hence, there are numerous by-products of the physiological response secreted extracellularly that could be used as cues by clone mates. For instance, blue light is coupled to the respiration (i.e. the function of mitochondria) as well as to protein synthesis and glycolysis in *P. polycephalum* [53,73]. Whether slime moulds recognize and respond to the substances released by clone mates that indicate a specific stressor remains to be investigated.

Monitoring and sampling environmental parameters are time- and energy-consuming, and might expose organisms to danger or competition [48,51]. For instance, when exploring a new environment, direct sampling of irradiation or nutrient levels require that slime moulds must be exposed, at least to a certain extent, to fluctuations in these stressors. The use of social information provides a way of gathering information about the environment and its potential dangers with a limited amount of risk for the individual. Individuals can use others (con- or heterospecifics), their presence, their actions and the consequences of their actions, as indirect sources of information to reduce the uncertainty of their world and make appropriate decisions [74,75]. For instance, being able to perceive the physiological state of a foraging conspecific might help an individual choose the best food [76].

Kin recognition, or the ability to identify, distinguish and classify kin versus non-kin, has been observed and extensively discussed in unicellular organisms such as bacteria [77–79] and cellular slime moulds [80–82]. Acellular slime moulds are also able to distinguish self from nonself to maintain their individuality and avoid fusion with conspecifics. In a supplementary experiment, we investigated if slime moulds could distinguish between substances released by kin (clone mates) or non-kin (conspecifics) in the context of nutrient stress (electronic supplementary material, S9). Using three different strains, we found that slime moulds were equally repelled by substrates explored by stressed clone mates than those explored by stressed conspecifics. Our results confirm previous studies showing that *P. polycephalum* does not or cannot discriminate between cues left by clone mates and cues left by conspecifics under exploration [36] and foraging context [44]. The process of recognition is often described as a three-component process: (i) a set of cues produced by the 'signaller', (ii) perception of these cues by the 'receiver', and (iii) a discriminatory response by the 'receiver' [82–84]. In our experiment, slime moulds were releasing chemical substances in response to stress. These substances were perceived and used to discriminate between substrates explored by stressed clone mates (or conspecifics) and substrates explored by undisturbed ones but could not be used to discriminate clone mates from conspecifics. However, non-discrimination does not necessarily indicate non-recognition. It would be necessary to test the slime moulds in multiple situations, as the fitness benefit of recognition may change depending on the context. In situations of high stress, it might not be adaptive to invest time in conspecific discrimination.

In biology, a terminological distinction exists between 'signals,' molecules that have, at least partly, 'evolved for' the purpose of transmitting information between a sender and a recipient, and 'cues', molecules released actively or passively that influence the behaviour or an organism but were not selected to communicate information. The results of the present study do not allow us to decipher if slime moulds are sending signals to warn conspecifics of danger or if they are inadvertently releasing cues in response to stressors. However, as suggested by Jablonka [85], cues from the physical world and an organism's social environment could be considered as informational sources even though they might not have evolved with the purpose of communicating information. Considerable work remains to be done, and the next step would be to identify the molecules that are released in stressful situations. Are they specific to a type of stress? Are they similar to the ones identified in cellular slime moulds? Understanding how slime moulds signal, sense and respond to cues left by conspecifics will help us understand how social behaviour and communication evolved.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. L.B. and A.D. conceived the study. L.B. and A.D. designed the study. L.B., C.G. and C.B. performed the experiments. L.B. carried out data acquisition and data analysis. L.B. and A.D. wrote the manuscript. A.D. secured funding. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests

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