Applied Microbiology and Biotechnology (2020) 104:6767–6777
https://doi.org/10.1007/s00253-020-10718-5

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Lactic acid accumulation under heat stress related to accelerated glycolysis and mitochondrial dysfunction inhibits the mycelial growth of *Pleurotus ostreatus*

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Received: 17 January 2020 / Revised: 19 May 2020 / Accepted: 1 June 2020 / Published online: 12 June 2020
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Abstract

High temperature is a major threat to *Pleurotus ostreatus* cultivation. In this study, a potential mechanism by which *P. ostreatus* mycelia growth is inhibited under heat stress was explored. Lactate, as a microbial fermentation product, was found unexpectedly in the mycelia of *P. ostreatus* under heat stress, and the time-dependent accumulation and corresponding inhibitory effect of lactate on mycelial growth was further confirmed. The addition of a glycolysis inhibitor, *2*-deoxy-D-glucose (2DG), reduced the lactate content in mycelia and slightly restored mycelial growth under high-temperature conditions, which indicated the accumulation of lactate can be inhibited by glycolysis inhibition. Further data revealed mitochondrial dysfunction under high-temperature conditions, with evidence of decreased oxygen consumption and adenosine triphosphate (ATP) synthesis and increased reactive oxygen species (ROS). The removal of ROS with ascorbic acid decreased the lactate content, and mycelial growth recovered to a certain extent, indicating lactate accumulation could be affected by the mitochondrial ROS. Moreover, metabolic data showed that glycolysis and the tricarboxylic acid cycle were enhanced. This study reported the accumulation of lactate in *P. ostreatus* mycelia under heat stress and the inhibitory effect of lactate on the growth of mycelia, which might provide further insights into the stress response mechanism of edible fungi.

Key Points

- *Lactate can accumulate in Pleurotus ostreatus mycelia under heat stress and inhibit its growth.*
- *The accumulation of lactate may be due to the acceleration of glycolysis and the dysfunction of mitochondria of P. ostreatus mycelia under high-temperature stress.*
- *The glycolysis and tricarboxylic acid cycle of P. ostreatus mycelia were accelerated under high-temperature stress.*

Keywords *Pleurotus ostreatus* · High-temperature · Mycelial growth inhibition · Lactate · Mitochondria

Introduction

Edible mushrooms are enjoyed worldwide for their considerable variety and appetizing flavor. *Pleurotus ostreatus*, as an edible mushroom preferred among Chinese people, ranks third in China's edible mushroom yield and thus has attracted increasing attention (http://www.cefa.org.cn/2018/12/27/10457.html). However, *P. ostreatus* is still cultivated in traditional agricultural way in China and other developing countries. The sheds used for cultivating *P. ostreatus* are simple in equipment and poor in environmental conditions control, thus the cultivation of *P. ostreatus* is always threatened by environmental stress, especially heat (Lei et al. 2019). When mushrooms experience high-temperature stress during cultivation in summer, the mycelia cease growth, become yellow or brown, and, most concerning, are easily contaminated, thus resulting in enormous economic losses (Qiu et al. 2017) and further necessitating the study of the mechanism by which heat stress affects edible fungi.

The existing research on the mechanism underlying the effects of heat stress describes a series of proteins that showed

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-020-10718-5) contains supplementary material, which is available to authorized users.

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increased expression after heat stress, and these proteins were named heat shock proteins (HSPs) although they had varying functions (Zhang et al. 2016b; Zou et al. 2018). In addition, signals such as nitric oxide (NO), which can alleviate heat stress-induced oxidative damage in *Pleurotus eryngii* (Kong et al. 2012), and calcium (Ca^{2+}), reactive oxygen species (ROS), and hydrogen sulfide (H_2S), which were induced under heat stress in *Ganoderma lucidum* (Liu et al. 2018b; Tian et al. 2019; Zhang et al. 2016b), have been reported to participate in the heat stress response. Moreover, further research has provided a new perspective on the heat stress response. Pyruvate, the last metabolite in glycolysis (Corbet et al. 2016; Plecita-Hlavata et al. 2016), was reported to accumulate in heat stress-treated *Metarhizium robertsii* and several other fungi (Zhang et al. 2017), and the accumulating pyruvate rapidly acted as an ROS scavenger, thus efficiently reducing protein carbonylation, stabilizing mitochondrial membrane potential, and promoting fungal growth. Another study (Zhang et al. 2016a) reported that *P. eryngii* cultured in bags increased oxygen consumption under heat treatment which resulting in an anoxic microenvironment. The accumulation of anaerobic respiration metabolites (ethanol, acetaldehyde, and lactate) were proved to have an inhibitory effect on mushroom mycelial growth.

Pyruvate can be converted to lactate and other metabolites depending on the metabolic state (Jha et al. 2016). The two studies above indicated an effect of heat stress on metabolism. However, the specific role of metabolic changes remained unclear, and the possible connection between metabolism and heat stress-induced mycelial growth inhibition became an interesting consideration. Moreover, both studies reported changes related to mitochondria (the metabolic hub) under heat stress, and there have been some reports on mitochondrial dysfunction caused by high-temperature stress in rats (Song et al. 2016), fish (Banh et al. 2016), and some plants (Rikhvanov et al. 2014). Yet, there are few reports about the effect of heat stress on the mitochondria of edible fungi and on the corresponding mechanism. Besides, studies have shown that metabolites such as lactate, pyruvate, octanoate, and ammonia synergized to culminate in mitochondrial impairment (Lockman et al. 2012). Lipid excess can also cause mitochondrial dysfunction (Chow et al. 2010). Meanwhile, mitochondrial dysfunction may lead to the accumulation of toxic lipid (Chow et al. 2010) and reduce energy metabolism and alter pyridine nucleotides and amino acid metabolism (Wang et al. 2017). Considering these complex relationships, the exploration of the effects of high-temperature stress on the mitochondria of edible fungi and the presence of an association between mitochondria and mycelial growth inhibition would be an interesting topic to study.

In this paper, we studied the mechanism of *P. ostreatus* mycelial growth inhibition under high-temperature conditions in a laboratory experiment, and the results revealed the substantial effect of accelerated glycolysis and mitochondrial dysfunction on mycelial growth inhibition and the stress resistance of edible fungi.

Materials and methods

Strains, culture conditions, and sample collection

P. ostreatus 389 (CCMSSC 00389) was obtained from the China Center for Mushroom Spawn Standards and Control. To culture *P. ostreatus* mycelia, an activated mycelial block (7 mm) was inoculated on complete yeast medium (CYM) (1% maltose, 2% glucose, 0.2% peptone, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.46% KH_2PO_4 at an initial pH of 5.5) and cultured at 28 °C in the dark for 6 days prior to use. After 6 days of culture, the mycelia were treated with heat at 40 °C for 0–48 h, and the mycelial samples were collected in tubes after different treatment times. The tubes were frozen in liquid nitrogen immediately after sample collection and finally stored at − 70 °C until use.

Lactate assay

Lactate was assayed by ultrahigh-performance liquid chromatography (UPLC) according to Zhang's method (Zhang et al. 2016a) with slight modifications. The frozen mycelia were ground into a fine powder in liquid nitrogen and weighed in a tube. Then, 4 mL of 80% alcohol solution was added to each tube, and the tubes were incubated at 80 °C for 10 min after homogenization. The homogenate was then centrifuged at 12,000 rpm for 10 min, and then the sediment was extracted twice with 3 mL and 2 mL 80% ethanol after absorbing the supernatant. Finally, 8 mL of supernatant was enriched using a rotary evaporator at 60 °C. The distillate was dissolved in 2 mL of sterile water and filtered through a 0.22μm pore membrane filter before UPLC analysis.

UPLC was performed on a Thermo UltiMate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) with a UV detector (210 nm) and a Waters ACQUITY UPLC@HSS T3 column (1.8 μm, 2.1 × 100 mm) (Waters Corporation, Milford, MA, USA). Mobile phase A was 10 mM $(NH_4)_2HPO_4$ at a pH of 3.2, mobile phase B was methanol (A/B = 94:6). The flow rate was 0.25 mL/min, the injection volume was 2 μL, and the column temperature was 30 °C. The lactate content was calculated according to the calibration curves. The lactate standard was purchased from Yuanye Biotechnology, Shanghai, China.

To identify lactate, two samples were chosen randomly for LC-MS detection by Qtrap 5500, ABsciex (AB Sciex, Singapore).

Enzymatic activity assay

The enzymatic activities of the frozen samples were detected using a commercial kit (Lactate Dehydrogenase Activity (LDH) Assay Kit (Sigma-Aldrich, Merck, Darmstadt, Germany)). Enzymatic activities were calculated via the protein concentration using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China).

Growth assays with lactate and HCl

To assay the effect of lactate and HCl on *P. ostreatus* mycelia, these reagents were added to CYM plates (diameter of 100 mm) at a variety of concentrations, and the plates were inoculated with a mycelial block. Plates supplemented with sterile water served as a control. The colony diameter was measured after 7 days of culture, and the inhibition percentage of mycelial growth was calculated as follows: Inhibition = (Control − Treatment)/Control × 100. The measurement was performed three times independently.

pH assay

To determine whether the pH value had an effect on mycelial growth, CYM medium with varying concentrations of lactate or HCl was used to measure the extracellular pH value of the mycelia, and the intracellular pH of the corresponding mycelia samples were evaluated after 6 days of growth.

The effect of 2-deoxy-D-glucose (2DG) and L-ascorbic acid on the lactate content

To confirm the effect of 2DG (Aladdin Biochemical Technology, Shanghai, China) on the lactate content, mycelia were cultured on CYM plates with 10 mmol/L 2DG for 6 days. To confirm the effect of L-ascorbic acid (VC, BBI Life sciences, Shanghai, China) on the lactate content, mycelia cultured for 6 days were transferred to new plates containing 2, 6, or 8 mmol/L VC. The control plates contained neither 2DG nor VC. Then, a heat treatment was performed to detect the change in lactate content. For 2DG, a growth test was also performed to determine its effect on *P. ostreatus* mycelia growth using the same method above.

ROS measurement

To confirm the source of ROS, mycelia grown at 28 °C and 40 °C were treated with a mixture of 10 μM DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate, Beyotime Biotechnology, Shanghai, China) and 1 μM Mito-tracker Red (Thermo Fisher Scientific, Waltham, MA, USA) (1:1) for 30 min in the dark and rinsed with water. Fluorescence was then observed using the Perkin Elmer Confocal System Ultra VIEW VOX (PerkinElmer Inc., Waltham, MA, USA) and analyzed by Volocity Demo 6.1.1 (PerkinElmer Inc., Waltham, MA, USA).

Oxygen consumption measurement

Oxygen consumption rates (OCRs) were measured using Hansatech Oxy-lab (Hansatech Instruments, Norfolk, UK) according to Liu's (Liu et al. 2018a) method with modifications. The homogenized mycelial pellets cultured in liquid medium were used in this measurement. A total of 0.5 mL of mycelial pellet from each treatment was added into a reaction chamber to measure the OCR, and another 0.5 mL was collected for the corresponding protein content assay.

Adenosine triphosphate (ATP) content measurement

The ATP content was measured using an ATP Assay Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's directions.

Mitochondrial copy number assay

The mitochondrial copy number was assayed by qRT-PCR according to Francis J. Miller's (Miller et al. 2003) method with slight modification. Genomic DNA was isolated using a Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China). The nuclear-specific gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the mitochondrial gene for NADH dehydrogenase 1 (ND1) were chosen as templates to compare the linear relationship and relativity between the CT (cycles times) value and DNA copies in the nucleus and mitochondria by a standard curve. DNA was diluted gradient to a series of concentrations to generate the standard curve. The mitochondrial copy number was represented as a CT value ratio between ND1 and GAPDH transcripts at a certain concentration. The primers used in the amplification are listed in Supplemental Table S1.

Metabolomic analysis by LC-MS

Metabolomic analysis was performed with heat-treated mycelia and carried out by Shanghai Sensichip Infotech (Shanghai, China). The general method is as follows: the freeze-dried samples were extracted with methanol and ground to a fine powder using a grinding mill at 65 Hz for 90 s, then treated with ultrasonic at 40 kHz for 30 min and left for 1 h at – 20 °C. After 15 min of centrifugation at 12000 rpm at 4 °C, 200 μL supernatant of each sample was transferred to vials to perform LC-MS analysis. LC-Q/TOF-MS (Agilent, 1290 Infinity LC, 6530 UHD, Accurate-Mass Q-TOF/MS, Agilent Technologies, Santa Clara, CA, USA) with Waters ACQUITY UPLC@HSS T3 column (2.5 μm, 2.1 × 100

mm) (Waters Corporation, Milford, MA, USA) was used in this test. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The automatic injector temperature was 4 °C, injection volume was 4 μL, and column temperature was 40 °C. The flow rate was 0.4 mL/min, and the drying gas flow was 11 L/min. Centroid data were collected from 100 to 1000 *m*/*z*.

Statistical analysis

All tests included at least three biological replicates, and the data are presented as the mean ± SEM. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using the GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA), SPSS 20.0 software (SPSS Inc., Chicago, IL, USA), and Excel 2010 software (Microsoft, Redmond, WA, USA).

Results

Time-dependent accumulation of lactate in *P. ostreatus* mycelia under high-temperature conditions

To test the universality of lactate production by edible fungi under heat stress, we evaluated lactate production in *P. ostreatus* mycelia within 48 h of heat stress (40 °C) or nonheat stress (28 °C) by UPLC (Fig. 1a). We found that lactate accumulated in a time-dependent manner in mycelia under high-temperature conditions, with a dramatic increase of approximately 2.4-fold at 3 h and a subsequent slow increase to 3.9-fold at 48 h. Surprisingly, we noticed that the production of lactate under nonthermal stress was in a stable state, which indicates that lactate is naturally present in mycelia but accumulates under heat stress. Further understanding of the significance of lactate in *P. ostreatus* mycelia is necessary to fully explain this finding. To confirm that the accumulation of lactic acid was caused by heat stress, we recultured the mycelia at 28 °C for recovery after 48 h of heat stress, and the lactate content was assayed after 6 h of recovery (48 + 6, Fig. 1a) and 36 h after recovery (48 + 36, Fig. 1a). The results showed that the lactate content in the recovered mycelia finally decreased after a long recovery period, even though there was a further increase in the lactate content after short-term recovery. When we determined the LDH (lactate dehydrogenase, an enzyme that produces lactate from pyruvic acid) activity, a similar trend was detected at the 48 + 6 time point (Fig. 1b), which showed that the newly recovered enzyme was more sensitive to temperature. In summary, lactate accumulates in *P. ostreatus* mycelia under high-temperature stress.

Lactate inhibits the mycelial growth of *P. ostreatus*

We aimed to determine the significance of lactate in *P. ostreatus* mycelia. Therefore, lactate was added to the CYM medium when culturing *P. ostreatus* mycelia to determine the effect of lactate on mycelial growth. The amount of lactate added to the medium was chosen based on data by Zhang et al. (2016a) and an additional larger concentration was added to account for species differences. Sterile water served as a control. We found a varying degree of decrease in the growth diameter of the mycelia (Fig. 2a, b and Table 1). The corresponding intracellular lactate content was also determined. Treatment with 0.434 mmol/L lactate had little effect on the mycelia (1.26 ± 0.88%), while treatment with 2.17 mmol/L lactate resulted in an intracellular lactate content of 69.62 ± 1.98 μg/g FW (fresh weight), which was slightly higher than normal intracellular lactate content (49.04 ± 8.47 μg/g FW), and had a slight inhibitory effect on the mycelia (14.47 ± 2.32%). Treatment with 21.7 mmol/L lactate resulted

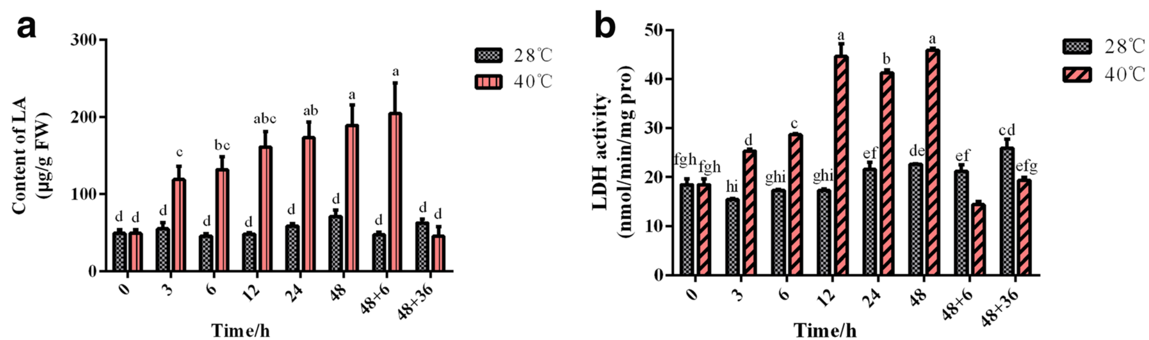

Fig. 1 Time-dependent lactate accumulation in *P. ostreatus* mycelia under high-temperature stress. The mycelia were cultured at 28 °C for 6 days and treated with heat at 40 °C for 0–48 h and recultured at 28 °C for 36 h recovery (48 + 6 means 6 h recovery after heat treatment 48 h, 48 + 36 means 36 h recovery after heat treatment 48 h). **a** Changes of lactate content in mycelia under different high-temperature stress time, $n = 3$. LA, lactate; FW, fresh weight. **b** The activity of LDH in mycelia under different high-temperature stress time, $n = 3$. LDH, lactate dehydrogenase; pro, protein. Unit enzyme activity is defined as the amount of enzyme required to catalyze the conversion of lactic acid to pyruvate to produce 1.0 μmol NADH/min/mg of mycelium protein at 37 °C. Data are expressed as the mean ± SEM. $P < 0.05$ by Duncan's multiple range test

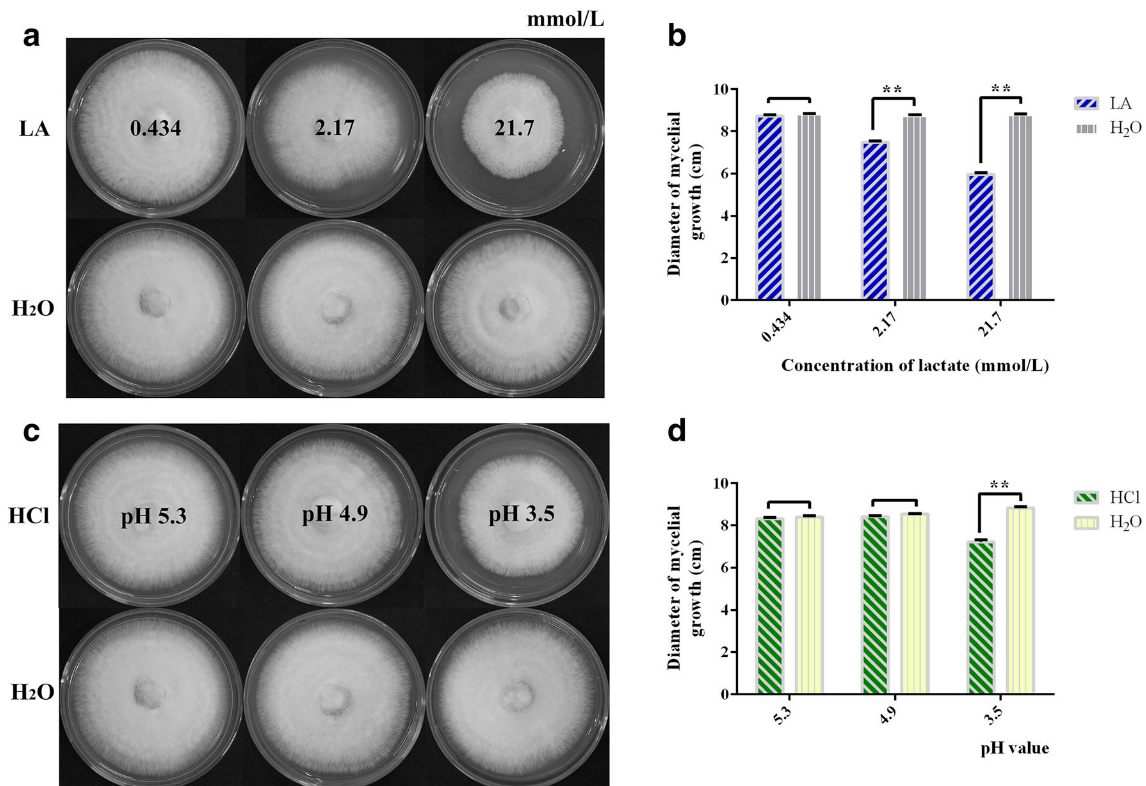

Fig. 2 Lactate inhibits the mycelial growth of *P. ostreatus*. Lactate or HCl were added to CYM plates at a variety of concentrations; sterile water served as a control. The mycelial blocks were inoculated on these plates and cultured at 28 °C for 7 days, and then the colony diameter of each plate was measured. **a** Effect of different concentration of lactate on mycelial growth, $n = 3$. LA, lactate. **b** Diameter quantification of **a**, $n = 3$. **c** Effect of HCl (with the same pH as lactate-containing medium) on mycelial growth, $n = 3$. **d** Diameter quantification of **c**, $n = 3$. Data are expressed as the mean ± SEM. $^{**}P < 0.01$ by Student's *t* test

in an intracellular lactate content of 188.33 ± 11.90 μg/g FW, which was close to the content under heat stress at 48 h (189.20 ± 46.27 μg/g FW), and reduced growth by approximately one third (32.19 ± 2.12%). Indeed, when taking the cellular mechanism of lactate import and export into consideration, this amount of exogenous addition may not have been sufficient to explain these effects but could at least explain the inhibition effect of lactate on the mycelial growth of *P. ostreatus*.

Lactate is a weak acid, and to determine whether the acidity of lactate caused mycelial growth inhibition, we tested the pH value of CYM medium with different concentrations of lactate (Table 1). An obvious reduction in the pH value was detected in the mycelia with the maximum growth inhibition rate (3.50 ± 0.04 to 5.44 ± 0.10). To further confirm the inhibitory effect of acidity on mycelial growth, we added HCl to medium to a pH equivalent to that of each lactate group for another growth test (Fig. 2c, d) and found that HCl had a limited effect when the pH was 5.3 and 4.9. Even when the pH was 3.5, the inhibition percentage was only 18.06 ± 4.95% (quantitative data not displayed), which was less than the inhibition rate of lactate-containing medium at the same pH (32.19 ± 2.12%),

Table 1 Lactate with different concentrations inhibits mycelial growth of *P. ostreatus* in varying degrees

Concentration of lactate (mmol/L)	Inhibition percentage (%)	Extracellular pH	Intracellular pH	Corresponding content of lactate in mycelia (μg/g FW)[b]
0	0	5.44 ± 0.10	6.44 ± 0.08[a]	42.79 ± 1.27
0.434	1.26 ± 0.88	5.34 ± 0.06	6.06 ± 0.02	45.29 ± 1.00
2.17	14.47 ± 2.32	4.95 ± 0.05	6.04 ± 0.05	69.62 ± 1.98
21.7	32.19 ± 2.12	3.50 ± 0.04	6.16 ± 0.06	188.33 ± 11.90

$n = 3$, data expressed as mean ± SEM

[a] The intracellular pH of mycelia without lactate

[b] Corresponding content of lactate in mycelia measured by UPLC

indicating that the inhibition of mycelial growth by lactate has other reasons besides its acidity. We also excluded the effect of lactate on intracellular pH because the measured values were close to each other (Table 1). Thus, we confirmed that heat stress causes lactate accumulation and depresses the mycelial growth of *P. ostreatus*.

Lactate accumulated under thermal stress may be due to glycolysis

We questioned why lactate was influenced by heat stress in *P. ostreatus* mycelia since lactate is generally the fermentation product of fungi (Xing et al. 2010). As lactate is also considered the end product of glycolysis in cancer cells (Heiden et al. 2009), we aimed to determine the role of lactate in edible fungi. Therefore, a glycolysis inhibitor 2DG (Moussaieff et al. 2015) was used to assay the variation in lactate and the growth state of *P. ostreatus* mycelia under heat stress. 2DG is a structural analogue of glucose, which can competitively inhibit glucose absorption. Therefore, the initial diameter of mycelia in 2DG treatment group is smaller than CYM control group (2DG-0 h compared with CYM-0 h in Fig. 3a, c). After heat treatment, the mycelia in the control nearly ceased to grow (CYM-6 h at 40 °C compared with CYM-48 h at 40 °C in Fig. 3a, c), while the mycelia with 2DG exhibited slight recovery at 48 h under heat stress (2DG-6 h at 40 °C compared with 2DG-48 h at 40 °C in Fig. 3a, c). In addition, the lactate content was significantly reduced at 48 h with 2DG under high-temperature conditions compared with the control at 48 h (Fig. 3b). Thus, the reduction in lactate restored the mycelial growth of *P. ostreatus*. The results may indicate that lactate accumulated under thermal stress may be due to glycolysis and show the possible relationship between mycelial growth inhibition and glycolysis under heat stress conditions.

Mitochondria may be involved in the accumulation of lactic acid under heat stress

Mitochondria play a central role in cellular metabolism because they are the site of oxidative phosphorylation, the tricarboxylic acid cycle (TCA), the β-oxidation of fatty acids, calcium flux, and heme biosynthesis (Mishra and Chan 2016) and are also involved in amino acid production and iron–sulfur cluster biogenesis (Pickles et al. 2018). We tested whether mitochondria were affected by heat stress and whether mitochondria were related to the

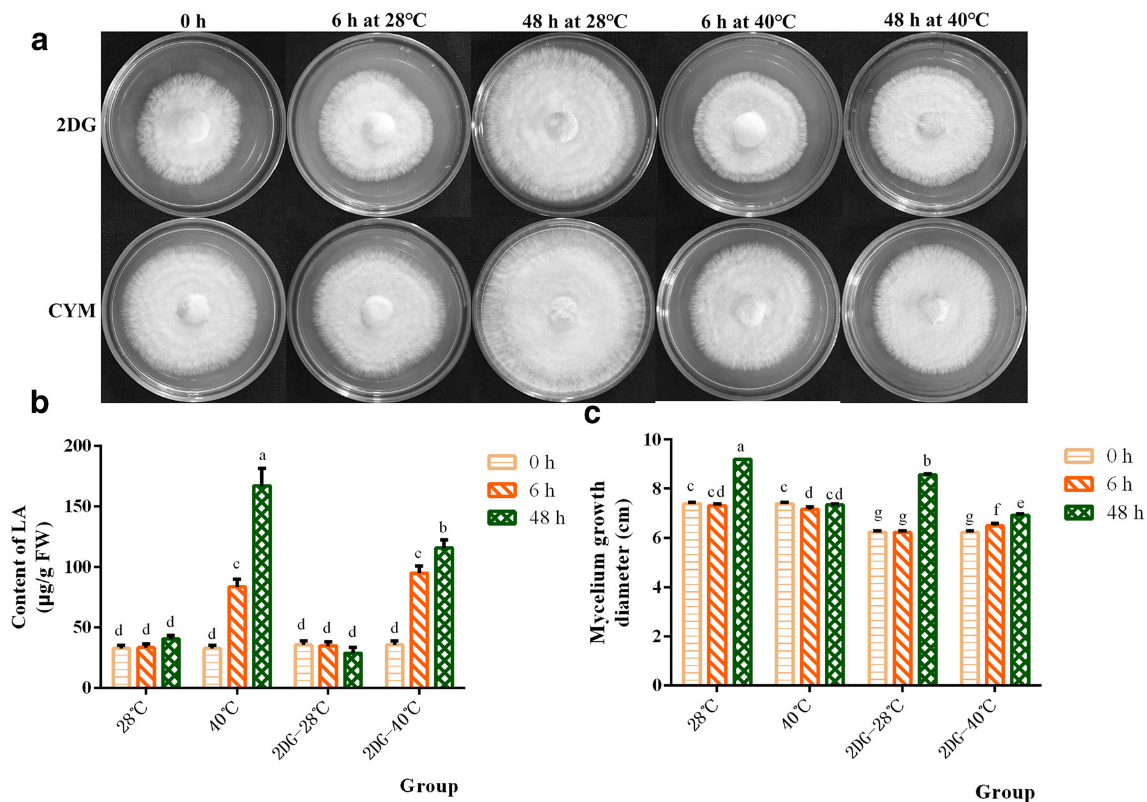

Fig. 3 2DG can alleviate the accumulation of lactate under heat stress and restore mycelial growth to a certain extent. The mycelia were cultured on CYM plates with or without 10 mmol/L 2DG at 28 °C for 6 days and then performed with heat treatment at 40 °C for 48 h. Effect of heat treatment on mycelia was compared respectively among the 2DG treatment group and among the CYM control group. **a** The effect of 2DG on the mycelial growth under different high-temperature stress time. **b** The influence of 2DG on the lactate content in mycelia under different high-temperature stress time, $n = 3$. LA, lactate; FW, fresh weight. Data are expressed as the mean ± SEM. $P < 0.05$ by Duncan's multiple range test. **c** Diameter quantification of **a**, $n = 3$. Data are expressed as the mean ± SEM. $P < 0.05$ by Duncan's multiple range test and $^*P < 0.05$ by Student's t test

accumulation of lactic acid. Lei et al. (2019) reported a massive accumulation of ROS in *P. ostreatus* mycelia at 40 °C. We also determined that ROS emerged in the mitochondria of mycelia at 40 °C by confocal microscopy (Fig. 4a), which indicated the occurrence of mitochondrial dysfunction. Moreover, the oxygen consumption of the mycelia (Fig. 4b) showed that, although the oxygen respiration rate at 40 °C fluctuated as much as that at 28 °C, the overall level declined. To determine whether the suppressed respiration in the mycelia impaired the ability of mitochondria to maintain cellular energetics, the content of ATP was measured (Fig. 4c). The ATP content exhibited a downward trend at 40 °C. Once recovered, the ATP content increased. These experiments indicated total mitochondrial dysfunction in *P. ostreatus* mycelia under heat stress. Here, heat stress did not significantly affect the number of mitochondrial copies (Fig. 4d), showing that 40 °C was not a lethal temperature for *P. ostreatus* mycelia.

When the ROS scavenger VC was added to the mycelia undergoing heat treatment, the symptoms of mycelial growth recovered in *P. ostreatus* (Lei et al. 2019), and the lactate produced by heat stress was significantly alleviated by 2 mmol/L VC at 48 h (Fig. 4e). Relatively higher concentrations (6 mmol/L and 8 mmol/L) mitigated the effects of heat stress at 6 h and reduced lactate production to very low levels at 48 h. These results suggested the presence of a connection between lactic acid accumulation and mitochondrial dysfunction

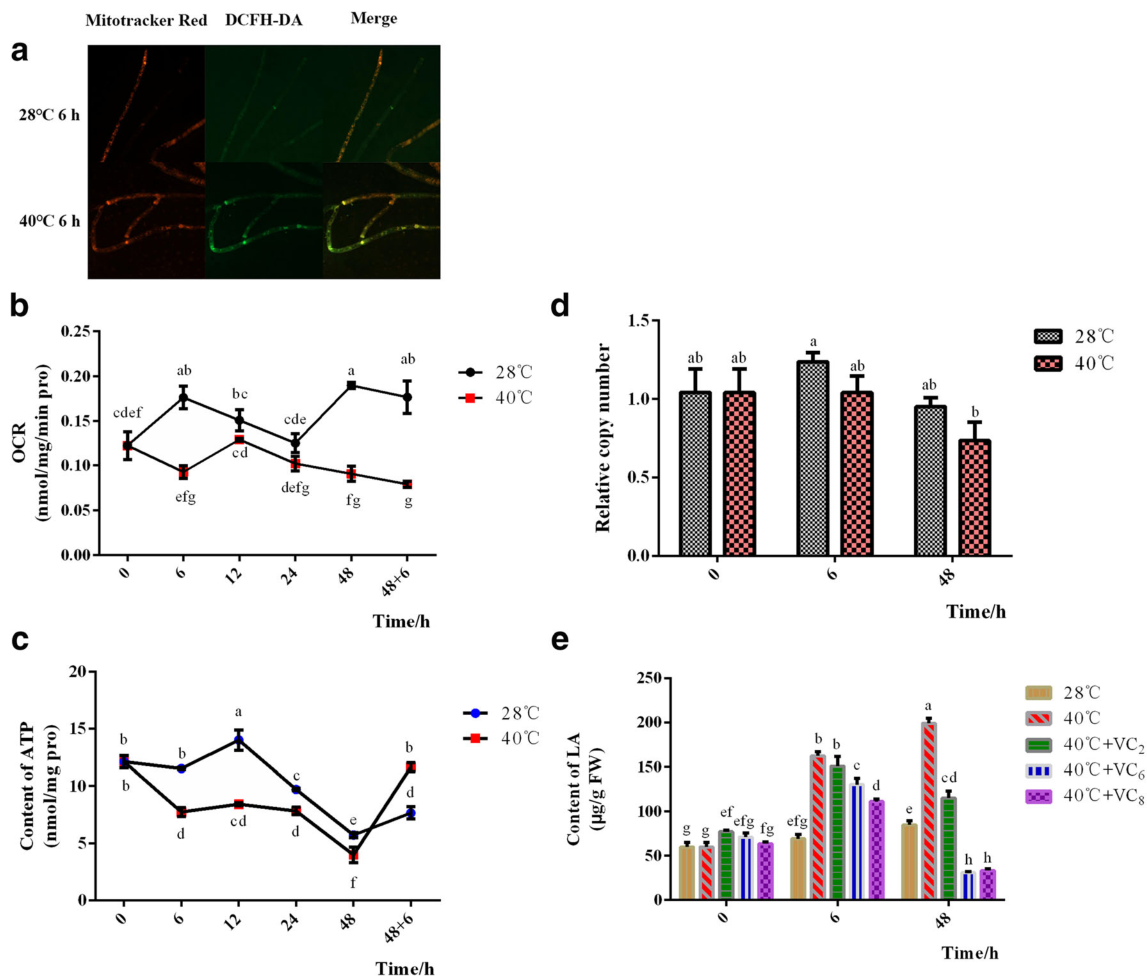

Fig. 4 Mitochondria may be involved in the accumulation of lactic acid under heat stress. **a** The mycelia were cultured at 28 °C for 6 days and treated with heat at 40 °C, and the mycelia were treated with a mixture of DCFH-DA and Mito-tracker Red to observe ROS enrichment in mitochondria, $n = 3$, Mito-tracker Red can stain mitochondria, DCFH-DA can stain ROS, and Merge exhibit ROS enrichment in mitochondria. **b** Homogenized mycelial pellets cultured in liquid CYM medium at 28 °C for 6 days were treated with heat at 40 °C for 0–48 h and used to measure OCR of *P. ostreatus* mycelia, $n = 3$. pro, protein. **c** The mycelia cultured at 28 °C for 6 days were treated with heat at 40 °C for 0–48 h and used to measure ATP content of *P. ostreatus* mycelia, $n = 3$. **d** The mycelia cultured at 28 °C for 6 days were treated with heat at 40 °C for 0–48 h and used to confirm the relative mitochondrial copy number change, $n = 3$. **e** The mycelia cultured on CYM plates at 28 °C for 6 days were transferred to new CYM plates containing 2, 6, or 8 mmol/L VC and treated with heat at 40 °C for 48 h to detect the effect of VC on the lactate content, $n = 3$. LA, lactate; FW, fresh weight; VC_2, 2 mmol/L VC; VC_6, 6 mmol/L VC; VC_8, 8 mmol/L VC. Data are expressed as the mean ± SEM. $P < 0.05$ by Duncan's multiple range test

after heat stress and further elucidated the link between mycelial growth inhibition and mitochondrial dysfunction under heat stress.

Accelerated glycolysis and tricarboxylic acid cycle of *P. ostreatus* mycelia under heat stress

To understand the changes in mycelial core carbon metabolism under heat stress, the metabolome of *P. ostreatus* mycelia under heat (40 °C) and nonheat (28 °C) stress was evaluated. We analyzed and counted all the different metabolites from the core carbon metabolic pathways in each treatment group and formed a time-course metabolite flux map (Fig. 5). First, in the glycolysis pathway, fructose 6-phosphate (F6P), glyceraldehyde 3-phosphate (3PG), and pyruvic acid (Pyr) presented an upward trend under heat stress with a maximum fold change of 1.9, 3.0, and 1.6, respectively. Lactate increased continuously (maximum fold change of 1.4). Although glucose did not exhibit a clear and consistent change (due to the sufficient amount of glucose provided in the medium), it can be concluded that glycolysis increased under heat stress. The results from the TCA pathway also showed an upward trend after heat stress for citric acid (Cit, maximum fold change of 5.4), cis-aconitic acid (cis-Aco, maximum fold change of 1.9), succinic acid (Suc, maximum fold change of 1.3), fumaric acid (Fum, maximum fold change of 2.5), and malic acid (Mal, maximum fold change of 1.8), which increased throughout the heat treatment and correspondingly decreased when mycelia resumed growth. This may indicate mitochondrial dysfunction of *P. ostreatus* mycelia under high-temperature stress, as the perturbation of the mitochondrial TCA cycle reflecting mitochondrial dysfunction (Schrauwen and Hesselink 2008; Szal et al. 2010). Moreover, high rates of the TCA cycle can promote ROS production (Kim et al. 2010), which is consistent with our results (Fig. 4a). Finally, only one key metabolite was detected as change in pentose phosphate pathway (PPP) under heat stress, namely 6-phosphogluconic acid (6-PG), which was decreased (maximum fold change of 0.6) (Fig. 5). In summary, the metabolomic data showed that accelerated glycolysis and

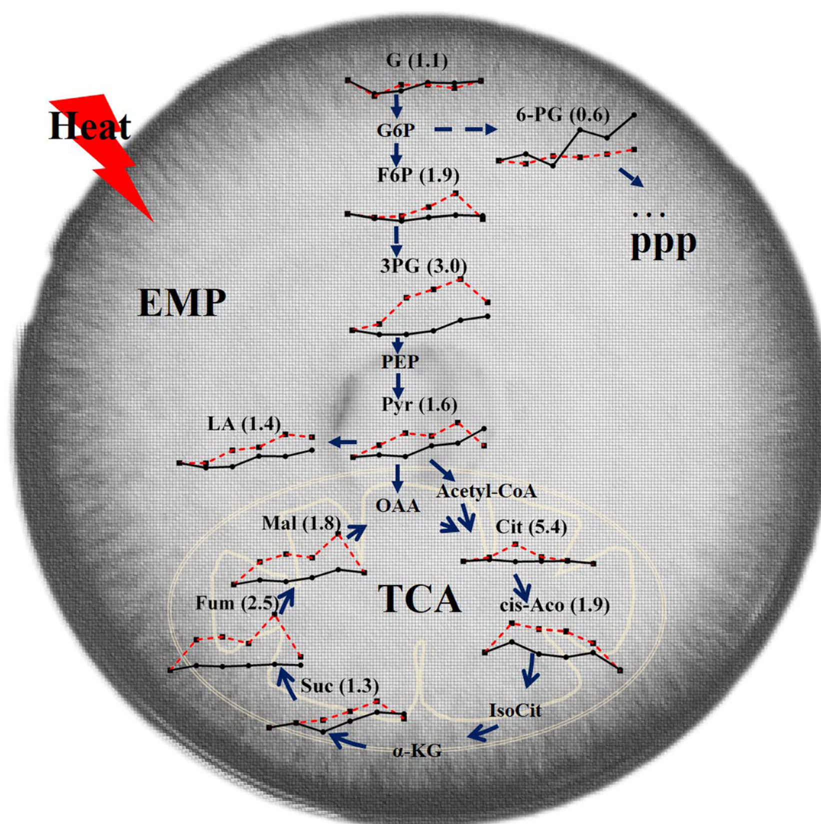

Fig. 5 Changes of core carbon metabolic flux in *P. ostreatus* mycelia under heat stress. The mycelia cultured at 28 °C for 6 days were treated with heat at 40 °C for 0–48 h and collected for metabolomic analysis. The metabolites in the 28 °C group are indicated with a black line (average), and the metabolites in the 40 °C group are indicated with a red line (average). The *X*-axis indicates the six time points (0, 6, 12, 24, 48, 48 + 6 h recovery), and the *Y*-axis indicates mean-normalized abundance levels of metabolites. The number in the parentheses next to each molecule name indicates the maximum fold change over the treatment time. EMP, glycolysis; G, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 3PG, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvic acid; LA, lactate; TCA, tricarboxylic acid cycle; OAA, oxaloacetic acid; Cit, citric acid; cis-Aco, cis-aconitic acid; IsoCit, isocitrate; α-KG, α-ketoglutarate; Suc, succinic acid; Fum, fumaric acid; Mal, malic acid; PPP, pentose phosphate pathway; 6-PG, 6-phosphogluconic acid

tricarboxylic acid cycle occurred in *P. ostreatus* mycelia under heat stress.

In brief, as shown in Fig. 6, when *P. ostreatus* mycelia was subjected to heat stress (40 °C), mitochondrial dysfunction occurred with increased TCA, decreased oxygen consumption and ATP synthesis, and accumulated ROS. Moreover, EMP (Embden–Meyerhof–Parnas) pathway was also enhanced and then lactic acid accumulated under heat stress.

Discussion

In this study, we detected the accumulation of lactic acid in *P. ostreatus* mycelia under heat stress and found that it inhibited the growth of mycelia. Further experiments showed that lactate accumulation under heat stress may due to accelerated glycolysis and mitochondrial dysfunction. Compared with previous studies on the mechanism of *P. ostreatus* mycelia responding to heat stress, this study proposed that accelerated glycolysis and mitochondrial dysfunction play important role in the response of mycelia to heat stress, which may provide a basis for studying the mechanism of heat stress in other edible and medicinal fungi and offer new insights into possible methods to prevent damage to mycelia under heat stress.

In fungi, lactate is generally the product of anaerobic fermentation (Xing et al. 2010). It seems that only *Rhizopus oryzae* can convert glucose into L(+)-lactate under aerobic conditions (Maas et al. 2008). Anyway, as far as we know, the source of lactate in fungi mainly comes from pyruvate transformation (Chen et al. 2019). Then, the direction of lactate in cells may include three ways, which are secretion to the outside of the cell, reconversion to pyruvate in the intercellular space, or transport into mitochondria and then reconversion to pyruvate to participate in the subsequent metabolic process (Mans et al. 2017; Passarella et al. 2008; Sørensen et al. 2009). In our research, the accumulation of lactate is accompanied by accelerated glycolysis under high-temperature stress, regardless of the oxygen respiratory state. It is similar to the phenomenon of lactic acid accumulation in cancer cells that an increased aerobic glycolysis in cancer cells produces too much lactate, an effect known as the Warburg effect (DeBerardinis and Chandel 2016). The accelerated glycolysis in cancer cells is to support mitochondrial oxidative phosphorylation and satisfy metabolic requirement of cell proliferation (Heiden et al. 2009). However, the accelerated glycolysis in *P. ostreatus* mycelia is the response to the high-temperature stress. We believe that heat stress accelerates glycolysis and impairs mitochondrial function of *P. ostreatus*, which may promote the subsequent accumulation of lactate. We also detected accelerated glycolysis and tricarboxylic acid cycle of *P. ostreatus* mycelia. Although metabolic changes play a considerable role in the stress response (Boone et al. 2017; Chiao et al. 2016; Corbet et al. 2016; Zhao et al. 2018), little attention has been given to such changes in edible fungi, and the response and mechanism of action, such as which signals cells enhance to cause metabolic response, should be further studied.

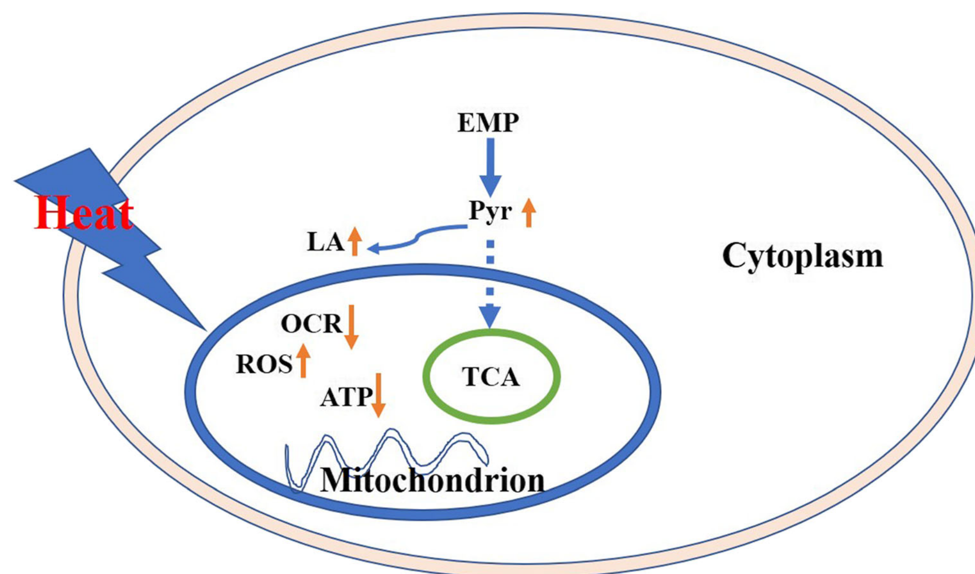

Fig. 6 The effect of heat stress on mitochondria function and glycolysis in *P. ostreatus* mycelia. *P. ostreatus* mycelia subjected to heat stress display mitochondria dysfunction, with increased TCA (tricarboxylic acid cycle), decreased OCR (oxygen consumption) and ATP (adenosine triphosphate) synthesis, and accumulated ROS (reactive oxygen species). Moreover, EMP (glycolysis) was also enhanced and then LA (lactate) accumulated under heat stress. Pyr, pyruvic acid. The orange arrow indicates the change trend of each index. The blue solid arrows in the chart indicate data supported by experiments performed in the present study, and the blue dotted arrow indicates data experimentally supported in other fungal systems

The specific mechanism by which lactate inhibits growth in addition to acidification is of substantial interest. As reported previously, lactate can form a complex with Fe^{3+} at a ratio of 2:1, and this compound can react directly with H_2O_2 to produce additional ·OH. Furthermore, lactate or lactate/Fe^{3+} can mediate the promotion of Fe^{3+}/Fe^{2+} redox cycling (Ali et al. 2000). These may function in edible fungi. Moreover, the transport and metabolism of lactate are also impacted. Lactate is secreted into the extracellular environment, therefore affecting a number of cells within the tumor microenvironment (Pavlova and Thompson 2016). In addition, lactate cannot only be secreted from the cell but also be imported into the cell through transporters (Pizzuto et al. 2012), and lactate can even enter mitochondria via transporters and continue to undergo metabolism (Atlante et al. 2007; Passarella and Schurr 2018; Paventi et al. 2007). The vectors transporting lactate to the cytoplasm and mitochondria are distinct and species specific (Passarella et al. 2008). We need to determine whether lactate-mitochondrial transporters function in *P. ostreatus* mycelia, which may explain this complex metabolic phenomenon in detail.

In conclusion, this paper, which initiated with the concept of mycelial growth inhibition and the functional study of mitochondria under heat stress, provides new insights into the research of high-temperature mycelial growth inhibition, but further research is necessary to clarify this problem, such as the study of the relationship between decreased ATP content and mycelial growth inhibition. In addition, lactate is converted from pyruvate, and the role of pyruvate in metabolism and mitochondrial dysfunction is a research focus. Yet, there are many sources and routes for pyruvate, which still need our further study.

Acknowledgments We sincerely thank Dr. Mingwen Zhao (Nanjing Agricultural University) for the critical comments on this manuscript.

Author Contributions ZY conceived, designed, and performed the experiments, analyzed the data, and wrote and revised the manuscript. XW and MZ designed and revised the manuscript. JZ conceived and designed the experiments.

Funding information This work was supported by the China Agricultural Research System (CARS-20) and Fundamental Research Funds for Central Non-profit Scientific Institution (No.1610132020033).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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