Stress response in terrestrial isopods: A comparative study on glycaemia

Jonathan A.C. Roques, Camille Houdelet, Freddie-Jeanne Richard

Stress response in terrestrial isopods: a comparative study on glycaemia

Jonathan A.C. Roques*, Camille Houdelet and Freddie-Jeanne Richard

Université de Poitiers, UFR Sciences fondamentales et appliquées, Laboratoire Ecologie et Biologie des interactions UMR CNSR 7267, Equipe Ecologie Evolution Symbiose, Poitiers, France.

*Current address: Department of Biological and Environmental Sciences and Swedish Mariculture Research Center (SWEMARC), University of Gothenburg, Box 463, SE-405 30, Gothenburg, Sweden.

*) Author for correspondence

E-mail address: Jonathan.roques@bioenv.gu.se (Jonathan A.C. Roques)

Abstract

Stressors are omnipresent in the environment and trigger specific physiological and/or behavioural responses that remain relatively less known in invertebrates. Among the parameters used to quantify the impact of stress on physiology, the circulating level of glucose is commonly used in aquatic crustacean species. Terrestrial isopods are small crustaceans commonly used as bioindicators of soil quality; however, their response to environmental stressors remains poorly documented. In laboratory conditions, we investigated how different stressors influence the physiological response of two terrestrial isopod species. Thus, we exposed *Armadillidium vulgare* and *Porcellio dilatatus* to a series of both acute (physical) and chronic (temperature, social isolation, and exposure to herbicide) stressors that mimic potentially stressful situations encountered in their environment. Our results revealed that, for both species, glycaemia is not impacted by thermic shock (1 h at 4°C), mechanical disturbances (1 minute of shaking or upside-down motion) or social isolation (4 weeks) at the population level. Nevertheless, we observed a significantly higher sensitivity in male *P. dilatatus* exposed to upside-down movements and in males of both species exposed to social isolation. Acute (96 hours) and chronic (21 days) exposure to glyphosate (1.8-2.5 $kg.ha^{-1}$) did not impact glycaemia levels in *A. vulgare*. However, at both tested doses, individuals exposed to glyphosate for 25 days and subjected to an additional stressor (haemolymph sampling at 96 hours) had significantly higher glycaemia in comparison to their control. Survival was also impacted by the joint application of chronic glyphosate exposure and intermediate haemolymph sampling. We conclude that glucose is a potentially good indicator to study the stress response of terrestrial isopods. Additional studies for this parameter, combined with other physiological indicators, are needed to better characterize this response.

Keywords: bioindicator**,** glyphosate, acute stress, social isolation, *Armadillidium vulgare*, *Porcellio dilatatus*

1. Introduction

In ecology, the ability of an organism or a population to respond to a given change can be used as a biological indicator, also called a bioindicator, of the quality of an environment (Whitfield and Elliott, 2002). For instance, bioindicator species can be used to measure and evaluate the impact of potentially harmful chemicals, such as heavy metals or pesticides, on the environment (Bargańska et al., 2016; Hopkin et al., 1993). Changes can be measured at different levels, such as specific diversity and abundance (Santorufo et al., 2012), or at the individual level with records of mortality and fecundity (Jones and Hopkin, 1996), physiology (Folguera et al., 2011) or behaviour (Zidar et al., 2005) to evaluate the direct impact of environmental changes. To date, several vertebrate and invertebrate species have been identified as reliable bioindicators of their natural habitat (Bargańska et al., 2016; Carravieri et al., 2013; Gerlach et al., 2013; Paoletti and Hassall, 1999; Park, 2015; Pearce and Venier, 2005; Souty-Grosset et al., 2005). Among invertebrate bioindicator species, terrestrial isopods have the potential to be used as indicators in both moist areas and grasslands (Gerlach et al., 2013; Souty-Grosset et al., 2005). Terrestrial isopods are important decomposers of leaf litter, and their integrity may be rapidly threatened when a disturbance occurs in the soil (Dallinger et al., 1992; Souty-Grosset et al., 2005).

When an organism is threatened, its resources are mobilized and redirected from functions such as growth or reproduction to face the challenges and preserve its integrity (Moberg and Mench, 2000). This response is known as the stress response. Invertebrates are frequently exposed to a number of stressors, including repeated predation attempts (Hawlena et al., 2011), adverse environmental conditions (Chown and Nicolson, 2004) and parasitism (Chang et al., 2005). As stressors are omnipresent in the environment, the stress response is essential for the survival of an individual and relatively well preserved during the course of evolution (Nesse et al., 2016). Reactions towards stress are highly variable not only among different species but also within individuals of the same species (Koehn and Bayne, 1989). As the stress response is initially adaptive and protective, prolonged exposure to a given stressor or a combination of stressors can further evolve into maladaptive responses and be detrimental for an organism or a community (Moberg and Mench, 2000). Stress can cause severe damage to an organism with a negative impact on its survival and fitness and potential consequences at the population level (Hoffmann and Hercus, 2000). As an example, the accumulation of various stressors resulted in the recent worldwide increase in colony failure rates, or ‘colony collapse disorder’, in honeybee (Even et al., 2012).

The stress response has been extensively studied in vertebrates and is the result of physiological changes in response to stressful stimuli (Kassahn et al., 2009). The stress response can be measured at three levels. The primary stress response is the release of stress hormones, such as corticosteroids and catecholamines, in the bloodstream (Tsalafouta et al., 2014). The main role of these compounds is to restore homeostasis following exposure to stressors. The secondary stress response acts at the cellular level by mobilizing and reallocating energy and increasing cardiac output and oxygen uptake and transfer. In mammals, for example, cortisol can facilitate the increase in the production and availability of metabolites such as glucose. Gluconeogenesis is promoted in the liver, while glucose use by the cells is limited to increase blood glucose availability to meet the needs of the organism in response to stressful stimuli (Desborough, 2000). The tertiary response affects the entire organism and can inhibit functions such as growth, reproduction, immune response and the ability to face additional stressors (Moberg and Mench, 2000; Sapolsky et al., 2000; Tsigos and Chrousos, 2002).

Although invertebrates are susceptible to stressors, the stress response in invertebrates has been studied to a lesser extent than it has in vertebrates. Therefore, fewer cellular and physiological indicators of stress in invertebrates are known. At the cellular level, the increased production or activation of antioxidant proteins and heat shock proteins is seen in response to a stressor, which is similar to the vertebrate response (Feder and Hofmann, 1999). Relatively few invertebrate species have been studied regarding the stress response. Among them, we find some model species for biological processes, such as *Caenorhabditis elegans* or *Drosophila melanogaster* (Feder and Hofmann, 1999), or economically and ecologically important species, such as the honeybee *Apis mellifera* (Even et al., 2012) and aquatic crustacean species, such as the American lobster *Hommarus gammarus* (Chang et al., 2005) or the white leg shrimp *Litopenaeus vannamei* (Chang et al., 2009). In aquatic crustacean species, it has been shown that exposure to harmful physical or chemical stimuli resulted in an elevation of glucose haemolymph (Lorenzon, 2005; Lorenzon et al., 1997). These physiological changes were shown to be under the control of the crustacean hyperglycaemic hormone (CHH) (Chang et al., 1998; Chang et al., 2005; Lorenzon, 2005; Lorenzon et al., 1997). This hormone is secreted from the sinus gland (Fanjul-Moles, 2006) in response to stressful stimuli in crustaceans (Chang et al., 2005). Moreover, this hormone has been described as the crustacean equivalent of cortisol and corticosterone (Elwood et al., 2009).

Terrestrial isopods have been used in tests of the toxicity of soil, and the parameters of evaluation were mainly the abundance of individuals (Faulkner and Lochmiller, 2000), reproduction rate (Niemeyer et al., 2009) and survival (Stanek et al., 2006). *Porcellio scaber* were studied most often regarding the metal bioaccumulation process (da Silva Souza and Fontanetti, 2011). In this study, we chose to investigate the impact of several potential stressors on the glucose levels of circulating haemolymph in two terrestrial isopod species, *Armadillidium vulgare* (Latreille, 1804) and *Porcellio dilatatus* (Brandt, 1833). These species belong to two different ecomorphological groups, with different defence strategies: *A. vulgare* is a roller and *P. dilatatus* is a clinger (Schmalfuss, 1984). In their habitat, these organisms can be exposed to various stressors that can be biotic or abiotic and acute or chronic. The main stressors encountered can be separated into three categories: 1) biological stressors (predators, parasites, fungi, bacteria and viruses); 2) chemical stressors (pesticides, fungicides, and herbicides), which can cause damage to non-target species; and 3) environmental stressors (agricultural practices, (micro)climate change, resource abundance fluctuation, social interactions, humidity, etc.). In our laboratory, we investigated the impact of five different stimuli that mimicked the whole range of potentially stressful situations encountered in the natural habitat or cultivated areas of these species (Swan et al., 2011; Szlavecz et al., 2018). The main objective of the present study was to assess the importance of three extrinsic factors (temperature, shaking, and falling), one intrinsic factor (gender) and social privation on the glycaemia level.

The approach of using more subtle endpoints, such as physiological and behavioural changes, to assess the toxicity of potentially harmful substances has become more popular and has been shown to be more accurate than the traditional survival, growth and reproduction responses (Drobne, 1997). In line with this approach, we use glycaemia as a physiological endpoint to measure the potential stress responses in two terrestrial isopod species.

2. Material and methods

2.1 Animals

The terrestrial isopods *A. vulgare* and *P. dilatatus* were derived from two natural populations collected in France (Nice, Alpes Maritimes, 1967, for *A. vulgare* and Rom, Deux-Sèvres, 1988, for *P. dilatatus*). The animals were maintained in standard laboratory conditions (in boxes of moistened compost, at 20°C, and with the natural photoperiod of Poitiers, France

46°40'N) and fed *ad libitum* fresh organic carrots and dried leaves of linden (*Tilia* sp.). For Experiments 1 and 2, we selected one-year-old adult *A. vulgare* and *P. dilatatus* of similar size (around 60 mg and 150 mg respectively). For Experiment 3, we selected a mixture of virgin 1- to 3-year-old *A. vulgare* individuals (60-150 mg). All animals used were in intermoult (di-ecdysis or beginning the pre-ecdysis stages C/D_0) (for growth cycle details, see Beauché and Richard, 2013 and Zidar et al., 1998). The period of each experiment was chosen to match the moult cycle of each species (at least 26 days for both species; Beauché and Richard, 2013; Zidar et al., 1998) in order to avoid moulting during the course of experiment. None of the tested individuals were moulting during the course of the experiment. Gravid females were excluded from the experimental pool. The experimental procedures and animal manipulations did not require an ethics statement. The stimuli used in these experiments were carefully selected to elicit a subtle response without causing any unnecessary distress.

2.2 Experimental procedure

All procedures were carried out in laboratory conditions during daylight hours between 10:00 and 16:00 in the spring and summer of 2016. We chose five different stimuli to be applied to the isopods: "cold", "shake", "upside-down" (Experiment 1), social isolation (Experiment 2) and chronic exposure to glyphosate (Experiment 3). The stimuli were chosen as they could mimic threats that the animals encounter in natural situations.

2.2.1 Experiment 1: biological stressor

Animals were randomly subjected to one of the three following acute stimuli: exposure to cold temperature, shaking or an upside-down movement. The cold stimulus was chosen as ectothermic animals, including terrestrial isopods, are sensitive to severe temperature changes as their body temperature is mostly dependent on the temperature of their environment (Dahlhoff and Rank, 2007). The shaking stimulus was used to mimic the impact of modern agricultural practices, such loud noises and vibration disturbances caused by heavy machinery (Schlacher and Thompson, 2007; Soane and van Ouwerkerk, 1994). The upside-down stimulus aimed to mimic the impact of predator attacks. In nature, terrestrial isopods can be targeted by numerous predators which could catch, move and drop them (Tuf et al., 2015).

For each species, 20 undisturbed individuals (10 males and 10 females) were randomly picked from the holding tanks and served as control groups. The 'cold' stimulus consisted of placing two individuals in one box (Ø 8 cm, H 5 cm) kept at 4°C for one hour. The light condition was kept constant during this period (refrigerator with a glass door), and the box was kept moist using water-dipped tissues in the bottom). The "shake" stimulus consisted of shaking the animals individually in a box (Ø 8 cm, H 5 cm) attached to an IKA KS 130 Basic 2D shaker (IKA, Staufen, Germany) for one minute at 560 rpm. The "upside-down" stimulus consisted of placing the animals in individual boxes (Ø 9.5 cm, H 5 cm) and overturning the box repeatedly by performing ample movements (180°) from right to left for one minute. This stimulus was carried out by the same experimenter at a constant speed (approximately 70 movements). Stimuli were applied randomly to different individuals.

2.2.2 Experiment 2: social isolation

We investigated the impact of social isolation, which could be considered a chronic stressor for the studied species. Terrestrial isopods are known to be gregarious organisms. Living in groups has been demonstrated to offer several advantages, such as the prevention of desiccation, the potential protection from predators and better access to mates and secondary food resources (Broly et al., 2013). For this stimulus, 36 animals per species (18 males and 18 females) were placed in individual boxes (Ø 8 cm, H 5 cm) and kept isolated for a period of 4 weeks. The boxes were visually isolated from each other by cardboard sheets. In parallel, 120 individuals 120 were sexed and kept in groups of 6 (20 boxes, Ø 11 cm, H 5 cm; 10 boxes for males and 10 boxes for females) and served as the controls. On the sampling day, the analyses of both the isolated and grouped individuals were performed simultaneously. In total, 20 animals (10 males, 10 females, one individual per box) were sampled.

2.2.3 Experiment 3: chemical stressor

The animals were chronically exposed to the glyphosate-based herbicide Roundup® (Monsanto, USA). Glyphosate-based herbicides are among the most widely used herbicides for weed control and have become one of the most commonly used agrochemicals worldwide (Shang et al., 2011). Despite studies of the impact of glyphosate on several non-target species (Balbuena et al., 2015; Giesy et al., 2000), the sublethal effect remains poorly studied. Terrestrial isopods have been proposed as good candidates to study the impact of pollutants in terrestrial environments (Drobne, 1997).

198

199 Animals (*A. vulgare*) were randomly exposed to one of three doses of glyphosate (0, 1.8 and
200 2.5 $kg.ha^{-1}$) for up to 25 days. These doses are related to the mg of glyphosate present in the
201 glyphosate-formulated herbicide Roundup®. In this study, we did not aim to observe high
202 mortality but rather to test the impact of the doses recommended by the manufacturer and
203 approved by the French Ministry of Agriculture and Food at the time of the experiment for
204 use against easy-to-control (1.8 $kg.ha^{-1}$) and difficult-to-control (2.5 $kg.ha^{-1}$) weeds. Animals
205 were subsequently placed in plastic boxes (Ø 10 cm, 5 cm H) containing 70 (±0.5) g of
206 moistened compost. Each box contained 5 animals of the same sex and same age class
207 (N=40/treatment). The animals were acclimated in the boxes for four days, during which they
208 received their usual food. After this acclimatization period, the uneaten food was removed.
209 The glyphosate used in our study was in the form of isopropylamine salt (170 $g.l^{-1}$) as
210 specified in the Roundup® formulation. Animals were exposed to the desired dose of
211 glyphosate (Roundup®) through the food and the soil. For each box, 5-6 pieces of thinly
212 sliced carrot and the equivalent of two linden leaves were soaked with a mixture of 7 ml of
213 water and 0 µl, 8.3 µl or 11.5 µl of Roundup® for 15 minutes prior to being placed in the
214 appropriate box. The remaining solution was subsequently pipetted homogeneously into the
215 box, where it was placed on the soil, not directly on the animals. This process was repeated
216 weekly until the completion of the experiment (25 days maximum). Mortality was recorded
217 during these processes, and dead individuals were removed from the experimental boxes. One
218 group was sampled after 96 hours (acute exposure), and one group was sampled after 21 days
219 (chronic exposure). In addition, after the puncture, the animals from the group sampled after
220 96 hours were kept moist in recovery boxes with water-dipped tissues at the bottom for 24
221 hours. Surviving individuals were placed back into their original experimental boxes and
222 sampled after 21 additional days (25 days in total). This was done to investigate the effect of
223 an extra stressor (haemolymph sampling, mimicking a wound) in addition to the chronic
224 exposure to glyphosate.

225 Immediately after each stimulus, circulating haemolymph was sampled by making a puncture
226 in the intersegmental membrane between the thorax and the abdomen. The respective control
227 groups from each experiment were also sampled for haemolymph at the same time. In total,
228 5-10 µl of haemolymph per individual was collected using a micropipette and stored at -20°C
229 until further analyses. After the puncture, the animals were kept moist in recovery boxes
230 using water-dipped tissues on the bottom for 24 hours. Surviving individuals (>50%) were

placed back into their original housing boxes with moistened compost litter and were not further used for another study.

2.3 Glucose determination

Haemolymph glucose was measured with a commercially available enzymatic test kit (Glucose HK, Sigma-Aldrich, St. Louis, USA), with protocols adapted to a 96-well microplate (Schram et al., 2010). Samples are first diluted in distilled water (1:6); then, 10 µl of the diluted sample or standard (5.55 mmol.l^{-1} glucose) are mixed with 200 µl of the reagent provided with the kit and incubated for 15 minutes at 20°C. Absorbance was read within 30 minutes at 340 nm (Berthold LB941 Multimode reader, Berthold Technologies, Zug, Switzerland).

2.4 Data analyses and statistics

Data are summarized by box and whisker plots that present first, second (median), and third quartiles as horizontal lines in the box and upper and lower values as "whiskers" extending from the boxes; In some instances, not all samples collected were analysed because of insufficient haemolymph quantity. For Experiment 1 (the "cold" and "shake" stimuli) and Experiment 3, there were no gender differences, and, therefore, the data were pooled. However, for the "upside-down" stimulus (Experiment 1) and Experiment 2, the data were split between males and females. The data were not normally distributed; therefore, the non-parametric Mann-Whitney test was used to assess statistical significance for all the experiments. We used the Chi-square test to compare the survival of individuals in Experiment 3.

3. Results

3.1 Experiment 1: biological stressor

There were no differences in the glucose haemolymph concentration regardless of the species or the type of stimulus tested in comparison with the control condition when the data were pooled (Fig 1A and 1B) (Mann-Whitney test; for *P. dilatatus* and *A. vulgare,* respectively: "cold": W=59, p=0.32; W=151, p=0.27; "shake": W=93.5, p=0.41; W=141, p=0.17; "upside-down": W=100, p=0.16; W=180.5, p=0.79). For *A. vulgare*, no gender-specific differences were found (Mann-Whitney test, p> 0.05 for all stimuli, Fig 1B and 2B). Similarly, *P. dilatatus* subjected to the cold and shake stimuli did not display gender differences regarding

their glucose haemolymph (Fig 1A). However, male *P. dilatatus* had significantly haemolymph glucose levels compared to those of their female counterparts for the upside-down stimulus only (Mann-Whitney test, W=20, p=0.03, Fig 2A). These levels were significantly higher than those of the male control group (Mann-Whitney test, W=13, p=0.02, Fig 2A).

3.2 Experiment 2: social isolation

There were no differences in the glucose haemolymph concentration for either species in comparison with their respective controls when the data were pooled (Mann-Whitney test; W=106.5, p=0.20 for *P. dilatatus* and W=157, p=0.08 for *A. vulgare*). However, we observed a gender difference for both species: male *P. dilatatus* and *A. vulgare* subjected to social isolation for a month had significantly higher haemolymph glucose levels compared to those of their respective control groups (Mann-Whitney test, W=56, p=0.04 for *P. dilatatus*,; and W=14.5, p<0.01 for *A. vulgare*, Fig 3A and 3B). No differences in glucose haemolymph were found between females subjected to social isolations and females kept in group for either species (Mann-Whitney test, W=98, p=0.96 for *P. dilatatus* and W=53, p=0.47 for *A. vulgare*).

3.3 Experiment 3: chemical stressor

There was no effect on the glucose haemolymph concentration when *A. vulgare* individuals were exposed to either dose of glyphosate for 96 hours (Mann-Whitney test, W=506.5, p=0.47 for the 1.8 $kg.ha^{-1}$ group and W=475, p=0.21 for the 2.5 $kg.ha^{-1}$ group, Fig 4A) or 21 days (Mann-Whitney test, W=353, p=0.45 and W=295.5, p=0.18, Fig 4B). However, individuals who were sampled after 25 days of exposure to either concentration and received another stressor (i.e., a haemolymph puncture after 96 hours) had significantly higher glucose haemolymph than that of the control group (Mann-Whitney test, W=57, p=0.01 for the 1.8 $kg.ha^{-1}$ group and W=64.5, p=0.05 for the 2.5 $kg.ha^{-1}$ group, Fig 4C). Survival tended to decrease naturally with time in all groups (non-significant; X^2=0.13, ddl=2; X^2=0.33, ddl=2; and X^2=0.58, ddl=2 for the control group and the 1.8 and 2.5 $kg.ha^{-1}$ glyphosate groups, respectively) (Fig 5). Survival was only negatively affected by the combination of chronic exposure to glyphosate and the application of an extra stressor (Fig 5). No differences in survival were observed between individuals from the control group samples after 96 hours and those from the group exposed to glyphosate for 96 hours + 21 days (X^2=3.63, ddl=1),

while survival decreased in both the 1.8 and 2.5 kg.ha^{-1} glyphosate groups (X^2=6.23, ddl=1 and X^2=7.41, ddl=1, respectively). There were no differences in survival among all groups after 96 hours and 21 days of exposure (X^2=0.58, ddl=1; X^2=0,01, ddl=1; and X^2=0.053, ddl=1 for the control group and the 1.8 and 2.5 kg.ha^{-1} glyphosate groups, respectively) (Fig 5).

4. Discussion

The elevation of circulating glucose levels is a common response to stressors and is found throughout the animal kingdom, including crustaceans. Crustaceans have been widely used for stress risk assessment and are used as bioindicators or biomonitors, especially in various aquatic systems (Rinderhagen et al., 2000). Among commercial aquatic crustaceans species, stress exposure, such as emersion, transport and handling, is common and causes hypoxia and variation in physiology (Lorenzon, 2005). For example, the decapods, *Liocarcinus depurator* and *Munida rugosa* showed a higher concentration of glucose when subjected to trawling in comparison to creel-caught crustaceans (Bergmann et al., 2001). Another study revealed that the CHH hormone concentration increased in the haemolymph in less than 1 hour after acute stress (handling) in lobsters, which is slower than the appearance of biogenic amines (Chang et al., 2005). The production and action of these hormones leads to mobilization of energy reserves, such as glucose. In the current study, we used two terrestrial isopod species to test the glucose level variation in response to different stressors.

We found that stress induced in *A. vulgare* and *P. dilatatus* adults by the cold and shake stimuli had no significant impact on the haemolymph glucose levels, which were collected right after the application of the stimuli to test the short-term impact of our stressors.

When exposed to the upside-down acute stressor, we observed that males of *P. dilatatus* had significantly higher glucose levels than those of their controls, while there was no effect on male *A. vulgare*. These two species have different defence strategies. *A. vulgare* can roll and may isolate themselves from the exterior (Schmalfuss, 1984). However, *P. dilatatus* corresponds to the clinger ecomorphological group (Schmalfuss, 1984) and is highly responsive to stimuli, but the duration of response (tonic immobility) is relatively short, with no difference between sex (Quadros et al., 2012).

In the second experiment, we observed a sex difference for both species when individuals were isolated for one month. The glucose levels of male are apparently affected, while those

of females are not. These results indicate that male isopods, regardless of their defence strategies, are more sensitive to non-physical and chronic stress, such as social isolation, than their female counterpart. Here, we showed that different types of stress have different impacts between species, but we also observed differences in sensitivity between females and males. Differences in sensitivity between sexes exist and have been previously observed in several invertebrates and in both aquatic and terrestrial systems (Sornom et al., 2010) and reference therein (McClellan-Green et al., 2007; Wilczek et al., 2008; Zeng et al., 2011). For example, under oxidative stress conditions, male flies ingested and excreted less food than females (Zeng et al., 2011). In two species of spider, cellular reactions against stress caused by metal pollutants seemed to be sex-dependant (Wilczek et al., 2008). In the terrestrial isopod *P. scaber*, a close relative of *P. dilatatus*, an exposure to dimethoate for 48 hours impacted the locomotor behaviour (increased hyperactivity and suppressed turning rate) of males only (Bayley, 1995).

In the third experiment, we evaluated the effect of two recommended concentrations of glyphosate used in agriculture (1.8 and 2.5 $kg.ha^{-1}$). Glyphosate ingestion might affect the learning and retrieval of memory for the recognition of food (Balbuena et al., 2015), and chronic exposure to traces of glyphosate reduces the responsiveness to sucrose in honeybee (Herbert et al., 2014). Glyphosate is also known to reduce growth in the earthworm (Springett and Gray, 1992) and affect reproduction and development in the freshwater snail (Tate et al., 1997). In our experiment, we observed no significant impact on *A. vulgare* glycaemia. However, individuals who were chronically exposed to glyphosate and an additional stressor (intermediate haemolymph sampling) had altered glucose levels in comparison with individuals that only received the additional stressor without glyphosate. No individual moulted between the two sampling sessions. Stressors are omnipresent in the environment and can potentially interact with each other and affect the capacity of an organism to cope with a specific stressor (Sornom et al., 2010). While assessing the sublethal impact of a contaminant on an organism, it is important to take into account that the potential of an organism to cope with stressors and survive is determined by the ability of all its biological systems to work in concert (Hebel et al., 1997). For instance, here, we observed no direct impact of exposure to the recommended doses of glyphosate, but when animals were exposed to an additional stressor (haemolymph puncture) that can be compared to a minor wound, we observed that its combination with glyphosate exposure resulted in stress.

In conclusion, changes in glycemia can be used to assess stress in terrestrial isopods. Males of both species seemed to be more sensitive than female to stressors such as upside-down movements or social isolation. In our study, we have focussed on the very short term impact of single stressor. The effect combined stressors and the impact of glyphosate exposure on the middle and longer term require further investigations.

Acknowledgements

We are grateful to Alexandra Laffite who is in charge of terrestrial isopods management and welfare in the Laboratory. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Balbuena, M.S., Tison, L., Hahn, M.-L., Greggers, U., Menzel, R., Farina, W.M., 2015. Effects of sublethal doses of glyphosate on honeybee navigation. J. Exp. Biol. 218, 2799-2805.

Bargańska, Ż., Ślebioda, M., Namieśnik, J., 2016. Honey bees and their products: bioindicators of environmental contamination. Crit. Rev. Environ. Sci. Technol. 46, 235-248.

Bayley, M., 1995. Prolonged effects of the insecticide dimethoate on locomotor behaviour in the woodlouse, Porcellio scaber Latr.(Isopoda). Ecotoxicology 4, 79-90.

Beauché, F., Richard, F.-J., 2013. The best timing of mate search in Armadillidium vulgare (Isopoda, Oniscidea). PloS one 8, e57737.

Bergmann, M., Taylor, A.C., Moore, P.G., 2001. Physiological stress in decapod crustaceans (Munida rugosa and Liocarcinus depurator) discarded in the Clyde Nephrops fishery. J. Exp. Mar. Biol. Ecol. 259, 215-229.

Broly, P., Deneubourg, J.-L., Devigne, C., 2013. Benefits of aggregation in woodlice: a factor in the terrestrialization process? Insect. Soc. 60, 419-435.

Carravieri, A., Bustamante, P., Churlaud, C., Cherel, Y., 2013. Penguins as bioindicators of mercury contamination in the Southern Ocean: birds from the Kerguelen Islands as a case study. Sci. Total Environ. 454, 141-148.

Chang, C.-C., Yeh, M.-S., Cheng, W., 2009. Cold shock-induced norepinephrine triggers apoptosis of haemocytes via caspase-3 in the white shrimp, Litopenaeus vannamei. Fish & Shellfish Immun. 27, 695-700.

Chang, E.S., Keller, R., Chang, S.A., 1998. Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, Homarus americanus, following various stresses. Gen. Comp. Endocrinol. 111, 359-366.

Chang, E.S., Stentiford, G., Neil, D., Chang, S.A., 2005. Crustacean hyperglycemic hormone and hemolymph metabolites: Stress responses in two lobster species. NOAA Tech. Rep. NMFS.

Chown, S.L., Nicolson, S., 2004. Insect physiological ecology: mechanisms and patterns. Oxford University Press, Oxford.

da Silva Souza, T., Fontanetti, C.S., 2011. Morphological biomarkers in the Rhinocricus padbergi midgut exposed to contaminated soil. Ecotoxicol. Environ. Saf. 74, 10-18.

Dahlhoff, E.P., Rank, N.E., 2007. The role of stress proteins in responses of a montane willow leaf beetle to environmental temperature variation. J. Biosci. (Bangalore) 32, 477-488.

Dallinger, R., Berger, B., Birkel, S., 1992. Terrestrial isopods: useful biological indicators of urban metal pollution. Oecologia 89, 32-41.

Desborough, J., 2000. The stress response to trauma and surgery. Brit. J. Anaesth. 85, 109-117.

Drobne, D., 1997. Terrestrial isopods—a good choice for toxicity testing of pollutants in the terrestrial environment. Environ. Toxicol. Chem. 16, 1159-1164.

Elwood, R.W., Barr, S., Patterson, L., 2009. Pain and stress in crustaceans? Appl. Anim. Behav. Sci. 118, 128-136.

406 Even, N., Devaud, J.-M., Barron, A.B., 2012. General stress responses in the honey bee.
407 Insects 3, 1271-1298.

408 Fanjul-Moles, M.L., 2006. Biochemical and functional aspects of crustacean hyperglycemic
409 hormone in decapod crustaceans: review and update. Comp. Biochem. Phys. C. 142, 390-
410 400.

411 Faulkner, B., Lochmiller, R., 2000. Increased abundance of terrestrial isopod populations in
412 terrestrial ecosystems contaminated with petrochemical wastes. Arch. Environ. Contam.
413 Toxicol. 39, 86-90.

414 Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress
415 response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61, 243-282.

416 Folguera, G., Bastías, D.A., Caers, J., Rojas, J.M., Piulachs, M.-D., Bellés, X., Bozinovic, F.,
417 2011. An experimental test of the role of environmental temperature variability on ectotherm
418 molecular, physiological and life-history traits: implications for global warming. Comp.
419 Biochem. Phys. A. 159, 242-246.

420 Gerlach, J., Samways, M., Pryke, J., 2013. Terrestrial invertebrates as bioindicators: an
421 overview of available taxonomic groups. J. Insect Conserv. 17, 831-850.

422 Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for
423 Roundup® herbicide, Reviews of environmental contamination and toxicology. Springer,
424 New York, pp. 35-120.

425 Hawlena, D., Kress, H., Dufresne, E.R., Schmitz, O.J., 2011. Grasshoppers alter jumping
426 biomechanics to enhance escape performance under chronic risk of spider predation. Funct.
427 Ecol. 25, 279-288.

428 Hebel, D., Jones, M., Depledge, M., 1997. Responses of crustaceans to contaminant
429 exposure: a holistic approach. Estuar. Coast. Shelf Sci. 44, 177-184.

430 Herbert, L.T., Vázquez, D.E., Arenas, A., Farina, W.M., 2014. Effects of field-realistic doses
431 of glyphosate on honeybee appetitive behaviour. J. Exp. Biol. 217, 3457-3464.

432 Hoffmann, A.A., Hercus, M.J., 2000. Environmental stress as an evolutionary force.
433 Bioscience 50, 217-226.

Hopkin, S.P., Jones, D.T., Dietrich, D., 1993. The isopod Porcellio scaber as a monitor of the bioavailability of metals in terrestrial ecosystems: towards a global 'woodlouse watch' scheme. Sci. Total Environ. 134, 357-365.

Jones, D., Hopkin, S., 1996. Reproductive allocation in the terrestrial isopods Porcellio scaber and Oniscus asellus in a metal-polluted environment. Funct. Ecol. 10, 741-750.

Kassahn, K.S., Crozier, R.H., Pörtner, H.O., Caley, M.J., 2009. Animal performance and stress: responses and tolerance limits at different levels of biological organisation. Biol. Rev. 84, 277-292.

Koehn, R.K., Bayne, B.L., 1989. Towards a physiological and genetical understanding of the energetics of the stress response. Biol. J. Linn. Soc. 37, 157-171.

Lorenzon, S., 2005. Hyperglycemic stress response in Crustacea. ISJ 2, 132-141.

Lorenzon, S., Giulianini, P.G., Ferrero, E.A., 1997. Lipopolysaccharide-Induced Hyperglycemia Is Mediated by CHH Release in Crustaceans. Gen. Comp. Endocrinol. 108, 395-405.

McClellan-Green, P., Romano, J., Oberdörster, E., 2007. Does gender really matter in contaminant exposure? A case study using invertebrate models. Environ. Res. 104, 183-191.

Moberg, G.P., Mench, J.A., 2000. The biology of animal stress: basic principles and implications for animal welfare. CABI, Wallingford.

Nesse, R., Bhatnagar, S., Ellis, B., 2016. Evolutionary origins and functions of the stress response system. Stress: Concepts, Cognition, Emotion, and Behavior: Handbook of Stress Series 1, 95-101.

Niemeyer, J.C., Santos, V., Araújo, P.B.d., Da Silva, E., 2009. Reproduction of Cubaris murina (Crustacea: Isopoda) under laboratory conditions and its use in ecotoxicity tests. Braz. J. Biol. 69, 137-142.

Paoletti, M.G., Hassall, M., 1999. Woodlice (Isopoda: Oniscidea): their potential for assessing sustainability and use as bioindicators. Agric. Ecosyst. Environ. 74, 157-165.

460 Park, K.J., 2015. Mitigating the impacts of agriculture on biodiversity: bats and their potential
461 role as bioindicators. Mammalian Biology-Zeitschrift für Säugetierkunde 80, 191-204.

462 Pearce, J., Venier, L., 2005. Small mammals as bioindicators of sustainable boreal forest
463 management. For. Ecol. Manage. 208, 153-175.

464 Quadros, A.F., Bugs, P.S., Araujo, P.B., 2012. Tonic immobility in terrestrial isopods:
465 intraspecific and interspecific variability. ZooKeys 176, 155-170.

466 Rinderhagen, M., Ritterhoff, J., Zauke, G.-P., 2000. Crustaceans as bioindicators,
467 Biomonitoring of Polluted Water-Reviews on Actual Topics. Trans Tech Publications-
468 Scitech Publications, Environmental Research Forum, Zurich, pp. 161-194.

469 Santorufo, L., van Gestel, C.A.M., Rocco, A., Maisto, G., 2012. Soil invertebrates as
470 bioindicators of urban soil quality. Environ. Pollut. 161, 57-63.

471 Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress
472 responses? Integrating permissive, suppressive, stimulatory, and preparative actions 1.
473 Endocr. Rev. 21, 55-89.

474 Schlacher, T.A., Thompson, L.M.C., 2007. Exposure of Fauna to Off-Road Vehicle (ORV)
475 Traffic on Sandy Beaches. Coast. Manage. 35, 567-583.

476 Schmalfuss, H., 1984. Eco-morphological strategies in terrestrial isopods, Symposia of the
477 Zoological Society of London. Cambridge University Press, Cambridge, pp. 49-63.

478 Schram, E., Roques, J.A.C., Abbink, W., Spanings, T., de Vries, P., Bierman, S., de Vis,
479 H.V., Flik, G., 2010. The impact of elevated water ammonia concentration on physiology,
480 growth and feed intake of African catfish (Clarias gariepinus). Aquaculture 306, 108-115.

481 Shang, Y., Shen, H.-m., Yang, S.-y., Wang, C.-y., Wu, Y.-b., 2011. Screening and Isolation
482 of Bacteria Degrading Herbicide Glyphosate and Its Degradation Efficiency [J]. Hubei
483 Agricultural Sciences 9.

484 Soane, B., van Ouwerkerk, C., 1994. Soil compaction problems in world agriculture. Soil
485 compaction in crop production 11, 1-2.

Sornom, P., Felten, V., Médoc, V., Sroda, S., Rousselle, P., Beisel, J.-N., 2010. Effect of gender on physiological and behavioural responses of Gammarus roeseli (Crustacea Amphipoda) to salinity and temperature. Environ. Pollut. 158, 1288-1295.

Souty-Grosset, C., Badenhausser, I., Reynolds, J., Morel, A., 2005. Investigations on the potential of woodlice as bioindicators of grassland habitat quality. Eur. J. Soil Biol. 41, 109-116.

Springett, J., Gray, R., 1992. Effect of repeated low doses of biocides on the earthworm Aporrectodea caliginosa in laboratory culture. Soil Biol. Biochem. 24, 1739-1744.

Stanek, K., Drobne, D., Trebše, P., 2006. Linkage of biomarkers along levels of biological complexity in juvenile and adult diazinon fed terrestrial isopod (Porcellio scaber, Isopoda, Crustacea). Chemosphere 64, 1745-1752.

Swan, C.M., Pickett, S.T., Szlavecz, K., Warren, P., Willey, K.T., 2011. Biodiversity and community composition in urban ecosystems: coupled human, spatial, and metacommunity processes. Handbook of urban ecology. Oxford University Press, New York, pp. 179-186.

Szlavecz, K., Vilisics, F., Tóth, Z., Hornung, E., 2018. Terrestrial isopods in urban environments: an overview. ZooKeys 801, 97-126.

Tate, T., Spurlock, J., Christian, F., 1997. Effect of glyphosate on the development of Pseudosuccinea columella snails. Arch. Environ. Contam. Toxicol. 33, 286-289.

Tsalafouta, A., Papandroulakis, N., Gorissen, M., Katharios, P., Flik, G., Pavlidis, M., 2014. Ontogenesis of the HPI axis and molecular regulation of the cortisol stress response during early development in Dicentrarchus labrax. Sci. Rep-UK 4.

Tsigos, C., Chrousos, G.P., 2002. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53, 865-871.

Tuf, I.H., Drábková, L., Šipoš, J., 2015. Personality affects defensive behaviour of Porcellio scaber (Isopoda, Oniscidea). ZooKeys 515, 159-171.

Whitfield, A.K., Elliott, M., 2002. Fishes as indicators of environmental and ecological changes within estuaries: a review of progress and some suggestions for the future. J. Fish Biol. 61, 229-250.

514 Wilczek, G., Babczyńska, A., Wilczek, P., Doleżych, B., Migula, P., Młyńska, H., 2008.
515 Cellular stress reactions assessed by gender and species in spiders from areas variously
516 polluted with heavy metals. Ecotoxicol. Environ. Saf. 70, 127-137.

517 Zeng, C., Du, Y., Alberico, T., Seeberger, J., Sun, X., Zou, S., 2011. Gender-specific prandial
518 response to dietary restriction and oxidative stress in Drosophila melanogaster. Fly 5, 174-
519 180.

520 Zidar, P., Božič, J., Štrus, J., 2005. Behavioral Response in the Terrestrial Isopod Porcellio
521 scaber (Crustacea) Offered a Choice of Uncontaminated and Cadmium-Contaminated Food.
522 Ecotoxicology 14, 493-502.

523 Zidar, P., Drobne, D., Štrus, J., 1998. Determination of moult stages of Porcellio scaber
524 (Isopoda) for routine use. Crustaceana 71, 646-654.

525

526 **Figure legend**

527 **Fig 1.** Haemolymph glucose concentrations (whisker plot) in *Porcellio dilatatus* (A) and
528 *Armadillidium vulgare* (B) in the control situation and following different stressful stimuli:
529 one hour at 4°C ("cold") and one minute of constant shaking at 560 rpm ("shake") (females
530 and males pooled). NS: not significant (Mann-Whitney test).

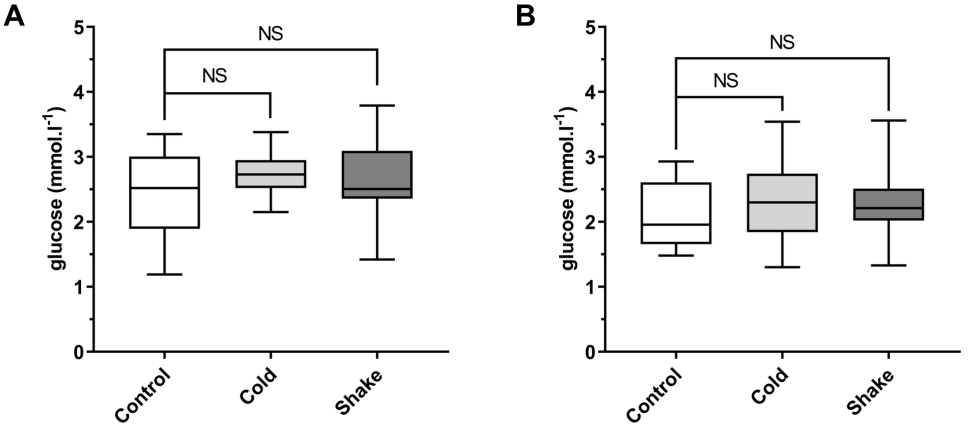
531 **Fig 2.** Haemolymph glucose concentrations (whisker plot) in *Porcellio dilatatus* (A) and
532 *Armadillidium vulgare* (B) in the control situation and one minute of repeated manual
533 overturns in a box ("upside-down"). NS: not significant, *: $p<0.05$ (Mann-Whitney test).

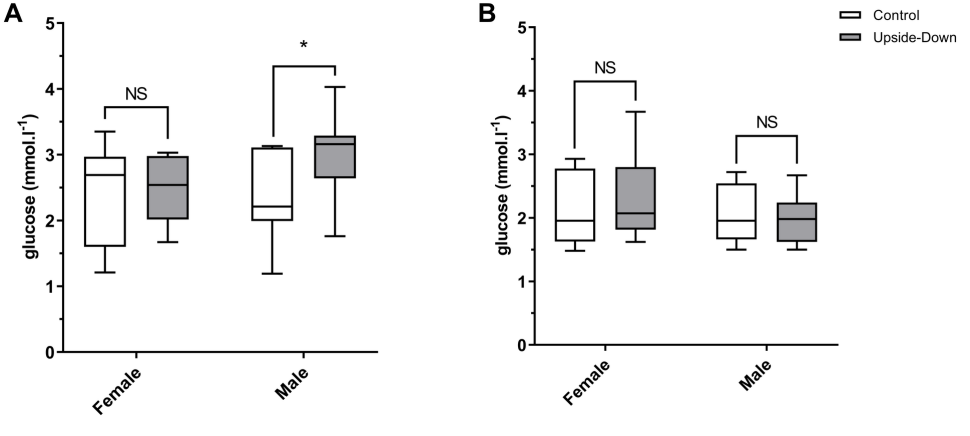
534 **Fig 3.** Haemolymph glucose concentrations (whisker plot) in *Porcellio dilatatus* (A) and
535 *Armadillidium vulgare* (B) kept in groups of 6 individuals ("control") or kept isolated
536 ("social isolation") for a period of 4 weeks. NS: not significant, *: $p<0.05$, **: $p<0.01$ (Mann-
537 Whitney test).

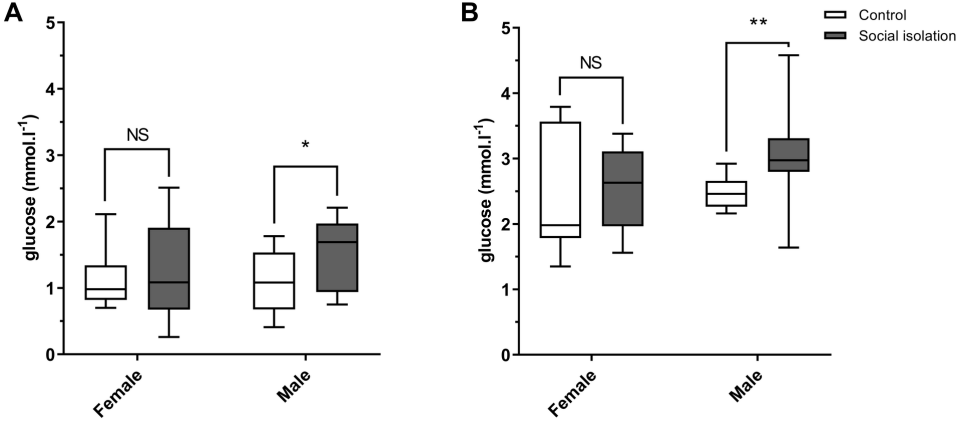
538 **Fig 4.** Haemolymph glucose concentrations (whisker plot) in *Armadillidium vulgare* exposed
539 to 0 (control), 1.8 and 2.5 $kg.ha^{-1}$ of glyphosate for 96 hours (A), 21 days (B) and 25 days
540 with an extra stressor (haemolymph sampling) after 96 hours (C) NS: not significant, *:
541 $p<0.05$, Mann-Whitney test).

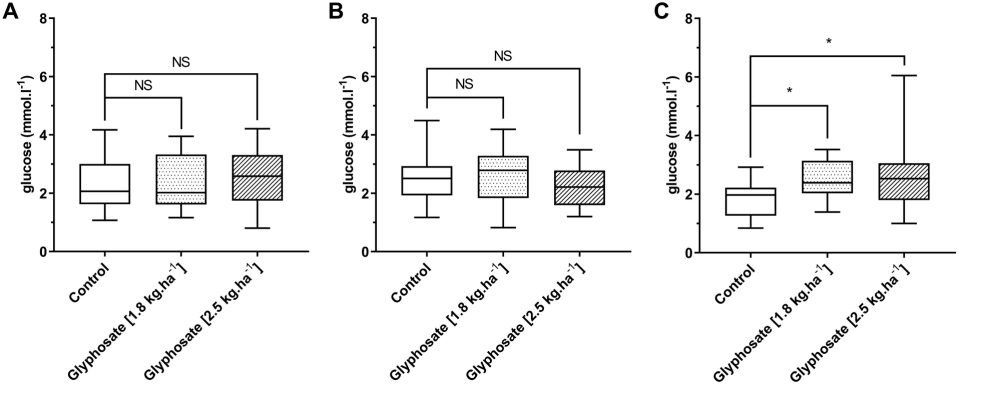
542 **Fig 5.** Survival (%) of *Armadillidium vulgare* exposed to 0 (control), 1.8 and 2.5 $kg.ha^{-1}$ of
543 glyphosate for 96 hours (T=96 H), 21 days (T=21 D) and 25 days with an extra stressor
544 (T=96 H + 21 D) after 96 hours. *: $p<0.05$ Chi-square test.

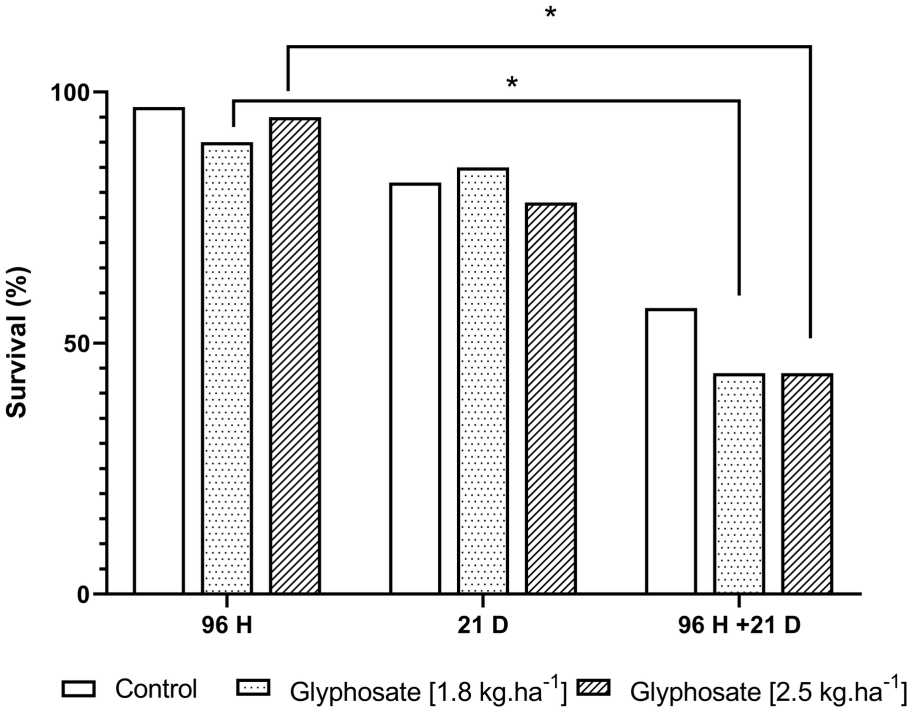
545


A
B
NS
NS
glucose (mmol.l-1)
0
1
2
3
4
5
Control
Cold
Shake


A
B
Control
Upside-Down
glucose (mmol.l-1)
NS
*
Female
Male
0
1
2
3
4
5


A
B
Control
Social isolation
glucose (mmol.l-1)
NS
*
**
Female
Male
0
1
2
3
4
5


A
B
C
glucose (mmol.l-1)
0
2
4
6
8
NS
NS
NS
NS
*
*
Control
Glyphosate [1.8 kg.ha-1]
Glyphosate [2.5 kg.ha-1]


*
*
100
50
0
Survival (%)
96 H
21 D
96 H +21 D
Control
Glyphosate [1.8 kg.ha-1]
Glyphosate [2.5 kg.ha-1]