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Physiological damage in Algerian mouse *Mus spretus* (*Rodentia*: *Muridae*) exposed to crude oil

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ABSTRACT

Small mammals have been used to predict ecotoxicological damage caused by metals in field studies and laboratory exposure. In natural ecosystems, rodents play an important role either as seed dispersers or food providers for various predators since they represent intermediate links in the food chain. Several studies have already focused on the effects of metals on wild rodents, but data provided on the effects of organic contaminants, such as crude oil, are scarce. Among the possible biological indicators, physiological parameters are useful because they reflect, accurately, the organism-environment interaction. The current study aimed: I) to evaluate the effects of the exposure to soil contaminated by crude oil in the *Mus spretus* mice and II) to select sensitive markers to crude oil pollution. Mice collected in free-contaminated areas were exposed to artificial soil contaminated by crude oil, and compared with animals housed in artificial non-contaminated soil (control soil). External signs such as lethargy and alopecia were observed in the first days of exposure. However, no changes in animals' body weight were recorded although changes in relative weight of some organs (liver, spleen and lungs) were observed. Furthermore, results also revealed increase in basal metabolic rate and decrease in exploratory and locomotor activity. Exposure to soil contaminated also caused dysfunction of the adrenal glands measured through fecal corticosterone levels. Data obtained highlight the relevance of using *ex situ* models, such as wild mice, and suggest a set of biological markers to predict and monitor environmental damage caused by crude oil exposure.

Key words: *Mus spretus*, petroleum, basal metabolism rate, open field test, fecal corticosterone

Introduction

Small wild mammals have been used to predict the environmental damage caused by chemical contaminants since the late 1970s (Beardsley et al., 1978; Roberts & Johnson, 1978). The initial concern was to investigate the bioaccumulation in different tissues thus determining the toxicokinetics of the toxic elements. Certain conditions contribute to use small mammals in environmental contamination researches, including distribution, abundance, site fidelity, longevity, and ecological representativeness (Tataruch & Kierdorf, 2003).

More recently, some studies have investigated the toxic effects of exposure to environmental contaminants through biomarkers of effect, such as genotoxic (Da Silva et al., 2000; Mitkovska et al., 2012) and histopathological biomarkers

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(Sánchez-Chardi et al., 2009), antioxidant and metabolism enzymes (Fouchécourt & Riviere, 1995), and immune system damage (Propst et al., 1999).

Mus spretus Lataste 1883 (*Rodentia: Muridae*) is a free-living rodent, popularly called Algerian mouse or "ratinho-ruivo" (in Portuguese), noncommensal preferably inhabits open habitats, sympatric (but non-syntopic) from *Mus musculus*, because it prevents anthropogenic sites. This rodent occurs in the Iberian Peninsula, southern France and northern Africa (Morocco, Algeria and Tunisia). They eat mostly seeds, plants and fruits, but also feed of insect larvae. On the other hand, *M. spretus* composes the diet of mammals, owls and snakes. Males and females of *M. spretus* occupy the same territory and male have reproductive sedentarism. They are nocturnal (except in winter that have diurnal behavior) and have a low water consumption compared to other rodents (Palomo et al., 2009).

This small mammal has been used in environmental assessment studies in industrial and mining areas in the Iberian Peninsula (Tanzarella et al., 2001, Nunes et al., 2001, Festa et al., 2003; Viegas-Crespo et al, 2003; Bonilla-Valverde et al., 2004), highlighting the effects of metals in genetic, biochemical and physiological disorders. In an ecological context, the physiological aspects are able to reflect the interaction between organism-environment through responses such as adaptation, tolerance and behavioral changes in adverse conditions (Mira & Mathias, 1993; Pouliquen-Young, 1994, Nunes et al., 2001; Homsi & Aulagnier, 2010), such as exposure to chemical contaminants.

The crude oil is a mixture of toxic contaminants, mainly aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). The toxic effects of oil were investigated in terrestrial invertebrates, plants (Dorn et al., 1998, Tang et al., 2011), marine animals (Engelhardt, 1983) and terrestrial animals, such as cattle, (Coppock & Christian, 2007). Some studies showed the crude oil effects in rodents (Easley et al., 1982; Lockard et al., 1982; Pryzgoda et al., 1999; Lemière et al., 2005; Patrick-Iwanyanwu et al., 2011), including wild mice (Fouchecourt and Riviere, 1995, Propst et al., 1999). On the other hand, no toxic effect of this contaminant was evaluated in *M. spretus* mouse.

Thus, the aim of this study was to investigate if the exposure to simulated *onshore* oil spill can affect some morphological and physiological parameters in the *M. spretus* mouse.

Materials and Methods

Animals and chemical treatment

In this study, 23 adult *M. spretus* (male and female, indistinctly) were captured in Sherman traps baited with a mixture of sardines, oil and flour. The animals (body weight: 13.9±3.5 g) were housed, individually, in cages in standard laboratory conditions: temperature (22.1±0.8ºC), humidity (36.3±3.3%) and photoperiod (12-h light/dark cycle). Food and water were provided *ad libitum* (Maintenance diet - Scientific Animal Food Engineering, France). Mice were collected between March and June, 2011 in a free-contaminated field (unpolluted area) in Amadora, Portugal (N38º45' W9º12'), and were acclimatized for at least one month, before the experiments began.

Fourteen individuals (eight male and six female) were exposed to soil artificially contaminated by crude oil (donated by Galp Energia, Portugal) (8% w/w) for 14 days, like a simulated *onshore* oil spill. The artificial soil was composed by a mixture of sand (70%), clay (20%) and vegetable organic matter (10%). Each animal was housed in an individual cage containing 1 kg of artificial soil. Nine control animals (six male and three female) were housed in similar cages containing the same soil mixture without crude oil. The control and exposed animals were kept in separate chambers because of the influence of volatile substances. After 14 days, the animals were anesthetized and sacrificed by exsanguination. The organs weight measurements and physiological parameters assessment was carried out in all the living animals after 14 days of exposure (ten exposed animals and nine control animals).

Health and body weight

Health external parameters, such as signs of lethargy, hair loss, locomotion difficulty and death, were daily monitored during the exposure period. Body weight was monitored on days 0, 4, 11 and 14.

Organ measurements

At the end of experiments, animals were sacrificed and the major organs (liver, lung, kidney, testis, heart, and spleen) were removed, cleaned and weighed. The organ weights were shown as relative weights (g/100 g body weight). The length and the width of the spleen were measured by a manual caliper (± 0.01 cm).

Hematology

The heparinized blood was collected by cardiac puncture

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and analyzed in a Coulter Counter Analyzer (three measurements were made for each animal and their average was used). The following parameters were measured: erythrocytes (RBC, x 10^6 mm^{-3}), leukocytes (WBC, x 10^3 mm^{-3}), hemoglobin concentration (HGB, g dl^{-1}), hematocrit (HCT, %), mean corpuscular volume (MCV, x 10^{-15}), mean corpuscular hemoglobin (MCH, 10^{-12}), mean corpuscular hemoglobin concentration (MCHC, g dl^{-1}), and platelets (Plt, mm^3).

Fecal corticosterone

After having been collected on day 11 of exposure, feces were stored in absolute ethanol at -30ºC, until processing. Hormone extraction was performed following Goymann's modified method (1999). Briefly, approximately 0.2g feces (Sartorius) was added to 4 ml methanol and pulverized by using a small pallet knife. The mixture was then vortexed for one hour at 500 rpm, followed by one-hour centrifugation at 600 rpm. The supernatant was then transferred to another tube and diluted with the buffer solution from the EIA Kit. The Corticosterone levels were determined by using Enzyme Immunoassay (EIA) kits (ADI-900-097, Assay Designs).

Oxygen consumption

At the 12th day, the oxygen consumption ($\dot{V}O_2$) was measured in an open-circuit respirometry system by using a Servomex oxygen analyzer (Series 4100; Servomex International Limited). Mice were put into a cylindrical chamber (0.30-m long x 0.08-m in diameter), and then placed inside the incubator (Sanyo Limited) at controlled temperature (28ºC). Inside the chamber, there was a wire-mesh grid to avoid the mice's contact with urine and feces. A flow of dried air passed through the metabolic chambers at a rate of 500 ml min^{-1}. Oxygen content was recorded every 30 seconds over a 2-h period, in two sessions (between 8 a.m. and 1 p.m. and between 1 p.m and 6 p.m.). Mice were not fasted prior to measurements but neither food nor water was available in the chamber.

Oxygen consumption was calculated as the average of the lowest five measurements of stable sections of each respirometry run, corresponding to 150 s the chamber. $\dot{V}O_2$ was obtained by Depocas & Hart (1957) as $\dot{V}O_2 = V_2 (F_1O_2 – F_1O_2) / (1-F_1O_2)$, where V_2 is the flow rate measured after the metabolic chamber, and F_1O_2 and F_1O_2 are the oxygen concentrations before and after the metabolic chamber. All $\dot{V}O_2$ measurements were corrected to standard temperature and pressure.

Open field test

The animals were individually placed in the upper left corner of an open field (28 x 21 cm) containing a floor divided into 12 equal squares and were observed over a period of five minutes. We observed the following parameters through capture videos: peripheral and central crossing (dislocation through the peripheral and central square), rearing (defined as the number of times that the animal stood on its hind legs), time in grooming, time in freezing, start time, and the number of fecal bolus. Before each test session, the animals were placed in the open field for a five-minute training session to get used to the environment (Archer, 1973). Test sessions occurred between 10 am and 14 pm in an isolated room at room temperature, around 24°C, on day 0 (before exposure), and days 4 and 14.

Statistical analysis

Results were expressed as mean ± standard deviation. The parametric data were analyzed by Student's t test and the nonparametric data were analyzed by Mann-Whitney U test. For comparison of neurobehavioral measurements considering the difference among the days of exposure, parametric data were evaluated by one-way ANOVA and non-parametric data were analyzed by Kruskal-Wallis nonparametric test. The p value < 0.05 was considered the critic value to statistical differences.

Results

The exposure to crude oil caused severe damage to treated mice. Signals, such as lethargy and bristly hairs, were common to the exposed animals. Three female animals exposed to crude oil had alopecia on their ventral portion, head, snout and near the tail (day 4) and two animals were affected on the locomotion, (leg muscle atrophy at the end of the experiment). Other four exposed animals died on the third day of exposure. On the other hand, in control animals no signs of health disturbance have been observed. The body weight in control and exposed animals did not change significantly over the exposure time (these data were not shown).

The relative measurements of organs are shown in Table 1. Liver weight was increased in animals exposed to crude oil, while their lungs weight was decreased. The weight of the other organs did not differ significantly from controls. The spleen was wider in the exposed animals.

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Table 1. *Relative weights (g /100 g body weight) of organs and measurements of the spleens (cm) of M. spretus mice exposed to soil contaminated by crude oil and to control soil for 14 days. SD – Standard Deviation.*

Control soil										
Organ	Liver	Spleen	Spleen length	Spleen width	Lungs	Heart	Right kidney	Left kidney	Right testicle	Left testicle
Mean	4.55	0.21	0.09	0.02	1.28	0.76	0.83	0.83	0.76	0.75
SD	0.32	0.05	0.01	0.004	0.26	0.13	0.08	0.05	0.14	0.10
Contaminated soil										
Organ	Liver	Spleen	Spleen length	Spleen width	Lungs	Heart	Right kidney	Left kidney	Right testicle	Left testicle
Mean	9.71*	0.25	0.10	0.03*	0.90*	0.71	0.88	0.83	0.82	0.87
SD	2.07	0.07	0.02	0.004	0.04	0.11	0.15	0.08	0.09	0.22

Legend: * significant differences in relation to control soil.

Table 2. *Hematological parameters of M. spretus mice exposed to soil contaminated by crude oil and to control soil for 14 days. SD – Standard Deviation; leukocytes (WBC, x 10^3 mm^{-3}), erythrocytes (RBC, x 10^6 mm^{-3}), hemoglobin (HGB, g dl^{-1}), hematocrit (HCT, %), mean corpuscular volume (MCV, x 10^{-15}), mean corpuscular hemoglobin (MCH, 10^{-12}), mean corpuscular hemoglobin concentration (MCHC, g dl^{-1}) and platelets (Plt, mm^3).*

Control soil								
Parameter	Leukocytes	Erythrocytes	Hemoglobin	Hematocrit	MCV	MCH	MCHC	Platelets
Mean	5.85	5.58	9.87	25.07	44.96	17.8	39.62	472.54
SD	1.88	0.97	1.13	4.3	1.10	1.65	3.40	123.24
Contaminated soil								
Parameter	Leukocytes	Erythrocytes	Hemoglobin	Hematocrit	MCV	MCH	MCHC	Platelets
Mean	4.26	6.08	9.81	27.49	45.14	16.29	36.11	506.83
SD	1.57	0.89	0.93	4.44	1.61	1.33	2.99	198.91

Finally, although it is not significant, there was a biased asymmetry in favor of the left kidney and the right testis.

No hematological parameters were altered after exposure to crude oil. The hematological values are shown in Table 2.

There was a stronger reduction in the fecal corticosterone levels of mice exposed to soil contaminated by crude oil. The results are shown in Figure 1. Another parameter affected by crude oil exposure was the measurement of O_2 consumption. Animals exposed to contaminated soil had higher oxygen consumption in relation to control animals (Figure 2).

In the open-field test, animals exposed to soil contaminated by crude oil reduced the crossing on the peripheral square after four days of exposure and the same scenario was sustained on day 14. On days 4 and 14, the exposed animals decreased the number of rearing when compared to control animals before exposure. After 14 days, the control animals also decreased the number of rearing. The time spent in grooming was lower in exposed animals, while there was no change in control animals.

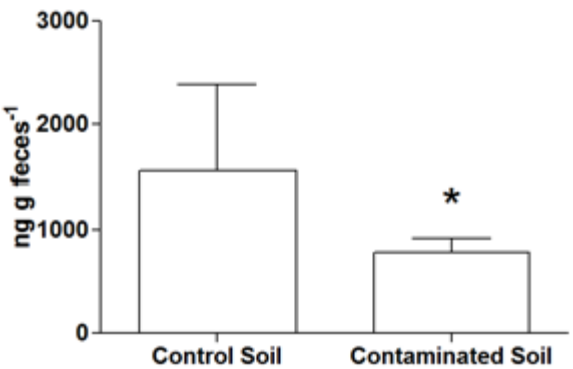

Figure 1. *Fecal corticosterone levels (ng/g wet feces) of M. spretus mice exposed to soil contaminated by crude oil and to control soil, after 11 days of exposure. * significant difference in relation to control soil.*

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After exposure, the time spent in freezing increased in animals exposed to contaminated soil, while on day 14, the freezing time of control animals reached zero. The other measured parameters did not differ significantly in any of the time periods under analysis. All results of open field parameters are shown in Table 3.

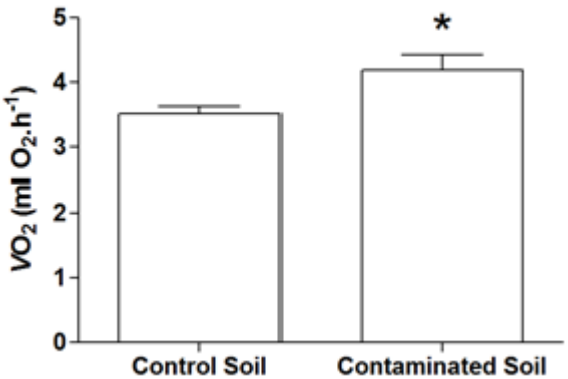

Figure 2. *Oxygen consumption of M. spretus mice exposed to soil contaminated by crude oil and control soil, after 12 days of exposure. * significant difference in relation to control soil.*

Discussion

In the current study, the results showed that crude oil could cause physiological damage in exposed animals. While the body weight and hematologic profile have not been affected by crude oil exposure, others parameters shown to be sensitive to this contaminant.

The exposure to soil contaminated by the simulation of an *onshore* oil spill caused clear signs of health disturbance in *M.spretus*. While control animals showed no external signs of decreasing health condition in oil-exposed animals apparent signs of compromised health were clear. Some of these signs, such as lethargy (Coppock & Christian, 2007) and hair loss (Gradiski et al., 1983), have already been associated in other studies to oil exposure. However, weight loss and other effects which are also commonly related to the effect of contamination by crude oil (Coppock & Christian, 2007), were not observed after 14 days of exposure.

Body weight was not affected by exposure to soil contaminated by oil, but the exposure affected the relative weight of the liver and lungs, besides the size of the spleen.

Table 3. *Open field parameters of M. spretus mice exposed to soil contaminated by crude oil and control soil for 14 days. Different lowercase letters indicate statistical difference in the line. Different capital letters indicate statistical difference in column.*

Measurement	Soil Samples	Day 0	Day 4	Day 14
Peripheral crossing	Control	53.4 ± 9.1 (aA)	66.7 ± 11.8 (aA)	63.8 ± 10.1 (aA)
	Contaminated	57.1 ± 10.9 (aA)	**30.4** ± 9.3 (bB)	**32.1** ± 9.1 (bB)
Central crossing	Control	6.8 ± 1.8 (aA)	5.1± 1.7 (aA)	4.0 ± 1.5 (aA)
	Contaminated	4.6 ± 1.1 (aA)	**1.0** ± 0.4 (bB)	4.3 ± 1.7 (aA)
Rearing	Control	16.9 ± 3.3 (aA)	10.7 ± 1.9 (aA)	**8.9** ± 1.4 (bA)
	Contaminated	10.6 ± 2.2 (aA)	**3.3** ± 1.8 (bB)	7.1 ± 2.9 (aA)
Feces	Control	0.6 ± 0.3 (aA)	1.1 ± 0.3 (aA)	1.9 ± 0.6 (aA)
	Contaminated	1.2 ± 0.3 (aA)	0.8 ± 0.3 (aA)	0.9 ± 0.5 (aA)
Start time	Control	26.1 ± 13.0 (aA)	41.6 ± 14.7 (aA)	8.6 ± 12.0 (aA)
	Contaminated	47.8 ±19.4 (aA)	9.4 ± 7.5 (aA)	18.0 ± 3.0 (aA)
Grooming time	Control	50.7 ± 10.0 (aA)	46.2 ± 9.3 (aA)	55.2 ± 7.5 (aA)
	Contaminated	76.2 ± 8.2 (aA)	**26.0** ± 10.3 (aA)	**28.8** ± 10.0 (aA)
Freezing time	Control	55.4 ± 16.1 (aA)	18.8 ± 8.7 (aA)	**0** (bA)
	Contaminated	48.2 ± 6.0 (aA)	**70.4** ± 41.3 (bA)	**70.0** ± 36.9 (bB)

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In fact, the relative increase in the liver weight was a measurement that was strongly altered by acute exposure to oil on the soil; this condition has been demonstrated in rodents exposed to petroleum and derivatives. Patrick-Iwanyanwu et al. (2011) studied the hepatotoxic and nephrotoxic effects of petroleum and kerosene and observed the increase in the liver relative weight in rats exposed for 12 weeks. In the present study, the increase in spleen measurements contrasts with the decrease in the spleen weight found by Nunes et al. (2001) in *M. spretus* living in sites contaminated by metals. Either way, the study shows that the morphology and the weight of the spleen are parameters that can be used as an indicator of physiological stress in animals exposed to chemical agents.

In contrast, there was a decrease in the lung relative weight. This result contrasts with the one found by Klonne et al. (1987). The initial decrease in the organ weight may be a cell death event (necrosis) in specific tissues, a fact which could cause a decrease in organ weight (Coppock & Christian, 2007), while the secondary increase could be related to adaptive response associated with the biotransformation of contaminants to combat systemic toxicity. Still, we may find biased asymmetry in organs, such as kidneys and testes, probably related to petroleum exposure, since this pattern is not a genetically controlled feature (Nunes et al., 2001).

Exposure to soil contaminated by crude oil caused no changes in the hematological profile of the exposed animals. The number of cells and the volume of blood cells were not affected by acute exposure to oil. In fact, the acute exposure to chemicals does not appear to alter hematological levels in small rodents (Hui et al., 2008). Khan et al. (2002) showed that hematological profile was not altered due to acute exposure of rats to petroleum hydrocarbons.

Another biological marker chosen to measure the damage caused by the exposure to soil contaminated by crude oil in *M. spretus* was the fecal corticosterone. This measure has been appreciated as a noninvasive method capable of monitoring environmental disturbances related to stress (Wasser et al., 2000, Touma et al., 2004, Hunt & Hambly, 2006). Lucas et al. (2006) showed that levels of fecal corticosterone in bird *Poecile carolinensis* accounted for differences between disturbed and undisturbed forested areas. In contrast, Schwartz et al. (2004) studied the effect of low concentrations of petroleum hydrocarbons in semi-aquatic mammal *Mustela vison* through contaminated prey but did not detect differences in fecal corticosterone levels between control and exposed animals.

In our study, fecal corticosterone levels in animals exposed to oil were low by comparison with the ones of control animals. This decrease may be associated with a dysfunction at some point in hypothalamic-pituitary-adrenal gland axis. In this study, we found alopecia in female mice and this syndrome can be caused by a dysfunction of the adrenal gland (Camacho-Martinez, 2009). This result, associated with low levels of fecal corticosterone, confirms the negative effect of crude oil exposure to the adrenal gland. However, we think that other studies of low levels of petroleum contamination in the soil are needed to validate the fecal corticosterone levels as biomarkers of stress resulting from oil pollution in wild rodent, such as *M. spretus*.

On the other hand, the oxygen consumption appeared to be a useful measure to evaluate damage to the health of animals exposed to oil. Alterations in $\dot{V}O_2$ measurements may reflect changes in the general metabolism of the animals; this parameter has been used for investigating the physiological adaptations related to habitat temperature (Mathias et al., 2003, Mathias et al., 2006). Animals exposed to the contamination increased their basal metabolic rate, thus contamination have an impact on general energy demand. After exposure to toxic substances, the short time strategies of dealing with the contaminants involve changes in animal behaviour, physiology and biochemistry. Detoxification processes are expensive mechanisms (Hawkins, 1991) and protein synthesis and high activity of detoxification enzymes require high levels of energy, which can take to an increase of total metabolic rate. In fact, the exposure of *M. spretus* to adverse conditions is able to affect to rate metabolism as mentioned by Pouliquen-Young (1994), studying the thermoregulatory behavior in response to cold in two species of mice, including *M. spretus*.

At a long term, inhabiting a contaminated area would require more feeding resources, and a reduce investment on grow and reproduction (Callow & Sibly, 1990). Higher metabolic rate also contribute for the increase of animal contamination (Maciak et al., 2011), thus animals inhabiting a contaminated area tend to increase their metabolic rate causing the advance of their inner contaminated status.

Finally, the use of behavioral tests (open field and elevated plus maze test) has been a recent strategy for the assessment of physiological and neurological responses in

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animals exposed to contaminated soils (Muccillo-Baisch et al., 2012). We have used the open field test to investigate neuro-behavioral effects resulting from the exposure to soil contaminated by crude oil. Our results showed a clear decrease in the locomotor and exploratory activity, which, associated with a decrease in grooming time and an increase in the freezing time. These associated responses seem to reveal a condition of lethargy and depression in animals.

In fact, many studies have shown that the *M. spretus* mouse has a peculiar and unique social behavior among murine. Among the behavioral responses-dependent ecological strategies are the spatial dispersion (Hurst et al., 1996; Gray & Hurst, 1997; Gray et al., 1998) and territoriality (Hurst et al., 1997), interspecific interactions (Hurst et al., 1994; Khidas et al., 2002), sexual behavior (Suchomelová et al., 1998; Cassaing & Isaac, 2007), hygiene (Hurst & Smith, 1995), and feeding (Muñoz & Bonal, 2008; Baraibar et al., 2011). Thus, in a hypothetical ecological scenario, the behavioral changes resulting from exposure to crude oil can result in serious hazards in the strategies of colonization, survival and reproduction of these small mammals.

Conclusion

Our results indicate that a simulation of an *onshore* oil spill (8% w/w) caused health hazards to *M. spretus* mice. The observation of external signs of health damage in mice can be used as an indicator of exposure to crude oil, even as the relative weight of internal organs (liver and lungs, spleen, kidneys). Besides, metabolic and behavioral parameters were changed by oil exposure and can also be used as biological markers of acute exposure in wild rodents. Therefore, we have concluded that *M. spretus* can be used as sentinel species for assessing damage caused by crude oil.

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