



Mobility parameters of *Tribolium castaneum* and *Rhyzopertha dominica* populations with different susceptibility to phosphine

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ABSTRACT

Phosphine (PH₃) is the most commonly used fumigant to protect stored products from arthropod infestations worldwide. Our knowledge about the behavioral differences between phosphine-resistant and -susceptible stored product pest populations is limited. This study evaluated differences in mobility and behavior of populations of two major stored product insects, *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (F.), which have different susceptibility to phosphine. In this regard, laboratory bioassays in Petri dish arenas were designed to determine if phosphine resistance has an impact on the walking and mobility behavior of adult beetles of both species. Results indicated that there were significant differences between resistant and susceptible populations for both species. Regarding velocity, *R. dominica* susceptible individuals moved faster than resistant ones. However, the resistant population showed reduced activity for several parameters tested compared to the susceptible population. Similar trends were also noted for *T. castaneum*. Knowledge of these parameters should be further utilized in management tactics, as resistant populations may behave in a different way in key management indicators such as trapping and sampling, as compared with susceptible ones.

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1. Introduction

Insect motility is a critical pattern that may be used as indicator of key biology parameters, such as the energy flow, and the ecology of distribution (Johnson et al., 1992; McIntyre, 1999). The behavioral and physiological responses of stored product insects present challenges for any insecticide used in agriculture. Demographic studies can provide certain information about fitness cost because they illustrate individual results on population-level responses. Exposure to some compounds can result in sensory precognition that compromises insecticide efficacy (Sousa et al., 2017). Insect locomotion activity is related to their spatial and temporal abundance and distribution, and ultimately the damage that they cause (Mazzi and Dorn, 2012). Smouse et al. (2010) noted that most

insects can acquire, store and use information about the habitats that have been visited. In this context, walking parameters in laboratory assays are useful to analyze the movement ability of the insects and to extract conclusions about their biology and management. Pimentel et al. (2012) found that exposure to phosphine can reduce walking activity due to the low metabolism rate during the fumigation which contributes to the lower uptake of phosphine.

Phosphine is the most commonly adopted fumigant worldwide for the control of stored product insects. Due to its extensive application, phosphine resistance has become a threat to food security all over the world. Several decades ago, Zettler and Cuperus (1990) reported the occurrence of phosphine resistance of the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) in Oklahoma, which later was found to overlap geographically in the same area (Opit et al., 2012). Currently, phosphine resistance has become a problem in many parts of the world such as US, Brazil, Australia, Morocco, India,

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China and, most recently, Greece (Collins, 2002; Benhalima et al., 2004; Pimentel et al., 2010; Song et al., 2011; Opit et al., 2012; Kaur et al., 2015; Agrafioti et al., 2019).

Resistance to phosphine coexists with a variety of physiological changes that may have considerable consequences in insects (Jagadeesan et al., 2012; Daghli et al., 2015). For example, fitness cost for resistant insects are associated with a decreased rate of development, fecundity, survival and mating competitiveness relative to susceptible insects. Pimentel et al. (2012), showed that lower walking activity is correlated with less respiration rate and as a result, reduced phosphine uptake.

Usually, most studies focused on sublethal exposure to phosphine to evaluate the fitness cost to the exposed insects (Ridley et al., 2012; Daghli et al., 2015), or the fitness costs in phosphine-free environments to understand behavioral responses of the individuals without exposure to the insecticide (Sousa et al., 2009; Malekpour et al., 2016). Moreover, some researches have been focused on the population growth of phosphine resistant and susceptible populations (Pimentel et al., 2007; Sousa et al., 2009; Daghli et al., 2015). Sublethal effects of phosphine resistance on walking behavior have been studied by Pimentel et al. (2012) for *R. dominica*. Similarly, Malekpour et al. (2016) studied flying and walking behavior of *T. castaneum* in phosphine-free environment and found that the resistant beetles fly less, walk slower, and locate resources less successfully versus susceptible beetles. Saxena and Bhatia (1980) have tested phosphine-resistant *T. castaneum* females, founding that they laid fewer eggs if compared to susceptible ones. Pimentel et al. (2007) showed that resistant populations of *T. castaneum* have lower respiration and reproduction rates than the susceptible populations. Moreover, Sousa et al. (2009) found that phosphine resistant populations of *T. castaneum*, *R. dominica* and the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) had lower population growth and developmental rates than the susceptible populations. In contrast, Daghli et al. (2015) did not record any fitness costs associated in weakly resistant populations in the absence of phosphine selection, which was considered indicative of the absence of the mutations that occur in strongly resistant populations. In this scenario, Kaur et al. (2013) reported that resistance alleles do not affect the movement ability of *R. dominica*. All these studies provide useful, and often dissimilar indications of fitness cost, and underline the need for additional experimentation.

The fitness cost of phosphine resistance is a subject of attention in stored product insects, as it reflects basic behavioral parameters that can be used as indicators in monitoring programs. In other words, a possible dissimilar response of insects to trapping or sampling protocols, may provide biased results, when attempting to monitor resistance frequency. Daghli et al. (2018) caught *R. dominica* in pheromone traps and then screened the captured individuals using molecular markers to determine the level of resistance according to the mutations that are associated with phosphine resistance. Nevertheless, it is unclear if susceptible and resistant individuals exhibit an equal response on trapping devices, or the occurrence of resistance is related with dissimilar behavioral aspects.

To shed light on possible behavioral changes in populations having different susceptibility to phosphine, we designed laboratory bioassays to determine walking behavior parameters in phosphine-free environments for *T. castaneum* and *R. dominica* adults. The fitness cost of phosphine resistance may be associated with different behavior patterns, which can impose important variations in mobility. We designed laboratory assays to determine if different phosphine resistance status can affect a variety of walking and movement parameters under phosphine-free environment. These parameters can be critical in understanding the

dynamics of each population and may provide the inferences necessary for improved management strategies.

2. Material and methods

2.1. Insects

Two strains of each of *T. castaneum* and *R. dominica* were used, one susceptible and one resistant to phosphine. The susceptible populations of both species, named LB *T. castaneum* and LB *R. dominica* are kept in the laboratory for more than 30 years without insecticide exposure, and their susceptibility to phosphine has been reconfirmed recently (Agrafioti et al., 2019; Athanassiou et al., 2019). The resistant populations of the two species were D1 *T. castaneum* and GA6 *R. dominica*, and had been initially collected from Bangladesh and Greece, respectively. Rearing of *T. castaneum* and *R. dominica* was carried out with adults on white flour and whole wheat, respectively, at 25 °C, 55% relative humidity (R.H.) and continuous darkness.

2.2. Detection of phosphine resistance

The Food and Agriculture Organization (FAO) protocol, as described by FAO Plant Protection Bulletin (FAO, 1975), and modified by Agrafioti et al. (2019), was used for the evaluation of the presence of phosphine resistance. Twenty adults of each of the tested species and strains were placed in a 1.5 L glass jar and exposed to phosphine concentration of 30 ppm for 20 h. After the termination of the exposure interval, active (capable of coordinated movement) and immobilized (not capable of coordinated movement) adults were recorded. The whole procedure was repeated three times (3 replicates), with three sub-replicates, each with new phosphine production performed on each replicate, as suggested by Agrafioti et al. (2019).

2.3. Behavioral trials

2.3.1. Bioassay series 1: Mobility behavioral analysis

Herein, the walking behavior was assessed for each species and population. The experiment was carried out in a plastic 90 mm-diameter Petri dish (63.58 cm² in surface) with the bottom of the dish covered with filter paper. Then, one adult of each species and population was released in the center of the dish (named hereafter as arena, with different arenas for each case). Each adult was left to acclimate to the arena for 3 min. Then, the following parameters were recorded visually: (1) duration of "walking" (time that the beetle was walking), (2) duration of "stops" (time that the beetle was not walking), (3) duration of "climb up" (time that the beetle was trying to climb upwards in the walls of the arena), (4) duration of "upside down" (time that the beetle was felt on its back after the climb up), (5) number of "short stops" (number of times that the beetle was stopping and beginning to walk), (6) number of climb up (number of times that the beetle was trying to climb up from the walls of the arena, named as "number of climb up"), (7) number of upside down (number of times that the beetle was on its back, named as "number of upside down"), and (8) number of "flight attempts" (number of times that the beetle was trying to fly inside the arena). For *R. dominica* there was one additional parameter that was estimated: the number of times that the adult opened the wings (named as "wing openings"). For each population, 30 replicates were conducted, and their behavior was monitored visually for 15 min. The experiments were carried out at 25 °C between 8 a.m. and 8 p.m.

2.3.2. Bioassay series 2: Mobility in presence of food

In this bioassay, the walking behavior of resistant and susceptible populations of both species was assessed when a patch of food, white flour for *T. castaneum* (0.3 g) and wheat kernels for *R. dominica* (6 kernels), was placed in the middle of the dish, which was considered as “the arena”. The insects were starved for 24 h before the bioassay. Each beetle was released in the periphery of the arena (walls of the dish), and the time to reach the food patch was recorded. Furthermore, the time that the insect spent on the food patch, and the number of times that the insect reached the food patch for subsequent times were also recorded. The conditions, number of replicates and observation interval was the same as above.

2.3.3. Bioassay series 3: Mobility in homocentric circles

Herein, the velocity of all populations was measured, as in bioassay series 1. However, in this series, we drew two homocentric circles around the center of the arena, the first with radius 19.3 mm and the second with radius 38.6 mm. The insects were released in the center of the arena, and the velocity was measured as the time between leaving the first circle and leaving the second circle. The two circles were drawn with a pencil, and the time was recorded in seconds. The conditions, number of replicates and observation interval was the same as above. Only the individuals that moved towards a straight line to the target circle were included in the analysis.

2.3.4. Bioassay series 4: High-speed analysis of walking behaviour

In this experiment, which was carried out only for *T. castaneum*, we recorded the frequency (i.e. number of steps of a recurring event per unit of time) of the walking behavior (calculating the time that the insect performed three steps). The frequency was measured using a tracking system with a HotShot® 512 SC high-speed video camera (NAC Image Technology Inc, Simi Valley, CA, USA). Thirty replicates were performed for each population. The total track period per video was about 8 s, and sequential images of *T. castaneum* behaviour were captured at a rate of 1000 fps. Video tracks were analyzed with the software of the high-speed camera (NAC Image Technology) to determine, frame by frame, the 3-step frequency over time.

2.4. Data analysis

Prior to analysis, all data were tested for normalization and homogeneity using Levene's or O'Brien's test. For all series of bioassays and each parameter, the data were analyzed separately for each species using ANOVA to indicate if there were differences among susceptible and resistant populations. When variances were not equal, the data were transformed to $\log(x+1)$. Means were separated by the Tukey's HSD test at 0.05. Untransformed means and standard errors are reported to simplify interpretation.

3. Results

3.1. Evaluation of phosphine resistance

The populations with code “LB” are referred in the text as the “susceptible” populations and the populations with code D1 and GA6 are referred in the text as the “resistant” populations. Regarding the susceptible populations no active adults were found after the termination of the exposure interval. In contrast, the vast majority of the exposed adults was still active after the termination of the 20 h-exposure for both resistant populations. More specifically, after 20 h, the immobilization for *T. castaneum* D1 and *R. dominica* GA6, was 22.0 ± 9.9 and 0.0 ± 0.0 , respectively (values

are given as means \pm SE).

3.2. Bioassay series 1: Mobility behavioral analysis

There were no significant differences between the two *T. castaneum* populations in the duration of walking and stops (Table 1). However, the susceptible population indicated a lower duration of upside down, number of upside down and number of flight attempts as compared with the resistant population. Conversely, the resistant population had lower number of short stops than the susceptible one (Table 1).

There were no significant differences between the two *R. dominica* populations in most of the parameters tested (Table 1). Nevertheless, the susceptible population showed a higher number of flight attempts than that of the resistant one (Table 1).

3.3. Bioassay series 2: Mobility in presence of food

No significant differences were noted between populations for any of the parameters tested for either *T. castaneum* or *R. dominica* (Table 2). In fact, in most of the cases, the measurements of the two populations were nearly identical, especially in the case of *R. dominica* (Table 3).

3.4. Bioassay series 3: Mobility on homocentric circles

The velocity of the susceptible population of *R. dominica* was significantly higher than that of the resistant one, while no differences were noted for the other parameters tested (Table 3). Moreover, no significant differences were noted between the two populations of *T. castaneum* for any of the parameters tested here (Table 3).

3.5. Bioassay series 4: High-speed analysis of walking behaviour

No significant differences were noted in frequency measurements between the two populations of *T. castaneum*. Specifically, the frequency, expressed as Hz (mean \pm SE), was 11.5 ± 1.8 and 14.2 ± 1.2 for the susceptible and the resistant population, respectively (Levene's test: $F = 1.59$, $P = 0.213$, ANOVA parameters: $F = 1.6$, $P = 0.206$, in all cases, $df = 1.53$).

4. Discussion

The current study investigated the potential impact of the presence of resistance on specific behavioral parameters in two major stored product beetles. The behavioral changes that insects exhibit in the presence of insecticides can give them additional abilities to adapt to environments treated with toxic compounds (Fardisi et al., 2013). Rigaux et al. (2001) showed a variation in mobility between susceptible and resistant populations of *T. castaneum* to diatomaceous earth (DE). In that work the resistant populations moved slower than the susceptible ones, resulting in reduced contact of the DE particles to the insect's cuticle. These results corroborate that, even for insecticides acting through desiccation, efficacy is directly related with the behavioral characteristics of the insect populations that are tested. Previous reports suggesting that this may be true in the case of phosphine as well (Kaur et al., 2013; Pimentel et al., 2012,2017).

Kliot and Ghanim (2012) noted that the type of the insecticide and the development of resistance is strongly related with fitness costs because resistance alleles correlate with high energetic cost or other disadvantages versus the susceptible populations. In order to handle the toxic agent, insects differ in their behavioral mechanisms that may affect their reproduction, their walking or flight,

Table 1
Walking parameters, expressed as mean \pm SE, of susceptible and resistant populations of *Tribolium castaneum* and *Rhyzopertha dominica*.

Parameters ¹	<i>T. castaneum</i>				<i>R. dominica</i>			
	Susceptible	Resistant	F	P	Susceptible	Resistant	F	P
Walking (s)	45.4 \pm 10.5	24.6 \pm 8.2	2.4	0.125	89.6 \pm 19.3	87.1 \pm 15.6	<0.01	0.902
Stops (s)	14.0 \pm 3.3	42.5 \pm 18.5	2.0	0.159	9.4 \pm 2.2a	7.4 \pm 3.6b	0.1	0.694
Climb up (s)	0.7 \pm 0.2a	4.0 \pm 0.7b	17.2	<0.001	7.3 \pm 2.4	3.0 \pm 0.6	3.0	0.088
Upside-down (s)	0.5 \pm 0.5a	17.5 \pm 4.2b	15.7	<0.001	10.9 \pm 4.3	4.3 \pm 1.7	2.0	0.163
Short stops (n)	22.2 \pm 2.3a	7.0 \pm 1.1b	35.9	<0.001	7.9 \pm 1.2a	4.5 \pm 1.0b	5.0	0.029
Number of climb up (n)	5.2 \pm 1.6a	37.8 \pm 4.1b	56.0	<0.001	10.8 \pm 2.0	10.0 \pm 2.0	0.1	0.791
Number of upside-down (n)	0.0 \pm 0.0a	2.7 \pm 0.6b	19.4	<0.001	1.3 \pm 0.3	0.7 \pm 0.3	2.0	0.158
Flight attempts (n)	0.0 \pm 0.0a	0.5 \pm 0.3b	4.2	0.459	3.4 \pm 0.8a	0.1 \pm 0.1b	16.9	<0.001
Wings opening (n)	–	–	–	–	1.7 \pm 0.5	1.0 \pm 0.7	0.6	0.442

Within each row and species, means followed by different letters are significantly different; where no letters exist, no significant differences were noted; Tukey's HSD test at 0.05, in all cases $df = 1, 59$.

¹For *T. castaneum*, Levene's test: $F = 3.3, P = 0.074$. For *R. dominica*, Levene's test: $F = 1.9, P = 0.175$; For *T. castaneum*, O'Brien test: $F = 1.6, P = 0.213$. For *R. dominica*, Levene's test: $F = 0.1, P = 0.772$; For *T. castaneum*, O'Brien test: $F = 2.3, P = 0.125$. For *R. dominica*, O'Brien test: $F = 2.8, P = 0.091$; For *T. castaneum*, data were x-square transformed O'Brien test: $F = 2.6, P = 0.012$. For *R. dominica*, O'Brien: $F = 2.6, P = 0.163$; For *T. castaneum*, data were $\log(x+1)$ transformed Levene's test: $F = 0.0, P = 0.811$. For *R. dominica*, Levene's test: $F = 0.7, P = 0.410$; For *T. castaneum*, data were $\log(x+1)$ transformed Levene's test: $F = 1.5, P = 0.217$. For *R. dominica*, Levene's test: $F < 0.01, P = 0.950$; For *T. castaneum*, O'Brien test: $F = 3.4, P = 0.070$. For *R. dominica*, Levene's test: $F = 3.4, P = 0.071$; For *T. castaneum*, O'Brien test: $F = 2.8, P = 0.091$. For *R. dominica*, data were transformed to x-square O'Brien test: $F = 3.1, P = 0.081$; For *R. dominica*, Levene's: $F = 0.1, P = 0.788$.

just to cite some examples. Our study indicates that there are specific behavioral patterns on which these populations differ substantially. Still, it is not clear if these differences can be considered as a direct consequence of the dissimilar susceptibility to phosphine, or to other parameters, which may or may not be linked with resistance.

For each species, both populations showed similarities and differences in their behavioral parameters. In some of the cases, the resistant population indicated a higher mobility than the susceptible one, while, logically, the reverse results were expected. Still, there are additional parameters that are responsible for different behavioral trends, such as geographical region and climatic conditions. For instance, in a previous study, Romano et al. (2016) found that geographical origin played a role in lateralization and mating behavior of populations of the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). Based on this, we assume that several conditions, including the geographical origin, and their interaction with the occurrence of resistance, may be responsible for these behavioral variations, rather than resistance alone. However, the fact that these populations have been reared in laboratory conditions for several generations, might have reduced the influence of other factors and strengthens resistance to phosphine as a key difference. For the same phenomenon, Beeman and Nanis (1986) showed no fitness cost between malathion-resistant and -susceptible populations of *T. castaneum*. Conversely, Haubruge and Arnaud (2001) showed that a malathion-resistant population of *T. castaneum* had higher fecundity than the susceptible population. Based on the above, in terms of mobility and fitness cost, it looks like insect populations sampled from different areas may not be directly comparable. In other words, it is likely that some

Table 3

Walking velocity, expressed mean \pm SE, of susceptible and resistant populations of *Tribolium castaneum* and *Rhyzopertha dominica*.

Species ¹	Parameter	Susceptible	Resistant	F	P
<i>T. castaneum</i>	Velocity (mm/s)	14.3 \pm 0.7	13.3 \pm 0.7	1.1	0.302
<i>R. dominica</i>	Velocity (mm/s)	2.6 \pm 0.3a	1.8 \pm 0.2b	4.5	0.037

Within each row and species, means followed by different letters are significantly different; where no letters exist, no significant differences were noted; Tukey's HSD test at 0.05, in all cases $df = 1, 59$.

¹ For *T. castaneum* Levene's test: $F < 0.01, P = 0.925$. For *R. dominica* O'Brien test: $F = 3.8, P = 0.057$.

behavioral attributes pre-existed before insecticidal exposure, and may be eventually correlated with insecticidal application, and not the opposite. In this context, in similar experiments, specific behavioral attributes may not be related with adaptation to a specific insecticide, being preexisting and can be utilized when it was needed. Consequently, the comparison of behavioral attributes of populations that have different susceptibility levels to phosphine does not necessarily mean that, apart from susceptibility to phosphine, all the other attributes are the same, unless these populations originate from the same parental individuals, as in the case of "lab selection" studies.

Previous studies have documented that walking behavior is also linked with respiration rate. Walking behavior is related with lower respiration rate for *R. dominica*, which leads to a reduction of phosphine uptake (Pimentel et al., 2012). Furthermore, Pimentel et al. (2007) measured the production of carbon dioxide in populations of *T. castaneum*, *R. dominica* and *O. surinamensis* with different resistance ratio and found that respiration rate decreases

Table 2
Walking parameters with a patch of food in the center of the arena, expressed as mean \pm SE of susceptible and resistant populations of *Tribolium castaneum* and *Rhyzopertha dominica*, when adults are released from the edge of the arena.

Parameters ¹	<i>T. castaneum</i>				<i>R. dominica</i>			
	Susceptible	Resistant	F	P	Susceptible	Resistant	F	P
Time to reach the food patch (s)	173.2 \pm 33.4	212.5 \pm 36.3	0.6	0.428	144.8 \pm 30.5	139.3 \pm 27.6	0.1	0.805
Time spent on the food patch (s)	140.0 \pm 37.7	106.1 \pm 30.8	0.5	0.489	522.1 \pm 59.3	536.8 \pm 60.6	<0.1	0.970
Number of times the insect reach the food patch again	1.6 \pm 0.3	1.9 \pm 0.4	0.4	0.513	0.3 \pm 0.1	0.5 \pm 0.1	1.0	0.317

No significant differences were noted for any of the pairs compared; Tukey's HSD at 0.05, in all cases $df = 1, 59$.

¹ For *T. castaneum* Levene's test: $F = 1.3, P = 0.264$. For *R. dominica* Levene's test: $F = 0.5, P = 0.478$.

For *T. castaneum* Levene's test: $F = 0.8, P = 0.378$. For *R. dominica* Levene's test: $F = 0.1, P = 0.800$.

For *T. castaneum* Levene's test: $F = 0.9, P = 0.338$. For *R. dominica* O'Brien test: $F = 2.4, P = 0.158$.

with the increase of phosphine resistance. The reduction of the respiration rate of the resistant population in comparison with the susceptible population is related to the reduced uptake of the fumigant by the resistant insects, which may be connected with energy production and flow, a critical parameter in phosphine's mode of action.

Different studies show a reduction of the population growth rates between resistant and susceptible populations (Sousa et al., 2009). Pimentel et al. (2012) reported that the walking activity was reduced for the more resistant population of *R. dominica*. However, Kaur et al. (2013) found no statistical differences in walking and flight behavior between resistant and susceptible populations. Considering our findings, for the vast majority of the parameters tested for all series of bioassays, there were no significant differences between populations for both species. This is in accordance with Kaur et al. (2013) that noticed that resistance to phosphine may not be related with behavioral changes, and, to some extent, resistance-linked mutations may not cause major differences in insect mobility.

The behavioral responses of phosphine-resistant and -susceptible populations in absence of phosphine have not been documented thoroughly. The hypothesis tested here was to examine different walking behavior parameters characterizing resistant and susceptible populations without any phosphine exposure, to associate the phosphine resistance level with the walking activity of the populations. Indeed, the present results showed some differences between susceptible and resistant beetle populations, but, considering the overall data, we found more similarities than differences. However, these results need to be investigated further, with phosphine sublethal exposure of the two populations, to determine differences in their mobility. Moreover, additional experimentation is required to illustrate the genetic background of the current populations. Notably, insect mobility should be taken into consideration when designing phosphine management plans, especially whenever mobility parameters are important for surveillance, such as in the case of trapping protocols.

Declaration of competing interest

The authors declare that have no conflict of interest.

CRedit authorship contribution statement

Maria K. Sakka: Formal analysis, Writing - original draft, Conceptualization. **Donato Romano:** Formal analysis, Writing - original draft, Conceptualization. **Cesare Stefanini:** Formal analysis, Writing - original draft, Conceptualization. **Angelo Canale:** Formal analysis, Writing - original draft, Conceptualization. **Giovanni Benelli:** Formal analysis, Writing - original draft, Conceptualization. **Christos G. Athanassiou:** Formal analysis, Writing - original draft, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jspr.2020.101593>.

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