DAILY RHYTHMS OF TOXICITY AND EFFECTIVENESS OF ANAESTHETICS (MS222 AND EUGENOL) IN ZEBRAFISH (*Danio rerio*)

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ABSTRACT

Although the chronotoxicity of xenobiotics is relatively well known in mammals, the existence of daily rhythms of drug toxicity and effectiveness in fish have been neglected to date. The aim of this research was to investigate the influence of the time (middle of the light phase, ML *vs* middle of the dark phase, MD) of exposure to two anesthetics (MS-222 or clove oil) commonly used in fish on the median lethal concentration (LC50) and swimming activity of zebrafish (*Danio rerio*). To this end, adult zebrafish were kept under a 12:12h LD cycle and exposed to different concentrations of anesthetic for 15 minutes at ML or MD. The LC 50 calculations were performed using the Spearman-Karber program, while swimming activity was video recorded and analyzed with specialized software. In the trials zebrafish exhibited a mostly diurnal activity pattern (77.9% of activity occurring during daytime). The acute toxicity and mortality caused by MS-222 and eugenol varied with the time of exposure. In the case of MS-222, the LC50 was 170. 6 ± 7.4 mg/L in fish exposed at ML, and 215.6 ± 3.9 mg/L at MD while the LC50 of eugenol was 70.3 ± 3.1 mg/L at ML, and 104.9 ± 5.4 mg/L at MD. Exposure to sublethal concentrations of MS-222 and eugenol also altered the swimming patterns of zebrafish in a different manner depending on the time of exposure. Thus, the time required for decreasing swimming activity during exposure to anaesthetics was shorter at ML than at MD whereas the recovery period was longer during the day. In conclusion, these results revealed that the toxicity and effectiveness of both anesthetics is highest during daytime, coinciding with the active phase of fish and suggesting a link between the daily rhythms of behaviour and toxicity.

Keywords: chronotoxicity, anaesthetics, eugenol, MS-222, zebrafish, daily rhythms, swimming activity.

INTRODUCTION

The physiology and behaviour of most organisms show rhythms that are synchronised to periodic environmental changes imposed by the earth's rotation. As part of the rhythmicity in physiology, drug efficacy and toxicity may also vary with time (reviewed in Paschos *et al.*, 2010). Chronotoxicology studies the existence of variations in the presence and severity of adverse effects when drugs are administered to an organism at different times of the day (reviewed in Bruguerolle, 1998). Over the past four decades, daily variations in drug absorption, distribution, metabolism and excretion have been extensively reported in mammals and later reviewed by several authors (Cambar and Pons, 1997; Lemmer, 1989, 1996; Reinberg, 1991; Smolensky and Peppas, 2007). However, the chronobiology of xenobiotics and administration-time differences in the effectiveness or toxicity of drugs are unknown to most investigators working with fish.

Some research practices in fish result in exposure of the experimental animals to a variety of stressors (Barton, 2000), causing physiological and behavioural alterations which may lead to unsatisfactory results and raising concerns about fish welfare. Anesthetics are often used in fish to reduce the stress intensity of these activities. As the concentration required to induce the desired stage of anaesthesia varies depending on the anaesthetic (Roubach and Gomes, 2001), toxicology tests need to be performed to establish acceptable limits and to determine the toxic effects of the chemical to which the fish will be exposed. In addition, since the adverse affects of chemicals vary with the time of administration (Rebuelto *et al*., 2002), daily changes in the toxicity and effectiveness of anaesthetics should also be investigated.

Anaesthetics such as tricaine methane sulfonate (MS-222) (Fig.1A), quinaldine sulfate, benzocaine, clove oil (Fig. 1B) and phenoxyethanol are commonly used in fish

farms (reviewed in Burka *et al.*, 1997; reviewed in Ross & Ross, 1999; King *et al.*, 2005). Among these, only MS-222 is licensed for its use in food fish in the United States and the United Kingdom (Velísek *et al.*, 2009) and so it is frequently used in fish as both sedative and euthanizing agent (Ackerman *et al.*, 2005). However, after being exposed to MS-222, fish cannot be consumed until 21 days have passed (Meinertz *et al.*, 1999). On the other hand, the use of anaesthetics from natural sources, such as clove oil, has been reported to offer several advantages (Grush *et al.*, 2004; King *et al*., 2005; Velisek *et al.,* 2005; Velisek *et al.,* 2006; Weber *et al.*, 2009). Clove oil is a natural product of low cost and high availability (King *et al*., 2005; Soto and Burhanuddin, 1995). Furthermore, eugenol (the main component of clove oil) has a wide safety margin for fish, is not toxic to the operator (Keene *et al.*, 1998) and is rapidly metabolised and excreted (Wagner *et al.*, 2002). Both anaesthetics (MS-222 and eugenol) cause hypoxemia, hypercapnia, respiratory acidosis and hyperglycemia in fish. However, eugenol induces anaesthesia quicker; the recovery period is longer and can produce ventilatory failure rapidly when using high concentrations (Sladky *et al.*, 2001).

The zebrafish (*Danio rerio* Hamilton) is one of the leading models of vertebrate organisms in biomedical research (Dooley and Zon, 2000; reviewed in Shin and Fishman, 2002), and is particularly used as a model in human disease investigations (Berghmans *et al.*, 2005; Guyon *et al.*, 2006) and for screening therapeutic drugs (reviewed in Rubinstein, 2003, 2006). This species has a number of attributes that make it particularly appropriate for experimental manipulation: it is a small but robust fish, and a large number can easily be maintained in the laboratory at low cost. Moreover, the genetic and embryological manipulation of zebrafish is easier than in mammals like mice, where such procedures are more complicated and expensive. This fish species is also widely used in other sorts of investigation, such as toxicology (reviewed in Lele &

Krone, 1996; Scalzo & Levin, 2004) or chronobiology studies, where it provides a useful tool to establish the molecular bases of the vertebrates circadian clock (Cahill 2002), and being a model for research on fish behavioural rhythms (Hurd and Cahill, 2002).

This study aims to determine the existence of daily rhythms in (1) the toxicity of MS-222 and eugenol, for which the LC50 at mid-light (ML) and mid-dark (MD) were calculated, and (2) the sublethal effect of both anaesthetics at ML or MD on the swimming activity of zebrafish.

MATERIAL AND METHODS

Animals housing

A total of 2040 mixed sex adult zebrafish of 0.35 ± 0.04 g body weight and 3.68 ± 0.07 cm body length were obtained from a retail store (Jumipez SL, Murcia, Spain). The fish were housed in 60 L aquaria (2 fish/L) in which water was recirculated by a filter pump (Eheim, Germany) and temperature was controlled (27±1°C) by a heater (150W). The aquaria were placed inside an isolated chamber (chronolab) with a photoperiod of 12 h light: 12 h darkness (LD). Light was provided by fluorescent tubes (F15W/GRO, Sylvania Gro-Lux, Germany) and fish were fed once a day "*ad libitum*" at random times during the daytime, with a commercial dry flake food (Tropical NUTRON Hi-Fi). The daily pattern of locomotor activity of zebrafish was recorded by means of infrared photocells (Omron, model E3S-AD62, Japan) connected to a USB data-acquisition board (Measurement Computing, USA) connected to a computer. Animals were acclimated to laboratory conditions for at least ten days before the beginning of the experiments and fasted for 24 hours before the assays. The experiments performed in the present research followed Spanish legislation on Animal Welfare and Laboratory

Practices and was approved by both the National Committee on Animal Welfare and the Bioethics Committee of the University of Murcia. In addition, the experimental design and methodology followed in this investigation were in accordance with the international ethical standards of the journal (Portaluppi *et al*., 2008).

Toxicity tests: mortality dose-response curve and LC50

To determine the LC50 at both ML and MD, fish were exposed to 7 or 10 different concentrations of MS-222 (0.612-0.918 mM=160-240 mg/L) and clove oil (0.274-1.218 mM=45-200 mg/L), respectively. For each concentration, 6 glass bottles containing 8 fish were filled with 0.6 L of MS-222/clove oil solution. Constant oxygenation was provided and throughout the experiments the glass containers were kept within a 100 L tank in which water temperature was controlled by a heater (27±1°C). The anaesthetic solutions were prepared immediately before the assays. To this end, an MS-222 stock solution was prepared (3.827 mM=1g/L) whereas eugenol was previously diluted in ethanol (1 eugenol: 9 ethanol) before being dissolved in water (Cooke *et al*., 2004). Nevertheless, a control test only with ethanol was not carried out in the present experiments. To determine day-night variations in LC50, the exposure to different concentrations of anesthetics were performed at both ML and MD. Each exposure was carried out for 15 minutes, then the anaesthetics were removed and the fish were transferred to other aquaria containing clean water to allow their recovery. After 1 h the mortality rate (%) was recorded.

Effect of sublethal concentrations on fish activity.

In this experiment zebrafish were exposed at both ML and MD to 0.229 mM (60 mg/L) MS-222 or 0.274 mM (45 mg/L) clove oil. For each assay, 7 independent replicates were used. To evaluate the effect of these anaesthetic concentrations on zebrafish activity, the experimental animals were video-recorded for 30 min before the

assay and 15 min during MS-222/clove oil exposure. Then, the anesthetic was removed, clean water was provided and swimming activity was recorded for an additional hour to monitor the recovery period. To record swimming activity, fish were placed inside an 8 L aquarium divided into seven compartments, where the anaesthetics exposure was carried out. An individual fish was placed in each compartment. During the experiment, temperature was kept constant (27±1°C). During the day, lighting was provided by fluorescent tubes (Grolux, 18W, Germany), whereas at night infrared lamps were used to allow video-filming, although this illumination was not detected by the experimental fish. Video-recording was performed with a digital video camera (SONY, Handycam, DCR-SR55E) equipped with "Nightshot plus" function to record in the absence of visible light.

Data analysis

The locomotor activity displayed by fish before the experiments was recorded by infrared photocells (OMRON 3S-AD62, Japan) placed in the aquaria and these records were analysed by a Chronobiology software (El Temps v. 1, 179, Dr. Díez-Noguera, Barcelona).

Mortality results were fitted with a three parameters logistic model using the least squares method (Sigmaplot Ver. 10, Systat Software, Inc, USA) (Guilhermino *et al*., 1997; Isnard *et al*., 2001), based on the logistic model:

$$Y = \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

where "Y" is the % mortality after exposure to a concentration "x" of anesthetic for 15 min, "a" represents the function maximum value, "b" is a measure of the

steepness of the rising portion of the curve and "x_o" the concentration at which the mortality rate is 50% (median lethal concentration "LC50").

Furthermore, a t-test was performed ($p<0.05$) to check the existence of significant differences in the mortality rate for each concentration at ML and MD. Prior to this the percentages of mortality were arcsine transformed.

The LC50 for MS-222 and eugenol at ML and MD was calculated by the trimmed Spearman-Karber method, with a confidence interval of 95%. To determine the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC), the mortality data were firstly normalised by arcsin transformation. Then, a one-way ANOVA followed by a Dunnett test was performed, with $P < 0.05$ taken as the statistically significant threshold (Isnard *et al.*, 2001).

The video analysis was performed using specialised software, Fish Tracker (Sánchez *et al.* 2010). The software tracked each fish from the videos and provided their position (X,Y) every second. The method consists of the following four main steps: image acquisition, video stabilization, image segmentation and tracking technique. This tool allowed the quantification of fish activity every second and generated a file that was exported to Excel for further analysis and graphics creation.

To calculate the induction time of anaesthesia and recovery after the anaesthetics exposure, the mean activity every minute was calculated and compared to the mean activity of fish before the exposure. To this end, a paired-samples t-test was performed ($p < 0.05$).

RESULTS

Daily activity rhythms

Locomotor activity was registered to test the light entrainment of the daily behavioral rhythms of zebrafish before the exposure to anesthetics. Under a 12h:12h

light-dark (LD) cycle, zebrafish showed a mostly diurnal pattern, with 77.9% of activity occurring during daytime (Fig. 2A). Moreover, the activity levels were higher during the first half of the photophase and sharply decreased once the lights were off (Fig. 2B).

Dose-response curves and LC50

The mortality data showed a good fit to the three parameter logistic model (Table I), which is routinely used in toxicology studies. The lethal effect of both anaesthetics differed between ML and MD, the mortality rate being higher at ML than at MD for most of the tested concentrations (t-test, $p<0.05$) (Fig. 3).

For MS-222, the LC50 at ML was 0.172 mM (45 mg/L) lower than at MD, whereas the NOEC and the LOEC were 0.153 mM (40 mg/L) and 0.192 mM (50 mg/L) lower, respectively (Table II). As regards eugenol, the LC50 at MD was around 0.211 mM (35 mg/L) higher than during the day. In addition, both the NOEC and LOEC were 0.06 mM (10 mg/L) lower at ML than at MD (Table II). Therefore, depending on the time of the day (ML or MD), the LC50, NOEC and LOEC differed, being higher during the night.

Sublethal effects of eugenol and MS-222

In addition to acute toxicity (mortality), the effectiveness of both anesthetics at sublethal concentrations also differed depending on the time of exposure.

During exposure to MS-222 at ML, shortly after adding the anesthetic to the water, fish tended to move towards the water surface and increased their swimming activity (figure 4 A, C). Such a response lasted for approximately 4 minutes, and was followed by a marked decrease in activity as fish went down to the bottom of the aquarium. During exposure at MD, however, fish showed no initial increase in activity, although they, too, tended to swim towards the water surface shortly after adding the

anesthetic (figure 4B, D). Immediately after MS-222 was added to the aquarium at ML fish significantly increased their activity, whereas at MD this increase was not observed. After 5 min of exposure the fish activity decreased significantly at both ML and MD (paired-samples t-test, $p<0.05$), although the activity levels were much lower at ML, compare to basal levels, than at MD. As for the recovery period, at both ML and MD fish did not reach basal levels of activity, not even at the end of the recording (paired-samples t-test, $p<0.05$).

In the case of eugenol, at ML a marked decrease in swimming activity as fish moved to the bottom was observed after 1 min of exposure (paired-samples t-test, $p<0.05$) (Figure 5 A, C). However, at MD fish did not reduce their activity or their vertical position in the aquarium (Fig.5B, D). Recovery after exposure at ML occurred after 4min (paired-samples t-test, $p<0.05$).

DISCUSSION

Our results revealed that both the toxicity and effectiveness of two common anesthetics used in fish had strikingly different effects, depending on the time of exposure: a given concentration of MS-222 (0.727 mM=190mg/L) killed 82% of fish at ML, but only 14% at MD, while eugenol (0.487 mM=80mg/L) killed 68% at ML and 22% at MD (Figure 2 and 3). Similarly, the LC50 for eugenol was 21% (MS-222) and 33% lower at ML than at MD. As regards the NOEC and LOEC, for MS-222 these concentrations were 20% and 23% lower at ML. These findings are in accordance with a recent study in gilthead seabream, which showed the existence of daily variations in the toxicity and effectiveness of MS-222 in this marine species (Vera *et al.*, 2010).

Despite the lack of chronotoxicity studies in fish, there are many examples of acute toxicity rhythms in mammals. For instance, Skubitz *et al.* (1986) reported that

75% of mice injected with a given dose of antibiotics at the beginning of the day survived in comparison to only 13% of those treated early at night. Zhang *et al*. (1993) also reported that mortality caused by drugs used in chemotherapy (AZT) was significantly higher in rats injected in the middle of the night. In general, although there are many rhythmic factors involved in the chronobiology of xenobiotics, lethality appears to be higher during the active phase of the animal (at night in nocturnal rodents). As regards the dosing-time effects of anaesthetics, in mice, the hypnotic duration of GABAergic drugs (pentobarbital, propofol, midazolam and ethanol) was larger in the early active phase (Sato *et al.*, 2005). On the contrary, some chronopharmacological studies in rodents and rabbits have shown that the effect of general anaesthetics is higher during the rest period (reviewed in Dispersyn *et al*., 2008). In both cases, the authors have linked these findings to circadian variations in the post-synaptic GABA receptor. In the case of zebrafish, our results are consistent with the hypothesis linking daily patterns of behaviour and toxicity, since toxicity of MS-222 and eugenol was highest in the middle of the day, coinciding with the diurnal activity rhythm displayed by zebrafish, which could be related to a higher anaesthetic absorption through the gills at ML. Actually, previous studies in sea bream have also found a relationship between diurnal activity and higher toxicity during the day (Vera *et al.*, 2010). Zebrafish is considered "day-active", as reported by López-Olmeda *et al*. (2006, 2009) and Blanco-Vives and Sánchez-Vázquez (2009), who showed that this species had diurnal locomotor activity and spawning rhythms under different environmental conditions (light and temperature cycles), in agreement with previous studies by Hurd *et al*. (1998), in which a strong confinement of zebrafish activity to the light phase was observed.

The mechanisms underlying toxicity rhythms are not fully understood, although drug pharmacokinetics is closely related to circadian variations in physiology driven by clock genes (reviewed in Paschos, 2010). In fish, circadian rhythms in many behavioural and physiological functions have been reported (reviewed in Kulczykowska *et al.*, 2010). Zebrafish has been used as a model species to study the vertebrate molecular clock system (Hirayama *et al.*, 2005). Moreover, recent research has found that zebrafish activity rhythms and clock gene expression can be modified by restricted feeding schedules (Sánchez and Sánchez-Vázquez, 2009; Sánchez *et al*., 2009). However, further research is required to understand how the circadian clock controls gastrointestinal, hepatic and renal processes, which, in turn, affects drug pharmacodynamics (absorption, distribution, metabolism and excretion).

The effects of anesthetics depend on a number of environmental factors such as anesthetic concentration, water temperature, dissolved oxygen, pH, etc. (reviewed in Burka *et al*., 1997; Hamackova *et al*., 2004; Myszkowski *et al*., 2003; reviewed in Ross & Ross, 1999; Soto and Burhanuddin, 1995; Weyl *et al.*, 1996). The response to anesthetics may also be influenced by biological factors such as the size and the species (Rombough, 2007), stress, individual fitness and skin thickness (Walsh and Pease, 2002). In the case of zebrafish, the LC50 of eugenol in our trials varied from 0.426 mM (70 mg/L) (ML) to 0.639 mM (105mg/L) (MD), which is higher than that found by Grush et al. (2004) and Mácová et al. (2008) for the same species: 0.128 and 0.116 mM (21 and 19 mg/L), respectively. Such differences between authors are most likely due to the different durations of exposure (96h *vs* 15min) to eugenol, a factor which is known to affect drastically the LC50 (Velisek *et al.*, 2006).

Because of the widespread circadian rhythms in physiology, fish might be expected to respond differently to anesthetics depending on their internal physiological

state. For instance, fish during the day may be more stressed, coinciding with the peak in cortisol levels, as observed in rainbow trout (Reddy and Leatherland, 2003). In this species, Holloway *et al.* (2004) evaluated the effect of eugenol and MS-222, finding a significant increase in hematocrit, plasma cortisol, glucose, tri-iodothyronine and thyroxine, accompanied by a decrease in potassium, while total protein and sodium levels remained unchanged. In the case of zebrafish, the higher toxicity found at ML in our trials may be related to increased metabolic activity during the day, which requires greater energy expenditure and might also increase the anaesthetic absorption through the gills. Fish exposed to anesthetics react by consuming more energy, which drives the response of excess catecholamine and cortisol secretion (Pickering, 1993). This overload may cause excessive stress in fish, leading to death. However, during the resting phase (MD in zebrafish) the metabolism is lower and the organism's efforts to maintain homeostasis is smaller, and so the stress and mortality are diminished. Furthermore, the increased metabolism during the active phase does not seem to be related with a higher activity of detoxifying enzymes, such as Cytochrome P450 in mice (Sato et al., 2005) or estearase and GST in *Drosophila* (Hooven *et al.*, 2009).

Treatment with excessive anaesthesia is very stressful to fish, causing abnormal behaviour, metabolic rate, oxygen consumption, blood pressure and blood physiological responses (Park *et al.* 2008). Moreover, these side-effects can last for hours after fish recover from anaesthesia (reviewed in Summerfelt and Smith, 1990). In our trial, immediately after MS-222 was added to the aquaria, zebrafish dramatically increased their activity and swam towards the water surface, particularly at ML (Figure 4). Changes in behaviour have previously been reported in zebrafish exposed to anaesthetics (Grush *et al.*, 2004), as well as in seabream *Sparus aurata* and seabass *Dicentrarchus labrax* (Mylonas *et al*., 2005), although the effect of time of day was not

considered. In experiment 2, the induction time of anaesthesia in zebrafish with eugenol was shorter at ML than at MD, which is in accordance with the toxicity results (exp. 1). Actually, fish exposed to eugenol at MD did not show any effect, and activity levels remained comparable to those displayed before the anaesthetic exposure. However, the same eugenol concentration (0.274 mM=45 mg/L) induced anaesthesia at ML in 1 min. The recovery times also differed between ML and MD, being longer during the day. These differences could also be explained by the fact that, for a given anaesthetic concentration, the toxicity (and therefore, effectiveness) would be higher at ML than at MD. Fish exposed to MS-222 showed lower activity levels after anesthesia than those treated with eugenol, suggesting that for zebrafish, MS-222 could be more toxic than eugenol, which contradicts other studies reporting that the recovery time is longer when fish are exposed to eugenol than to other anesthetics (reviewed in Svobodova *et al.*, 2007). However, other factors, such as differences in the physiological status of fish should also be considered (Weber *et al.*, 2009).

In conclusion, the present results showed that the lethality of MS-222 and eugenol, and their effectiveness to induce anaesthesia in zebrafish, depended on the time of day at which the exposure was carried out. These findings should be used to synchronize behavioural and physiological rhythms and the dosage of medication to increase efficacy and safety. Although the importance of the time of drug administration has been well established in mammals, husbandry and/or experimental protocols using anaesthesia in fish should also take into account the existence of daily rhythms at which the effectiveness is optimum and the toxicity is lowest.

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FIGURE LEGENDS

Figure 1. Chemical structure of MS-222 and eugenol.

Figure 2. Representative actogram of locomotor activity from one aquarium (A) and average diel profile (B) from three independent replicates, each one containing 25 zebrafish reared under a 12:12 h LD cycle and showing a diurnal pattern. The white and black bars at the top of each graph indicate the light and dark periods, respectively. The actogram is double-plotted for better visualization. Data in (B) represent the mean (continuous line) + SEM (vertical lines) during a sixteen day period.

Figure 3. Zebrafish mortality at different MS-222 (A) and eugenol (B) concentrations after 15 min of exposure at ML (white circles) or MD (black circles). The continuous and dotted lines indicate the best fit to the logistic function at ML and MD, respectively. Each point represents the mean mortality rate in 6 independent replicates (8 zebrafish/replicate). Asterisks above the white circles indicate statistical differences between the mortality rate at ML and MD for a given concentration (t-test, $p<0.05$).

Figure 4. Zebrafish mean position (cm) (A, B) and swimming activity (cm/s) (C, D) in the water column prior, during and after a 15 min MS-222 exposure (n=7 fish). Video-recording were performed at ML and MD. MS-222 exposure period is indicated by a grey rectangle. Black lines represent fish activity every second whereas the white line represents the mobile average of fish activity every 15 seconds. The blank gaps correspond to the few minutes needed to remove the anaesthetic from the aquarium and provide clean water.

Figure 5. Zebrafish position (cm) (A, B) and swimming activity (cm/s) (C, D) in the water column prior, during and after a 15 min eugenol exposure at ML and MD (n=7 fish). Eugenol exposure period is indicated by a grey rectangle. Graph definitions as given for figure 4.

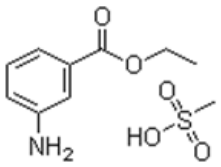
Table I. Adjr^2 and parameter estimates ± SEM of the three parameter logistic model for % mortality of zebrafish exposed to MS-222 and eugenol at ML and MD.

	Adjr^2		**a**		**b**		**X_0**	
	ML	MD	ML	MD	ML	MD	ML	MD
MS-222	0.9591	0.9416	99.82±6.16	211.68±246.81	-14.40±3.61	-10.64±4.50	0.65±0.01	0.94±0.19
Eugenol	0.9683	0.9704	98.60±4.96	106.72±8.50	-5.39±0.87	-4.45±0.72	0.42±0.02	0.66±0.03

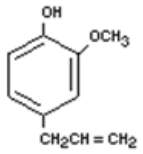
Table II. MS-222 concentrations (mM) corresponding to the LC50, NOEC and LOEC for zebrafish exposed to MS-222 and eugenol at ML and MD. LC50 was calculated by the trimmed Spearman-Karber method, the NOEC and LOEC with ANOVA I followed by a Dunnett test ($p<0.05$).

	LC50 (95% CL)		**NOEC**		**LOEC**	
	ML	MD	ML	MD	ML	MD
MS-222	0.653±0.03	0.825±0.01	0.612	0.765	0.650	0.842
Eugenol	0.426±0.02	0.639±0.03	0.305	0.365	0.365	0.426

Figure 1



MS-222



Eugenol

Figure 2

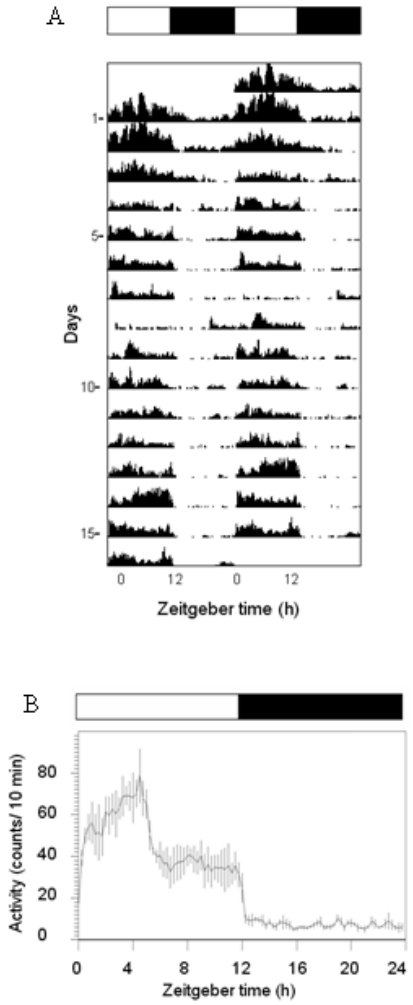

A
1
5
10
15
Days
0
12
0
12
Zeitgeber time (h)
B
Activity (counts/ 10 min)
80
60
40
20
0
0
4
8
12
16
20
24
Zeitgeber time (h)

Figure 3

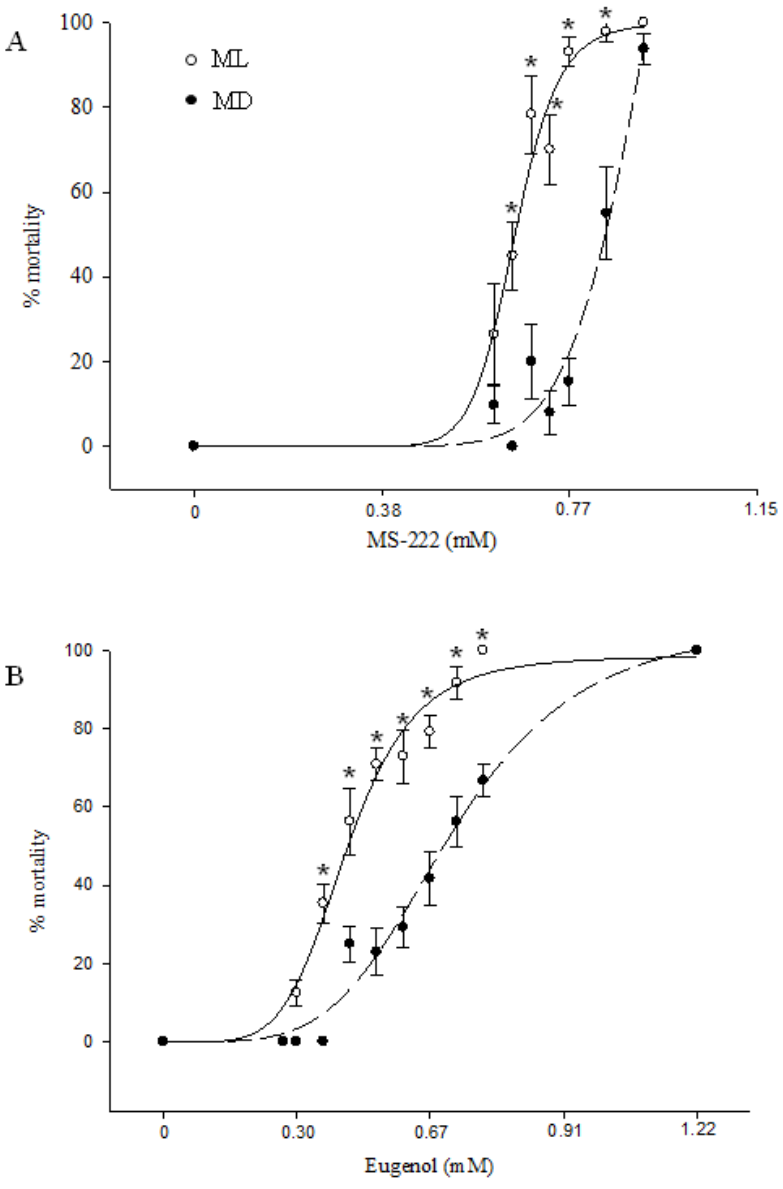

A
ML
MD
% mortality
100
80
60
40
20
0
0
0.38
0.77
1.15
MS-222 (mM)
B
% mortality
100
80
60
40
20
0
0
0.30
0.67
0.91
1.22
Eugenol (mM)

Figure 4

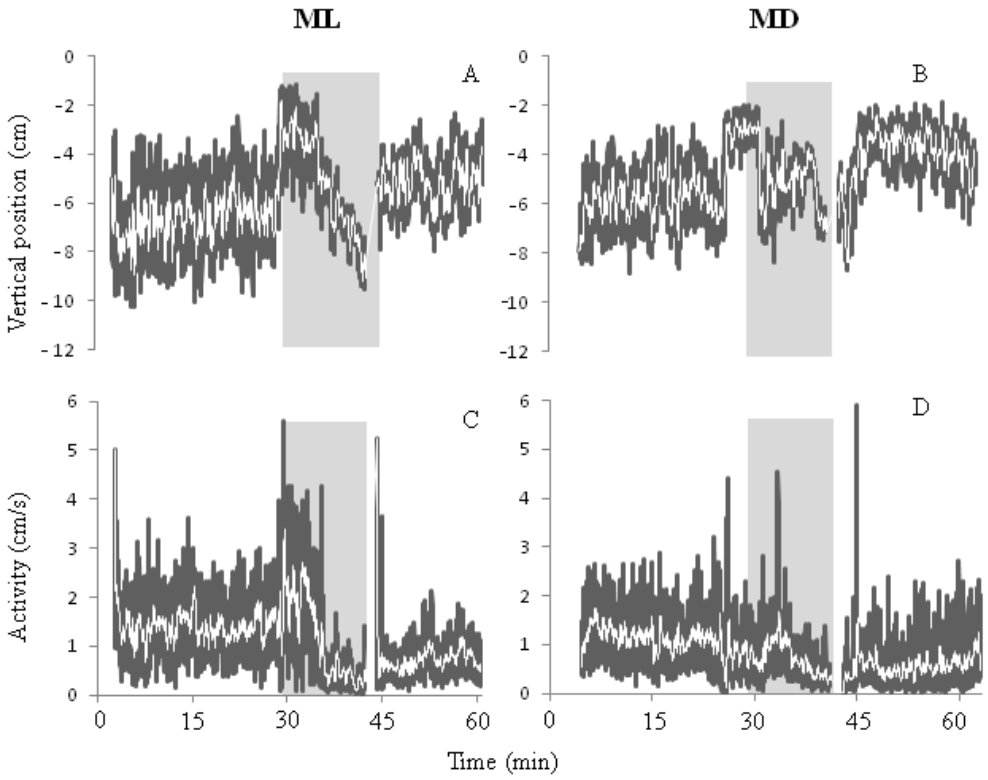
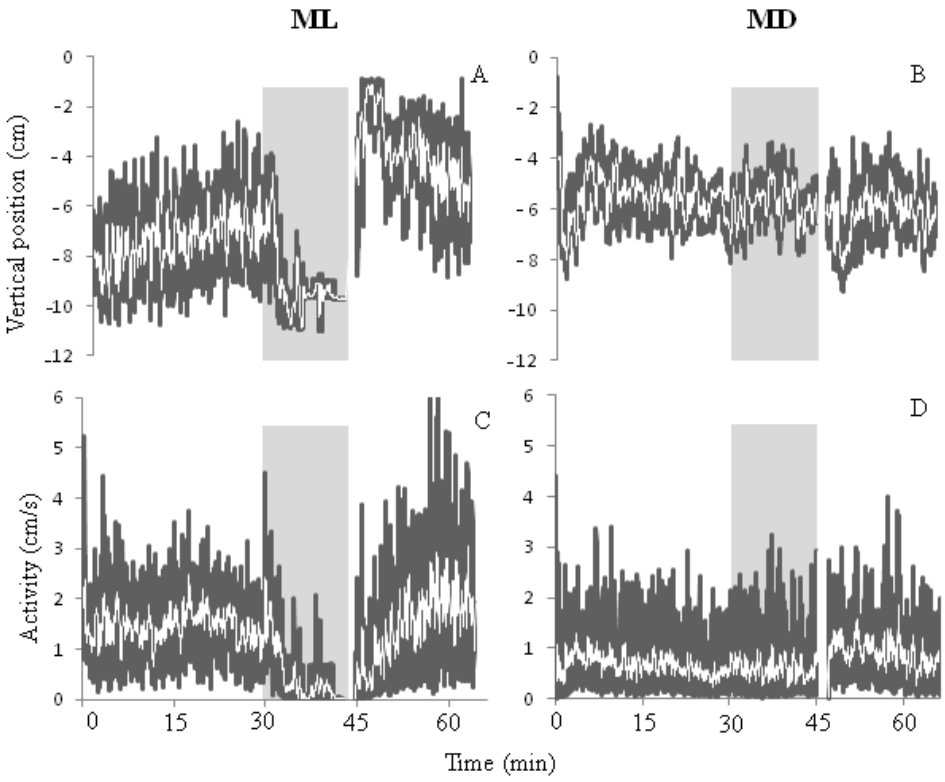

ML
MD
A
B
C
D
Vertical position (cm)
Activity (cm/s)
Time (min)
0
-2
-4
-6
-8
-10
-12
1
2
3
4
5
6
15
30
45
60

Figure 5


ML
MD
A
B
C
D
Vertical position (cm)
Activity (cm/s)
Time (min)
0
-2
-4
-6
-8
-10
-12
1
2
3
4
5
6
15
30
45
60