MS-222 TOXICITY IN JUVENILE SEABREAM CORRELATES WITH DIURNAL ACTIVITY, AS MEASURED BY A NOVEL VIDEO-TRACKING METHOD

Vera, L.M.[a]; Ros-Sánchez, G.[b]; García-Mateos, G.[b]; Sánchez-Vázquez, F.J.[a]

[a] Department of Physiology, Faculty of Biology, University of Murcia, 30100, Spain.

[b] Faculty of Computer Science, University of Murcia, 30100, Spain.

Keywords: *Sparus aurata*, video-tracking, swimming activity, chronotoxicity, MS-222, anaesthesia.

Corresponding author:
Luisa M. Vera
Department of Phyisiology
Faculty of Biology
Campus of Espinardo
30100 – Murcia (Spain)
Tel. +34 868884931
Fax +34 868883963
E-mail: lmvera@um.es

ABSTRACT

Fish are frequently exposed to anaesthetics since their use is necessary in several aquaculture procedures. The aim of this study was to investigate the existence of day-night differences in toxicity and effectiveness of a common fish anaesthetic (MS-222) in juvenile gilthead seabream (*Sparus aurata*), determining the induction time of anaesthesia and subsequent recovery by a novel video-recording system. Our results showed that MS-222 toxicity was significantly higher at ML (mid-light) (LC50=85.5 mg/L) than at MD (mid-darkness) (LC50=107.6 mg/L) (trimmed Spearman-Karber method). In addition, when fish were exposed to a sublethal but effective MS-222 concentration (65 mg/L), 7 min passed before 50% a reduction in swimming activity was observed at ML compared to the 9 min required at MD. As regards recovery, fish showed activity levels similar to basal levels 10 min after MS-222 removal at ML, but only 6 min at MD. These results indicated that both toxicity and effectiveness were higher during the day than at night, coinciding with the diurnal activity pattern displayed by seabream, which should be taken into account when designing and applying daily protocols for anaesthesia in aquaculture.

INTRODUCTION

The development of accurate methods to quantify behavioural changes in fish is of great interest in order to characterise the sublethal effects caused by exposure to xenobiotics. Besides traditional methodologies applied in ecotoxicology studies, there is a need to find systems which link toxicology data with swimming activity alterations (Vogl et al., 1999). Although several commercial tools are available for performing behavioural analysis, these expensive packages are designed for terrestrial animals and have serious constraints when used in fish, which resulted in the design of costumised alternatives by various research groups (Kane et al., 2004, Kato et al. 1996, Miller at al., 1982).

Aquaculture fish species are exposed to several stressful conditions during handling and transportation (Gatica et al., 2010), as well as to a great number of xenobiotic substances (e.g. antibiotics, disinfectants, vaccinations and anaesthetics). The toxicity and effect of anaesthetics is of special interest since they are frequently used in research and routine aquaculture procedures to immobilise fish and minimise their stress responses (King et al., 2005). These operations include the grading of fish, measurement, sampling, labelling, injection of vaccines, medical treatment and spawning induction, among others (Park et al., 2008; Weber et al., 2009). An appropriate anaesthetic for aquatic organisms should satisfy the following requirements: short induction and recovery time, non-toxicity to fish and humans, no lasting physiological effects, rapid elimination from exposed animals, high solubility in water, high chemical stability, non-foaming and cost effective (King et al., 2005; Shoettger and Julin, 1967). Tricaine methanesulphonate (MS-222) is a commonly used anaesthetic for fish and the only one approved by the US Food and Drug Administration for use on aquatic organisms (Barreto et al., 2007). MS-222 is a benzocaine analogue, but its

higher solubility in water makes it a better option for fish anaesthesia (Ortuño et al., 2002). Furthermore, recent investigations in Nile tilapia (*Oreochromis niloticus*) have demonstrated the absence of genotoxic activity induced by MS-222, under both *in vivo* and *in vitro* conditions (Barreto et al., 2007). In gilthead seabream, MS-222 did not depress humoral or cellular immune responses (Ortuño et al., 2002). However, other studies have reported higher plasma cortisol and glucose levels after exposing chinook salmon (*Onchorhynchus tshawytscha*) to tricaine (Cho and Heath, 2000). The toxicity of MS-222 has been reported to decrease with fish age in zebrafish (Rombough, 2007). The influence of other environmental conditions (temperature, body weight, pH, etc.) on the toxicity of anaesthetics has also been investigated (Park et al., 2008; Zahl et al., 2009).

Most biological functions of animals show circadian rhythmicity (period ~24 h), including those influencing pharmacokinetic parameters (Bruguerolle et al., 2008). Several studies in mammals have revealed that the pharmacokinetics of xenobiotics may differ depending on the timing of application, which might be caused by the rhythms of absorption, distribution, metabolism and elimination of these substances (Lemmer, 1996; Reinberg, 1991). However, in fish, as opposed to mammals, there is a lack of research dealing with the differential toxicity of xenobiotics depending upon the time of day.

Therefore, the aim of the present paper was to investigate the existence of day-night differences in (1) lethal toxicity of MS-222 in juvenile gilthead seabream and (2) to develop novel video-recording and fish-tracking software to determine daily variations of MS-222 sublethal effects on seabream swimming activity and vertical position in the water column.

MATERIALS & METHODS

1. *Animals and housing*

A total of 430 juvenile gilthead seabream (3.7 ± 0.1 g weight and 6.6 ± 0.1 cm length) were used for the present experiments. Fish were obtained from Predomar S.L. (Carboneras, Spain) and housed at the marine fish facilities of the University of Murcia at ENA ("Estación Naval de la Algameca", Cartagena, Spain), in 150 L glass fibre tanks in a semi-open system. The photoperiod was 12 h L (light): 12 h D (darkness) and the temperature was maintained at around 23 ºC throughout the study. The animals were fed once a day at random times to avoid feeding synchronization of both activity and toxicity rhythms. The fish used for each experiment were not fed during the 24 h prior to each assay.

2. *Experimental design*

Before beginning the chronotoxicity experiments, the existence of a daily activity rhythm and its synchronisation to the light-dark (LD) cycle was checked. To this end, locomotor activity was recorded by an infrared photocell (E3Z-D67, OMRON, China) placed in each tank. The photocells were connected to a computer, and every time a fish interrupted the infrared light beam it produced an output signal that was recorded and stored in 10 minute bins using specialized software (DIO98USB, University of Murcia, Spain).

2.1. MS-222 toxicity: mortality test

In the first experiment, the mortality caused by MS-222 at ML (mid light) and MD (mid darkness) was investigated and the LC50 was assessed. To this end, fish were exposed to six different anaesthetic concentrations (60, 80, 90, 100, 120 and 200 mg/L). Anaesthetic exposure lasted 15 min in 6 L plastic boxes containing sea water plus the corresponding amount of MS-222. A control test without anaesthetic was also

performed. Water was aerated during the entire assay and temperature was kept constant. For each concentration, 6 boxes were used, each one containing 10 fish (total number of fish per concentration = 60). After exposure to MS-222, fish were transferred to similar plastic boxes which contained only clean water, to monitor their recovery, and after 30 min the mortality rate was recorded. At MD, the tests were performed in darkness with a dim red light to record fish mortalities.

2.2. Anaesthesia effectiveness: activity recording

In a second experiment, the time of induction to anaesthesia and recovery were investigated at ML and MD, using a sublethal concentration of MS-222 (65 mg/L), a sublethal concentration chosen based on the toxicity tests, in which the mortality rates were estimated by a logistic fit (at ML and MD) for MS-222 concentrations between 60 and 200 mg/L. The locomotor activity of fish prior, during and after MS-222 exposure was filmed to study the time of induction and recovery. For this, fish were transferred to a 30 L glass aquarium divided into 6 individual compartments with plastic separators. The fish activity was first filmed under control conditions, at both ML and MD, for comparative purposes. In the toxicity tests, activity was recorded during the 30 min prior to the assay, 15 min during MS222 exposure and 30 min after exposure to record the recovery phase, for which purpose the anaesthetic was washed off and clean sea water provided. Special care was taken to avoid exposing fish to air during water renewal. At ML, light was provided by a fluorescent lamp (F15W/GRO, Sylvania Gro-Lux, Germany), whereas at MD the aquarium was equipped with infrared LEDs (light emitting diodes) (monocolor diode, model L- 53F3BT, 5 mm), which were not detected by the seabream but which allowed video recording and further analysis to be made. Filming was carried out with a video camera (SONY, Handycam, DCR-SR55E) provided with a “Nightshot plus” function for night recording (Fig. 1).

3. *Data analysis*

The mortality data from ML and MD were fitted with a three parameter logistic model (Guilhermino et al., 1999; Isnard et al., 2001), using the least squares method (Sigmaplot Ver. 10, Systat Software, Inc, USA), 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 MS-222 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").

To calculate the LC50 of MS-222 at ML and MD, the trimmed Spearman-Karber method was applied, with lower and upper 95% confidence interval endpoints (Hamilton et al., 1977). To determine the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) a non-parametric statistical test was used: Jonckheere-Terpstra, with $P < 0.05$ taken as the statistically significant threshold (Isnard et al., 2001). The LC5 (concentration at which the mortality rate is 5%) is also indicative of the NOEC (Muller et al., 2008), and so its value at ML and MD was calculated from the regression model.

To analyse the locomotor activity videos, specialised software was developed and validated. This software, named Fish Tracker, offers an in-house alternative to other methods. 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:

1) Image acquisition**.** This step is responsible for acquiring images of *F* frames at regular intervals (normally 1 frame per second), thus permitting exhaustive analysis.

2) Video stabilization. This step, which is performed by template matching using correlation, corrects small displacements or vibrations of the camera, adapting each captured image with respect to a reference image.

3) Image segmentation. By using an adaptive background model, the scene is segmented into background and foreground, the latter representing moving objects. Each pixel is modeled by a mixture of Gaussian distributions.

4) Tracking technique. With a robust and lightweight tracker based on the median operator, this step determines the current position of each fish by its centre of mass.

At the end of the video analysis, the software generated a file that could be exported to Microsoft Excel for further analysis. In our experiments, a high success rate of 98% correctly tracked positions in daylight videos and 94.8% in night videos was obtained.

To study how activity changed under control conditions (ML & MD), the mean activity every 15 min was calculated and the existence of statistical differences between these means was checked by a GLM repeated measures ($p < 0.05$), in which the within-subjects factor was “time” (with 5 levels: 15, 30, 45, 60 and 75 min) and the between-subjects factor was “group” (ML/MD).

To calculate the recovery time after MS-222 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 ($p < 0.05$). All statistical tests were carried out with the SPSS v16.0 program (SPSS Inc., USA).

RESULTS

1. MS222 toxicity

The juvenile gilthead seabream showed a diurnal rhythm in their levels of activity, with the highest levels (76%) occurring during the photophase (Fig. 2). One hour before lights on, the fish increased their activity significantly with respect to the nocturnal levels, indicating their anticipation of the beginning of the photophase. Furthermore, the number of registers remained stable during the first part of the day but decreased gradually towards the end, anticipating the arrival of night (Fig. 2).

For all the MS-222 concentrations tested in this experiment, the mortality rate was always higher at ML than at MD (Table I). The mortality data showed a good fit to the three parameter logistic model (Table II), which is routinely used in toxicology studies. MS-222 toxicity was higher at ML, so that, depending on the time of the day (ML or MD) the LC50, NOEC, LOEC and LC5 differed, being higher during the night. Thus, the MS-222 LC50 at ML was 22 mg/L lower than at MD (trimmed Spearman-Karber method). As for the NOEC and LOEC, the MS-222 concentrations were higher at MD than at ML. NOEC was 20 mg/L lower at ML, whereas LOEC was 10 mg/L lower (Jonckheere-Terpstra test, $p<0.05$). Finally, the LC5 values calculated from the adjusted logistic model were in accordance with the NOEC obtained from the Jonckheere-Terpstra test, and the MS-222 concentration causing a lethal effect in 5% of the exposed fish was 26 mg/L higher at MD (Table III).

2. Anaesthesia effectiveness

Under control conditions, seabream activity was similar in both groups at the beginning of the recording. However, the evolution of activity significantly differed between groups: increasing during the ML assay and decreasing in MD (GLM repeated measures, $p < 0.05$) (Fig. 3). Fish position in the water column did not differ between ML and MD, the fish remaining in the middle most of the time.

When 65 mg/L of MS-222 was added to the experimental aquaria, both ML and MD fish reduced their activity but resumed normal activity levels when new clean water was provided (Fig. 4A, 4B). The fish position prior to the anaesthetic exposure was close to the middle of the water column, although slightly lower at ML (Fig. 4C). During the day, 5 min after the addition of MS-222, the fish moved to around 5 cm from the bottom of the aquarium and did not return to the middle until 10 min after the anaesthetic had been removed (Fig. 4C). However, at MD the fish only moved to the bottom after 12 min of exposure and returned to the middle position immediately after the anaesthetic was eliminated (Fig. 4D). The time of exposure needed to reduce fish activity to 50% of basal levels was 7 min at ML (Fig. 5A) and 9 min at MD (Fig. 5B). Moreover, while the reduction in activity was gradual at ML, two inflection points were observed at MD, the first 1 minute after the anaesthetic was added and the other 9 minutes later (Fig. 5B). As regards the recovery time, the ML seabream needed 10 min to display activity levels comparable to those registered before the addition of MS-222 (3.40 ± 0.20 cm/s) (Fig. 5A), but the MD fish only 6 min (2.55 ± 0.28 cm/s) (Fig. 5B) (paired-samples t-test, $p < 0.05$).

DISCUSSION

The present investigation in gilthead seabream showed that the newly developed system for recording and analysing fish activity, “Fish Tracker”, constitutes a suitable tool for studying the swimming activity of anaesthetised fish which revealed the existence of day-night differences in the effect of MS-222 exposure in this species.

The importance of water temperature, oxygen content, salinity or pH on anaesthesic effectiveness has been widely reported (Burka et al., 1997; Ross and Ross, 1999). The size and life cycle status of anaesthetised fish is also recognised as a factor

influencing the concentration of anaesthetic needed to induce anaesthesia within an acceptable time (from a welfare point of view) (Rombough, 2007). Recent studies have also focused on the pharmacokinetics, effectiveness and stress response to anaesthetics (Kiessling et al., 2009), which, in trout (*Onchorhynchus mykiss*) exposed to MS-222, seems to be reduced by water aeration (Conde-Sieira et al., 2009). However, the administration time factor has been neglected until now. To our knowledge, this is the first study focusing on the chronotoxicity of anaesthetics in fish. Our results showed that MS-222 toxicity and effectiveness in seabream was higher at ML than at MD and, consequently the time needed to induce anaesthesia by means of a sublethal concentration was shorter during the day, while the recovery time was longer.

Conventional toxicological studies involve the determination of LC50 (or LD50), NOEC, LOEC and EC_x for a given xenobiotic, while chronotoxicity investigations determine these parameters taking into account the existence of circadian changes in host tolerance (Dridi et al., 2005). In seabream, the MS-222 LC50 during the night was 22 mg/L higher than during the day. This difference might be the result of several factors, such as the anaesthetics absorption, distribution, excretion and metabolism (Hooven et al., 2009). Other authors have reported that the immediate cause of death in fish exposed to MS-222 is asphyxiation due to the gills ventilation blocking, which causes hypoxia and finally death (Cornish & Moon, 1986; Rombough, 2007; Soivo et al., 1977). However, when lethal concentrations are used but the gills are artificially ventilated, fish can be kept alive for a longer period (Brown, 1987). The rate of anaesthetics elimination during recovery also increases with artificial ventilation (Kiessling et al., 2009). The gilthead seabream used for our experiments showed a diurnal activity pattern and therefore anaesthetic absorption through the gills might have also been higher at ML. Thus, for a given concentration, toxicity would be greater

during the day since not only the swimming activity would be higher but also the respiration and metabolic activities, as observed in two fish species exposed to the pesticide lindane (Walton et al., 1997). Another effect of high concentrations of MS-222 seems to be heart failure, since this anaesthetic affects ion transport, blocking Na^+ and K^+ conductance (Frazier and Narahashi, 1975). Potential day/night differences in cell permeability might also have an influence, although this should be studied further and corroborated in sea bream.

The metabolism and elimination of xenobiotics are affected by circadian oscillations. In mice, Inoue et al. (1999) reported the existence of a circadian variation in detoxification systems, such as hepatic glutathion S-transferase (GST), which showed higher enzyme activity at ML and lower activity at MD, although the variation was sex-related and showed seasonal differences. A seasonal effect on the GST activity has also been described in several Brazilian fish (Da Rocha et al., 2009). In contrast, *Drosophila* GST activity remained constant throughout the 24h cycle, although there were daily changes in the expression of GST genes and in the enzyme activity of P450s and UGTs (5′-diphosphoglucosyltransferase) (Hooven et al., 2009). During evolution, the appearance of detoxifying rhythms (enzymes and metabolising genes) could be related to the existence of daily feeding rhythms, since several toxins may be ingested with food (Hooven et al., 2009; Shea et al., 2005). In the present study, fish were fed at random times but always during the photophase, which could have had an effect on the fact that toxicity was higher during the day. Indeed, previous investigations carried out in gilthead seabream have shown that both behavioural and physiological parameters can be food-entrained, including plasma cortisol and glucose, which are used as stress indicators (Sánchez et al., 2009). In seabream, cortisol and glucose display daily rhythms that are also dependent on the feeding behaviour of fish (López-Olmeda et al.,

2009). Taken altogether, these results suggest that fish exhibit different physiological status depending on the time of day, so that daily variations in xenobiotics toxicity might also be expected. Nevertheless, to ascertain the impact of fish behaviour and feeding time, further research should be carried out.

The fact that the induction time of anaesthesia in seabream was shorter at ML than at MD (exp. 2) is in accordance with the toxicity test results (exp. 1). Moreover, the induction of anaesthesia was gradual during the day, whereas at night a sharp decrease of activity was observed after 9 minutes of exposure to MS-222. On the other hand, the recovery times also showed marked differences: 6 min at MD and 10 min at ML. These day-night variations could also be due to the existence of diel changes in both the intake and detoxifying/excretion mechanisms. Our results highlighted the fact that dose-time considerations should be taken into account when designing anaesthesia protocols for use in the aquaculture industry, since MS-222 effectiveness in seabream substantially varied during course of the day. However, our results refer to juvenile fish and both the toxicity and effective concentrations of MS-222 might be different in adult individuals. Other authors have suggested that optimum anaesthetic concentrations should induce anaesthesia within 3 min and recover within 5 (Weber et al., 2009) or 10 (Park et al., 2008) minutes, depending on the source. In adult seabream, 50 mg/L of MS-222 caused a loss of sensation and equilibrium in less than 1 min, whereas recovery took more than an hour (Ortuño et al., 2002). However, in adult black seabass (*Centropristis striata*) the induction of anaesthesia with 70 mg/L of MS-222 took around 4 min and total recovery almost 3 min (King et al., 2005). And finally, juvenile chinook salmon (*Onchorhynchus tshawytscha*) exposed to 50 mg/L of MS-222 reached stage 5 of anaesthesia within 2 min (loss of reflex activity) (Cho and Heath, 2000). This wide variety of data supports the idea that anaesthesia protocols should be optimised

taking into account the species, life cycle status, weight and other environmental conditions, as well as the time of day, as shown in the present investigation.

In conclusion, "Fish Tracker" is a novel video-tracking system that could be used to quantify and characterise swimming activity of fish in a variety of environmental and/or experimental conditions, e.g. during exposure to toxic substances. Our findings revealed for the first time that when assessing the optimum concentrations to induce anaesthesia in aquaculture fish species the time of day factor should also be considered. Therefore, anaesthesia protocols should be designed as a function of (a) the time of day at which the effectiveness is optimum for the species of interest and (b) the time of day at which the toxicity is the lowest (better tolerance). Considering these dose-time issues would imply that husbandry and/or experimental protocols should be modified accordingly. In the case of diurnal sea bream, caution must be taken to anaesthetise fish during daytime, when toxicity of MS222 is highest.

ACKNOWLEDGEMENTS

The authors would like to thank Viviane Felizardo for helping during the toxicity assays, Ander Montoya for his support during fish transportation before the experiments and José Antonio Oliver for helping in daily fish management. Financial support was provided by the project AGL2007/66507/C02/02 from the Spanish Ministry of Education as well as by "Fundación Séneca" for the postdoctoral contract awarded to L.M. Vera. Part of these results was presented at the COST Action 867 "Welfish" (Iceland, June 2009). Thanks are also due to the Spanish Ministry of Defense for letting us use the ENA aquaculture facilities.

REFERENCES

Barreto, R. E., Gontijo, A. M. M. C., Alves-de-Lima, R. O., Raymundi, V. C., Pinhal, D., Reyes, V. A. V., Volpato, G. L., Salvadori, D. M. F., 2007. MS222 does not induce primary DNA damage in fish. Aquac. Int. 15, 163-168.

Brown, L.A., 1987. Recirculation anaesthesia for laboratory fish. Lab. Anim. 21, 210-215.

Bruguerolle, B., Boulamery, A., Simon, N., 2008. Biological rhythms: a neglected factor of variability in pharmacokinetic studies. J. Pharmac. Sci. 97, 1099-1108.

Burka F.J., Hammell, K.L., Horsberg, T.E., Johnson, G.R., Rainnie, D.J., Speare, D.J., 1997. Drugs in salmonid aquaculture – a review. J. Vet. Pharmacol. Ther. 20, 333-349.

Cho, G. K., Heath, D. D., 2000. Comparison of tricaine methanesulphonate (MS222) and clove oil anaesthesia effects on the physiology of juvenile chinook salmon *Oncorhynchus tshawytscha* (Walbaum). Aquac. Res. 31, 537-546.

Conde-Sieira, M., López-Patiño, M.A., Muñoz, J.L.P., Aguilar, A., Soengas, J.L., Míguez, J.M., 2009. Efecto del anestésico MS-222 en parámetros sanguíneos indicadores de estrés en trucha arco-iris (*Onchorhynchus mykiss*) y su atenuación mediante oxigenación intensa del medio. Proceedings of the XII Congreso Nacional de Acuicultura. Madrid, Spain.

Cornish, I.M.E., Moon, T.W., 1986. The glucose and lactate kinetics of American eels, *Anguilla rostrata* (LeSueur), under MS 222 anaesthesia. J. Fish Biol. 28, 1-8.

Da Rocha, A. M., Salompo de Freitas, D. P., Burns, M., Vieira, J. P., de la Torre, F. R., Monserrat, J. M., 2009. Seasonal and organ variations in antioxidant capacity, detoxifying competence and oxidative damage in freshwater and estuarine fishes from Southern Brazil. Comp. Biochem. Physiol. C, Pharmacol. Toxicol. Endocrinol. 150,512-520.

Dridi, D., Boughattas, N. A., Aouam, K., Reinberg, A., Ben Attia, M., 2005. Circadian time-dependent differences in murine tolerance to the antihistaminic agent loratadine. Chronobiol. Int. 22,499-514.

Frazier, D.T., Narahashi, T., 1975. Tricaine (MS-222): effects on ionic conductances of squid axon membranes. Eur. J. Pharmacol. 33, 313-317.

Gatica, M.C., Monti, G.E., Knowles, T.G., Gallo, C.B., 2010. Muscle pH, rigor mortis and blood variables in Atlantic salmon transported in two types of well-boat. Vet. Rec. 166, 45-50.

Guilhermino, L. C., Sobral, O., Chastinet, C., Ribeiro, R., Gontalves, F., Silva, M. C., Soares, A. M. V. M., 1999. A *Daphnia magna* First-Brood Chronic Test: An Alternative to the Conventional 21-Day Chronic Bioassay? Ecotoxicol. Environ. Saf. 42,67-74.

Hamilton, M. A., Russo, R. C., Thurston, R. V., 1977. Trimmed Spearman-Karber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. Environ. Sci. Technol. 11,714-719.

Hooven, L. A., Sherman, K. A., Butcher, S., Giebultowicz, J. M., 2009. Does the clock make the poison? Circadian variation in response to pesticides. PloS One 4,e6469.

Inoue, N., Imai, K., Aimoto, T., 1999. Circadian variation of hepatic glutathione S-transferase activities in the mouse. Xenobiotica 29,43-51.

Isnard, P., Flammarion, P., Roman, G., Babut, M., Bastien, P., Bintein, S., Esserméant, L., Férard, J. F., Gallotti-Schmitt, S., Saouter, E., Saroli, M., Thiébaud, H., Tomassone, R., Vindimian, E., 2001. Statistical analysis of regulatory ecotoxicity tests. Chemosphere 45,659-669.

Kane, A.S., Salierno, J.D., Gipson, G.T., Molteno, T.C.A., Hunter, C., 2004. A video-based movement analysis system to quantify behavioral stress responses of fish. Water Res. 38, 3993-4001.

Kato, S., Tamada, K., Shimada, Y., Chujo, T., 1996. A quantification of goldfish behavior by an image processing system. Brain Res. 80, 51-55.

Kiessling, A., Johansson, D., Zahl, I.H., Samuelsen, O.B., 2009. Pharmacokinetics, plasma cortisol and effectiveness of benzocaine, MS-222 and isoeugenol measured in individual dorsal aorta-cannulated Atlantic salmon (*Salmo salar*) following bath administration. Aquaculture 286, 301-308.

King, W. V., Hooper, B., Hillsgrove, S., Benton, C., Berlinsky, D. L., 2005. The use of clove oil, metomidate, tricaine methanesulphonate and 2-phenoxyethanol for inducing anaesthesia and their effect on the cortisol stress response in black sea bass (*Centropristis striata* L.). Aquaculture Res. 36,1442-1449.

Lemmer, B., 1996. The clinical relevance of chronopharmacology in therapeutics. Pharmacol. Res. 33,107-115.

López-Olmeda, J.F., Montoya, A., Oliveira, C., Sánchez-Vázquez, F.J., 2009. Synchronization to light and restricted-feeding schedules of behavioral and humoral daily rhythms in gilthead sea bream (*Sparus aurata*). Chronobiol. Int. 26, 1389-1408.

Miller, D.C., Lang, W.H., Graeves, J.O.B., Wilson, R.S., 1982. Investigation in aquatic behavioral toxicology using a computerized video quantification system, in: Pearson, J.G., Foster, R.B., Bishop, W.E. (Eds.), Aquatic Toxicology and Hazard Assessment: Fifth Conference. STP 766. American Society for Testing and Chemicals, Philadelphia, PA, pp. 206-220.

Muller, W.J., Palmer, C.G., Slaughter, A.R., 2008. A chronic toxicity test protocol using *Caridiдna nilotica* (Decapoda: Atyidae) and the generation of salinity toxicity data. African Journal of Aquatic Science 33, 37-44.

Ortuño, J., Esteban, M. A., Meseguer, J., 2002. Effects of four anaesthetics on the innate immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 12,49-59.

Park, M. O., Hur, W. J., Im, S. Y., Seol, D. W., Lee, J., Park, I. S., 2008. Anaesthetic efficacy and physiological responses to clove oil-anaesthetized kelp grouper *Epinephelus bruneus*. Aquac. Res. 39,877-884.

Reinberg, A.E., 1991. Concepts of circadian chronopharmacology, in: Hrushesky, W.J.M., Langer, R., Theeuwes, F. (Eds.). Temporal control of drug delivery. Ann. NY Acad. Sci., pp. 102-115.

Rombough, P. J., 2007. Ontogenetic changes in the toxicity and efficacy of the anaesthetic MS222 (tricaine methanesulfonate) in zebrafish (*Danio rerio*) larvae. Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 148,463-469.

Ross, L.G., Ross, B., 1999. Anaesthetic and sedative techniques for aquatic animals. Blackwell Science, Oxford.

Sánchez, J.A., López-Olmeda, J.F., Blanco-Vives, B., Sánchez-Vázquez, F.J., 2009. Effects of feeding schedule on locomotor activity rhythms and stress response in sea bream. Physiol. Behav. 98, 125-129.

Shea, S.A., Hilton, M.F., Orlova, C., Ayers, R.T., Mantzoros, C.S., 2005. Independent circadian and sleep/wake regulation of adipokines and glucose in humans. J. Clin. Endocrinol. Metab. 90, 2537-2544.

Shoettger, R.A., Julin, A.M., 1967. Efficacy of MS-222 as an anaesthetic on four salmonids. US Fish & Wildlife Investigations in Fish Control 13, 1-15.

Soivo, A., Nyholm, K., Huhti, M., 1977. Effects of anaesthesia with MS 222, neutralized MS 222 and benzocaine on the blood constituents of rainbow trout (*Salmo gairdneri*). J. Fish Biol. 10, 91-101.

Vogl, C., Grillitsch, B., Wytek, R., Hunrich Spieser, O., Scholz, W., 1999. Qualification of spontaneous undirected locomotor behavior of fish for sublethal toxicity testing. Part I. Variability of measurement parameters under general test conditions. Environ. Toxicol. Chem. 18, 2736-2742.

Walton, W. J., Brown, K. L., Lydy, M. J., 1997. Diurnal fluctuations in toxicity in two fish species: *Gambusia affinis* and *Notropis ludibundis*. Bull. Environ. Contam. Toxicol. 59, 414-421.

Weber, R. A., Peleteiro, J. B., García Matín, L. O., Aldegunde, M., 2009. The efficacy of 2-phenoxyethanol, metomidate, clove oil and MS-222 as anaesthetic agents in the Senegalese sole (*Solea senegalensis* Kaup 1858). Aquaculture 288, 147-150.

Zahl, I.H., Kiessling, A., Samuelsen, O.B., Hansen, M.K., 2009. Anaesthesia of Atlantic cod (*Gadus morhua*) – Effect of pre-anaesthetic sedation, and importance of body weight, temperature and stess. Aquaculture 295, 52-59.

Table I. Mortality rate (%) of gilthead seabream exposed to different MS-222 concentrations at ML and MD. Data are expressed as mean ± SEM.

MS-222 concentration (mg/L)	% mortality ML	% mortality MD
60	0.0 ± 0.0	0.0 ± 0.0
80	36.7 ± 6.7	5.0 ± 3.4
90	68.3 ± 7.9	11.7 ± 4.0
100	71.7 ± 12.2	63.3 ± 7.0
120	98.3 ± 1.7	73.3 ± 2.1
200	100 ± 0.0	96.7 ± 2.1

Table II. Adjr^2 and parameter estimates ± SEM of the three parameter logistic model

	Adjr^2	**a**	**b**	**X_0**
ML	0.9797 ± 6.0303	99.97 ± 5.15	-8.61 ± 1.86	85.04 ± 2.05
MD	0.9569 ± 8.4632	85.34 ± 6.07	-26.16 ± 8.99	96.20 ± 1.75

for the % mortality of juvenile gilthead seabream exposed to MS-222 at ML and MD.

Table III. MS-222 concentrations (mg/L) corresponding to the LC50, NOEC, LOEC and LC5 for juvenile gilthead seabream exposed to the anaesthetic at ML and MD. LC50 was calculated by the trimmed Spearman-Karber method, the NOEC and LOEC with the Jonckheere-Terpstra test ($p<0.05$) and LC5 was estimated based on the logistic model. CL = confidence level.

	LC50 (95% CL)	**NOEC**	**LOEC**	**LC5**
ML	85.5 (82.6-88.5)	60.0	80.0	60.5
MD	107.6 (102.4-113.1)	80.0	90.0	86.2

FIGURE LEGENDS

Figure1. Schematic representation of the video-recording system (A) and Fish Tracker interface showing the analysis of fish position (B).

Figure 2. Average diel profile of locomotor activity for juvenile gilthead seabream reared under a 12:12 h LD cycle, showing a diurnal pattern. The white and black bars at the top of the graph indicate the light and dark periods, respectively. Data represent the mean (continuous line) + SEM (vertical lines) of one tank during a sixteen day period.

Figure 3. Evolution of seabream activity (cm/s) registered by video-recording during a 75 min period at ML (white circles) and MD (black circles), under control conditions. Each point represents the average activity every 15 min. Activity levels of each experimental group changed over time in different ways: increased during the ML assay and decreased for MD (GLM repeated measures, $p < 0.05$).

Figure 4. Seabream activity (cm/s) (A, B) and position (cm) (C, D) in the water column prior, during and after a 15 min exposure to MS-222. Video-recording was performed at ML and MD. MS-222 exposure period is indicated by a grey rectangle. The white line represents the mobile average (n=15). The blank gaps correspond to the minutes needed to remove the anaesthetic from the aquarium and provide clean water.

Figure 5. Induction time of anaesthesia and subsequent recovery in juvenile gilthead seabream exposed to MS-222 at ML (A) and MD (B). White bars indicate the average activity level during the 30 minutes prior to anaesthetic exposure, black bars represent fish activity during MS-222 exposure and grey bars during the recovery phase. Each bar corresponds to the mean activity of fish during the previous minute ± SEM. An asterisk (*) over a dark grey bar indicates a reduction of activity to 50% of basal levels. Two asterisks (**) over a grey bar indicate activity levels similar to those displayed before the MS-222 exposure (paired-samples t-test, $p < 0.05$).

Figure 1:

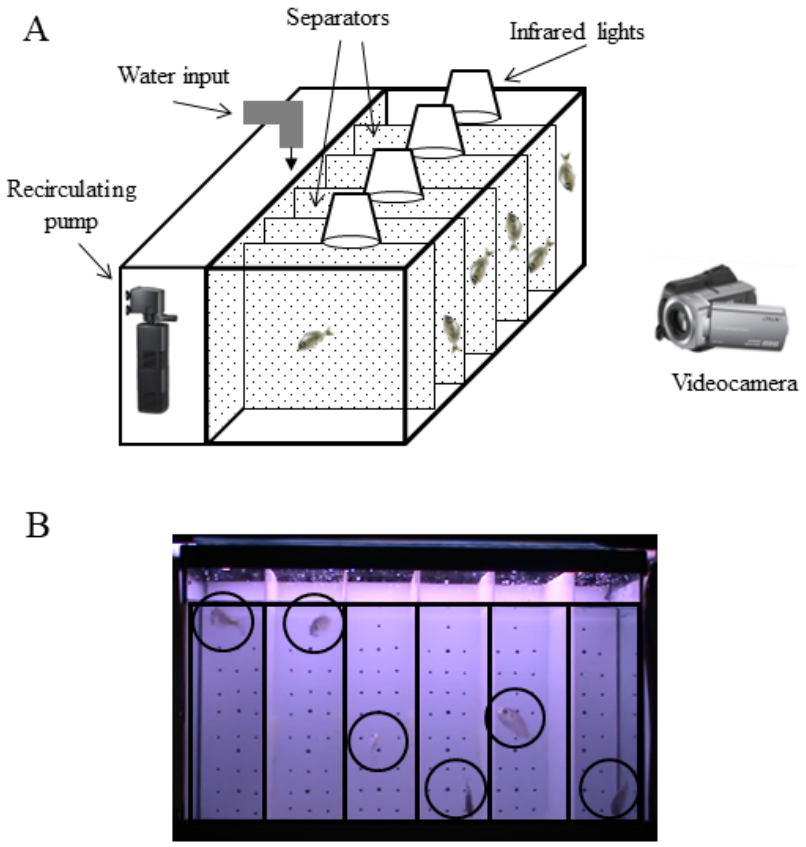

A
Separators
Infrared lights
Water input
Recirculating pump
Videocamera
B
Fish Tracker interface

Figure 2:

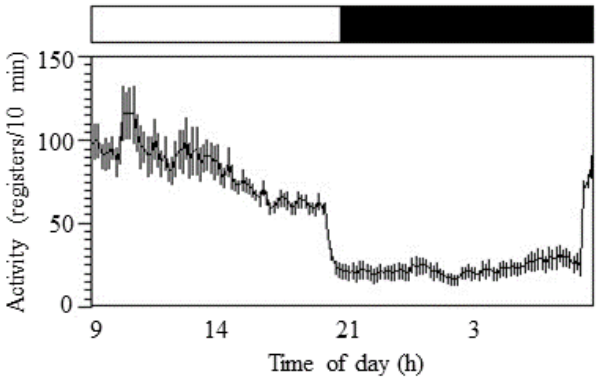


Figure 3:

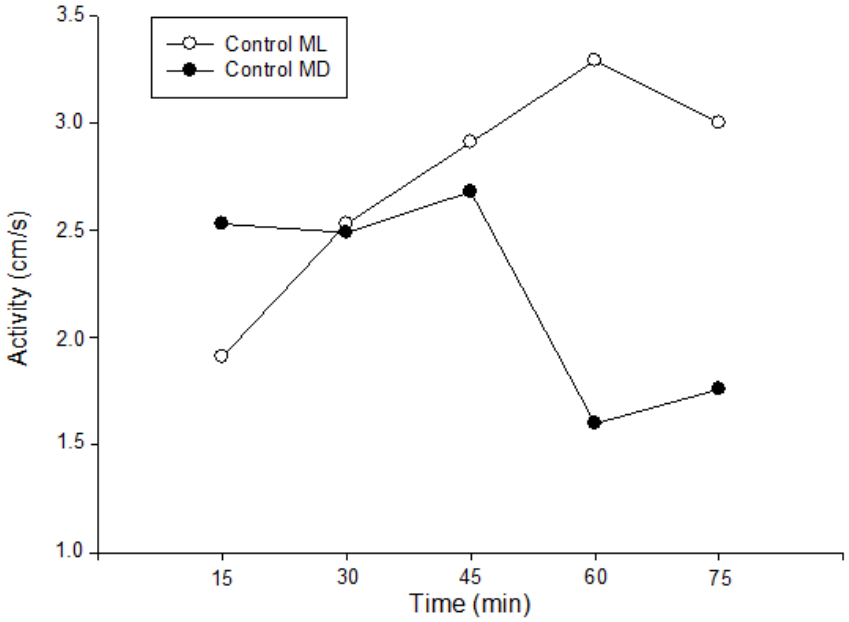

Control ML
Control MD
Activity (cm/s)
3.5
3.0
2.5
2.0
1.5
1.0
15
30
45
60
75
Time (min)

Figure 4:

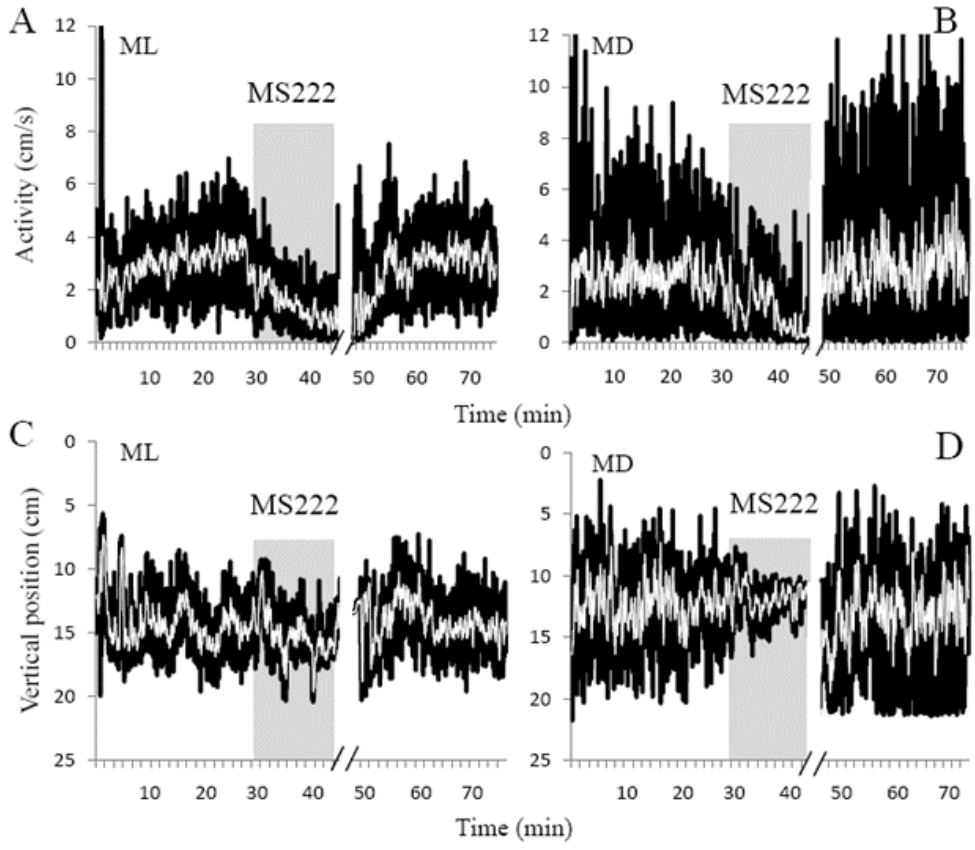
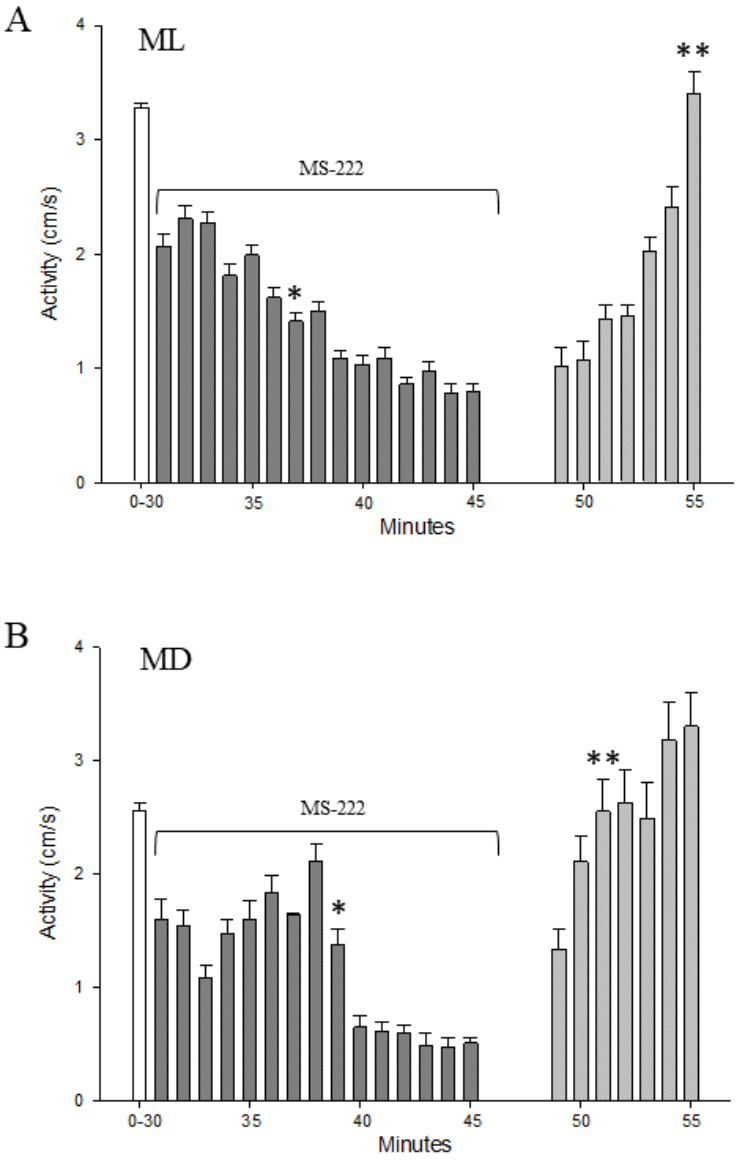

A
ML
MS222
B
MD
MS222
Activity (cm/s)
Time (min)
C
ML
MS222
D
MD
MS222
Vertical position (cm)
Time (min)

Figure 5:


A
ML
MS-222
Activity (cm/s)
Minutes
0-30
35
40
45
50
55
*
**
B
MD
MS-222
Activity (cm/s)
Minutes
0-30
35
40
45
50
55
*
**