UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN

Polish Journal of Natural Sciences

39

(3/2024)

PUBLISHER UWM
OLSZTYN 2024

The Polish Journal of Natural Sciences is indexed and abstracted
in Biological Abstracts and Biosis Previews

The print edition is the primary version of the Journal

The Journal is also available in electronic form on the websites
http://www.uwm.edu.pl/polish-journal/ (home page)
https://czasopisma.uwm.edu.pl/index.php/pjns/about (electronic platform; submissions)

PL ISSN 1643-9953

Publisher UWM Olsztyn
ul. Jana Heweliusza14
10-718 Olsztyn-Kortowo, Poland
tel.: +48 89 523-36-61
fax: +48 89 523-34-38
e-mail: wydawca@uwm.edu.pl

Edition 56 copies; publisher's sheets 4.62; number of printed sheets 3.75
Print Zakład Poligraficzny UWM w Olsztynie
order number 1365

TABLE OF CONTENTS

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 39(3): 143–150, Y. 2024

DOI: 10.31648/pjns.10220

INFLUENCE OF SELECTED PARAMETERS OF MATING VOCALIZATION OF PHEASANTS *(PHASIANUS COLCHICUS)* ON REPRODUCTIVE SUCCESS*

***Piotr Czyżowski*[1], *Piotr Nawłatyna*[2], *Sławomir Beeger*[3]**

[1] ORCID: 0000-0002-8587-246X
[2] ORCID: 0009-0001-5979-1594
[3] ORCID: 0000-0003-4569-8273
[1–3] Department of Animal Ethology and Wildlife Management
University of Life Sciences in Lublin, Lublin, Poland

Key words: pheasant, vocalization, reproductive behaviour.

Abstract

The purpose of this study was to analyze the mating vocalizations of pheasant roosters (time between successive vocalizations, peak frequency of sound amplitudes) and to demonstrate the relationship between vocalization parameters and the number of hens accumulated by the roosters (reproductive success). The research was conducted based on recordings of the sounds of 6 rolling roosters. A total of 79 isolated vocalizations constituted the study material. The recordings were made using a Zoom H1 portable sound recording device based on two microphones in an X/Y arrangement, enabling correct recording of stereophony. Roosters leading two or one hen were characterized by more active vocalizations, which increases their attractiveness to hens choosing them. Cooks leading two or one hen were characterized by a lower peak frequency of the vocalization sound produced than roosters without hens. This may indicate that roosters making sounds at lower frequencies are more attractive to hens.

Introduction

The study and analysis of bird sounds are essential in studying bird ecology and behaviour (SLABBEKOORN and SMITH 2002, NEAL et al. 2011). There are few studies on the acoustic characteristics of tooting pheasants *Phasianus colhicus* (HEINZ and GYSEL 1970, SHIREN et al. 1996), although the species is easy to observe and is characterized by a spectacular mating

Address: Sławomir Beeger, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, e-mail: slawomir.beeger@up.lublin.pl

* The research was financed from the statutory funds of Department of Animal Ethology and Wildlife Management, University of Life Sciences in Lublin, Poland.

ritual and loud vocalizations. With the arrival of spring, male pheasants mark out territories by demonstrating this with vocalizations (drinking). This behaviour attracts females and informs other males that a territory has been occupied. The sound of a rooster provides pheasant hens with acoustic information about the fitness of males, which indirectly translates into the male's choice of a particular habitat. The most attractive males to hens show greater territoriality – they move less during the day, have better territories and moult more often (GRAHN et al. 1993, MATEOS 1998). The more attractive and diverse the territory, the better the access to food and nesting sites, and the easier the escape from possible danger (JOHNSGARD 2017). Females make their choice among tooting males probably based on morphology (GÖRANSSON et al. 1990, VON SCHANZT et al. 1994), courtship behaviour (MATEOS and CARRANZA 1999), and based on significant tissue compatibility system (MHC) genes (BARATTI et al. 2012). The sound of a rooster can also provide pheasant hens with acoustic information on male fitness, which indirectly translates into the male's choice of a particular habitat (SLABBEKOORN and SMITH 2002, HARMA 2003).

The purpose of this study was to analyze the mating vocalizations of pheasant roosters (time between successive vocalizations, peak frequency of sound amplitudes) and to demonstrate the relationship between vocalization parameters and the number of hens accumulated by the roosters (reproductive success).

Materials and Methods

The study was based on recordings of the sounds of 6 tooting roosters in the Lublin area, including allotment gardens, a city park and ruderal areas. The area is located in the valley of the Bystrzyca River and includes a section from the allotments near Muzyczna Street to the former Riding Club. The dominant habitat in the club's vicinity was shrubbery and woodland adjacent to an area of meadows and formerly functioning pastures, while the allotment gardens are a mosaic of cultivated and ornamental plants.

The sounds were recorded in 2023, from April to June. A total of 79 isolated vocalizations constituted the study material. The total length of the recordings made in the different terms ranged from 12 to 60 minutes. The recordings were made with a Zoom H1 portable sound recording device based on two microphones in an X/Y arrangement, enabling correct recording of stereophony. Due to later analysis and archiving, the files with recorded vocalizations of roosters were divided into 3-minute recordings,

which were analyzed in Cool Edit Pro 2.1 software. Individual vocalizations of roosters were extracted and evaluated in terms of peak amplitude frequency, the duration of the first and second syllables, and pauses between syllables. Pheasants were divided into three groups based on the number of hens they had (0, 1, 2), and the average values of the sound frequency of peak amplitudes and the duration of pauses between vocalizations (vocalization frequency) were calculated for each group. For each song, the duration of the first syllable (in seconds), the duration of the second syllable (in seconds), the duration of the pause between syllables (in seconds), the interval between vocalizations (in minutes) and the peak frequency for the first and second syllables (in Hz) were determined. The duration of syllables and pauses between syllables and vocalizations were determined by spectrogram evaluation (Figure 1). At the same time, direct observations of roosters were made using binoculars to determine the number of hens led by males.

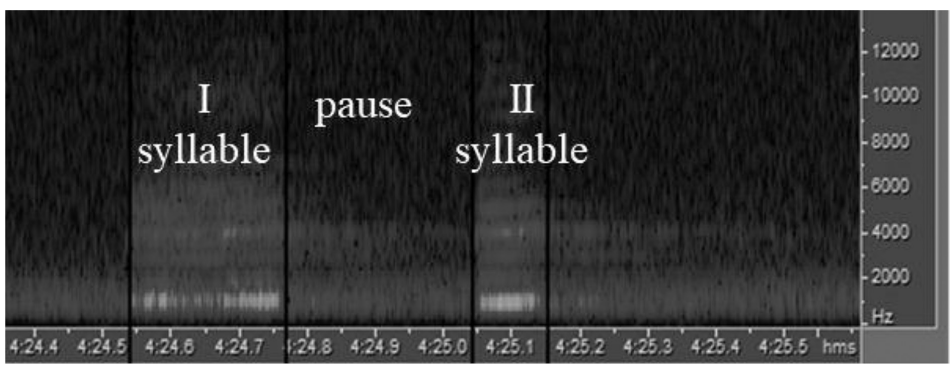

Fig. 1. Screenshot from Cool Edit Pro with spectrogram and highlighted rooster song phrases

Statistical analysis of the study results was carried out using the Statistica 13.3 PL statistical package. Since the distributions of the analyzed traits significantly deviated from normality, nonparametric (rank) tests were used to test the significance of differences between their distributions. For comparisons of mean values of sound frequencies (independent variables) between groups (number of hens gathered by the rooster), a nonparametric analysis of variance – Kruskal-Wallis ANOVA and a multiple comparisons test of mean ranks for pairwise comparisons were performed. The significance of differences between groups was determined by nonparametric analysis of variance – Kruskal-Wallis ANOVA.

Results and Discussion

The mean time between vocalizations of roosters leading two hens was significantly lower compared to roosters in which no hens were present (Table 1), and these differences assessed by nonparametric ANOVA were statistically significant (Figure 2).

Table 1

Average values of analyzed parameters of pheasant cock depending on the number of hens [$\overline{x}$ / n (min–max)]

Number of hens by the pheasant cock (n)	The time between vocalizations [(min : sec)/n min–max)]	Peak amplitude frequency of syllable I [(Hz/n) min–max)]	Peak amplitude frequency of syllable II [(Hz/n) min–max)]
0	02:53/29 (00:18–06:00)	1731/29 (1041–4190)	1635/29 (933–4508)
1	01:54/26 (00:30–03:00)	1085/26 (1015–1181)	1092/26 (967–1261)
2	01:36/24 (00:12–06:00)	1176/24 (968–4110)	1190/24 (958–4092)

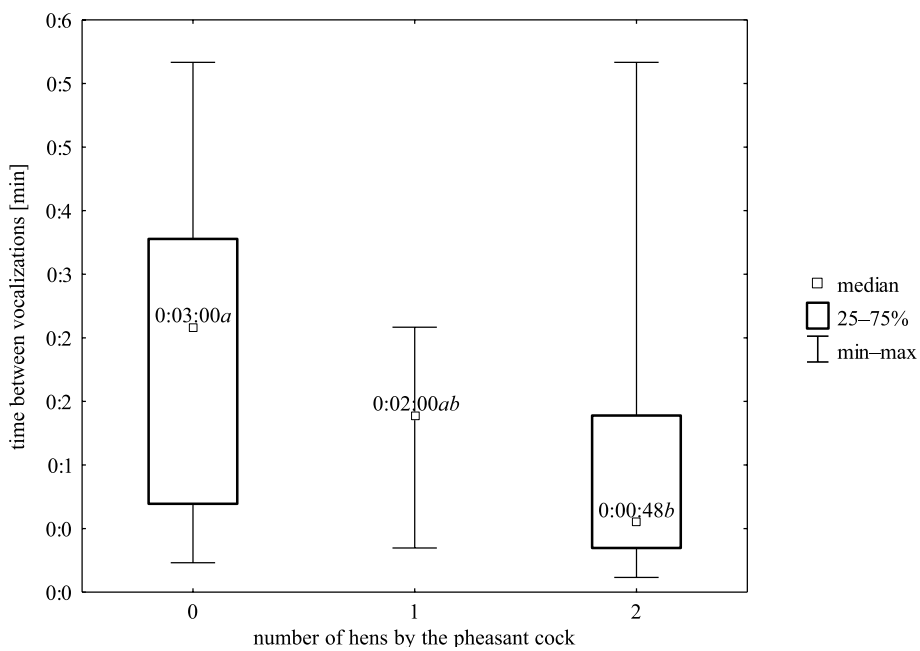

Fig. 2. The time between vocalizations [min] due to the number of hens at the pheasant cock

The total sound frequency range of pheasants' vocalizations was from 900 to 4500 Hz, and 50% of all observations (between quartiles I and II) were in the 1000–1300 Hz range. According to studies by other authors (HEINZ and GYSEL 1970, SHIREN et al. 1996), in pheasants, the frequency of vocalizations is in the range of 250–10500 Hz, and the most distinct frequency of pheasant singing syllables is usually in the range of 800 Hz to 1000 Hz.

The highest peak amplitude of the first syllable of vocalization was characterized by roosters at which no hens were observed. Roosters that gathered 1 or 2 hens were characterized by lower peak amplitude values (Table 1), and these differences were statistically significant (Figure 3). Similar results were obtained by analyzing the peak amplitudes of the second syllable – the highest amplitude was characterized by roosters at which no hens were observed, and these were also statistically significant differences (Figure 4). A lower sound frequency means that sound waves carry less energy, reducing their susceptibility to absorption by obstacles. Low-frequency sounds propagate further and can be heard by more females. (COSENS and FALLS 1984, SLABBEKOORN et al. 2002). Vocalization behaviour is significantly influenced by hormonal factors, as evidenced by the relationship between vocalization frequency and gonad mass, and thus testosterone levels (HEINZ and GYSEL 1970), which fluctuate cyclically (KIM and YANG 2001). The singing of male birds is a secondary sexual trait under the control of gonadal steroids, so it increases and disappears with the seasonal cycle of testicular growth and regression (MARLER 1988). Thus, analysis of the sounds made by birds can be an indirect determinant of the individual quality of roosters. This is also supported by studies on Gallus gallus, which have shown that vocalization quality is an indicator of body size and individual fitness (HAO et al. 2022). According to GREIG et al. (2013), low vocalization frequency is more strongly associated with larger body size, indicating that morphological limitations also affect the acoustic quality of vocalizations.

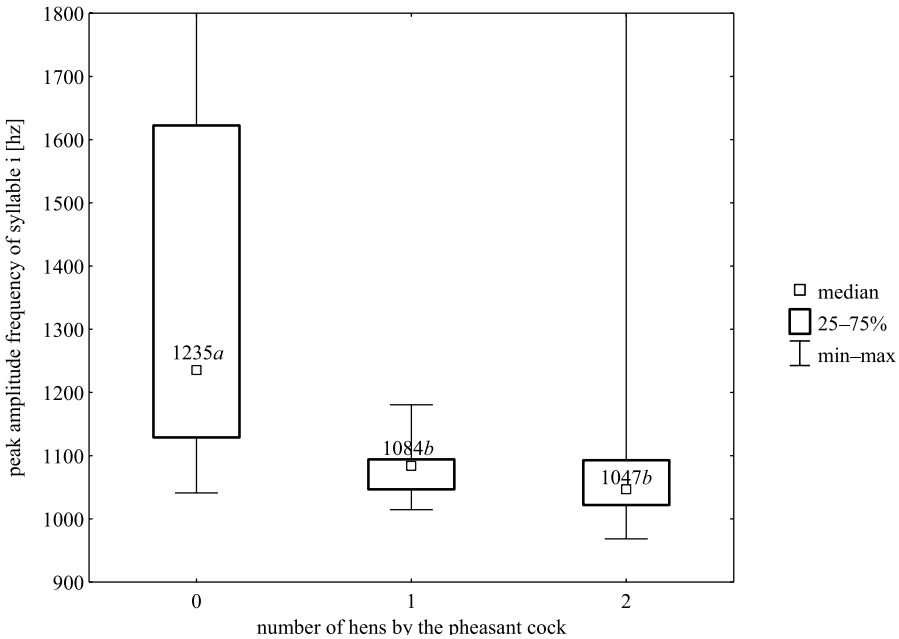

Fig. 3. Peak amplitude frequency of syllable I [Hz] due to the number of hens at the pheasant cock

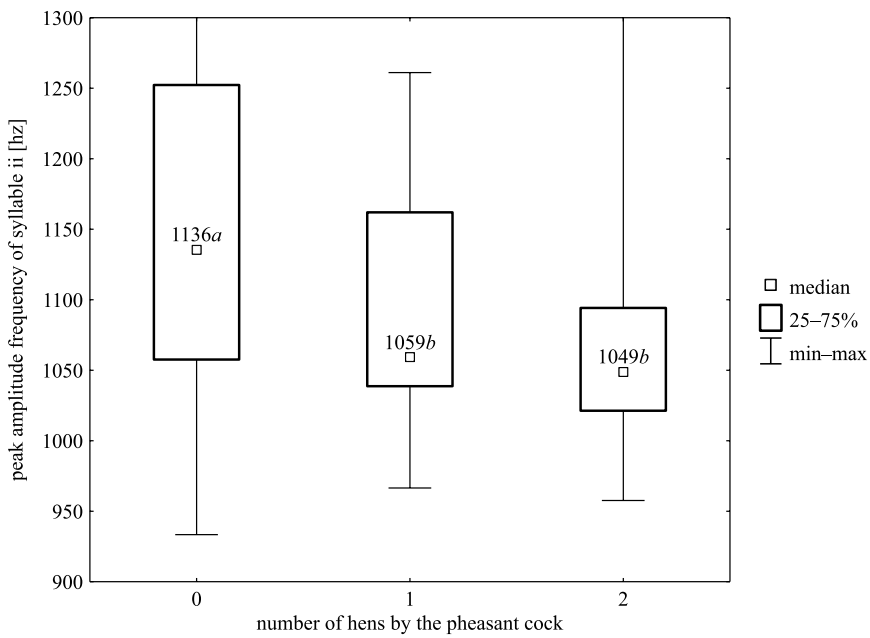

Fig. 4. Peak amplitude frequency of syllable II [Hz] due to the number of hens at the pheasant cock

Conclusion

1. Cooks leading two or one hen were characterized by higher vocalization activity, which increases their attractiveness to hens choosing them.

2. Cooks leading two or one hen were characterized by a lower peak frequency of the vocalization sound compared to roosters without hens. This may indicate that roosters making lower-frequency sounds are more attractive to hens.

3. Our work may provide a basis for further research on the relationship of vocalization with reproductive success and testosterone levels.

References

Baratti M., Dessì-Fulgheri F., Ambrosini R., Bonisoli-Alquati A., Caprioli M., Goti E., Matteo A., Monnanni R., Ragionieri L., Ristori E., Romano M., Rubolini D., Scialpi A. Saino N. 2012. *MHC genotype predicts mate choice in the ring-necked pheasant Phasianus colchicus*. J. Evol. Biol., 25(8): 1531–1542. https://doi: 10.1111/j.1420-9101.2012.02534.x.

Cosens S.E., Falls J.B. 1984. *A comparison of sound propagation and song frequency in temperate marsh and grassland habitats*. Behav. Ecol. Sociobiol., 15: 161–170.

Göransson G., Von Schantz T., Fröberg I., Helgee A. Wittzell H. 1990. *Male characteristics, viability and harem size in the pheasant, Phasianus colchicus. Anim. Behav.,* 40(1): 89–104. https://doi.org/10.1016/S0003-3472(05)80668-2.

Grahn M., Göransson G., von Schantz T. 1993. *Spacing behaviour of male pheasants, Phasianus colchicus, in relation to dominance and mate acquisition. Anim. Behav.,* 45(1): 93–103. https://doi.org/10.1006/anbe.1993.1009.

Greig E.I., Price J.J., Pruett-Jones S. 2013. *Song evolution in maluridae: Influences of natural and sexual selection on acoustic 407 structure*. Emu, 113: 270–281.

Hao P., Zhao K., Huang X., Rao X., Liang W., Zhang Y., 2022. *The vocalization of the red junglefowl is a signal of body size and 410 individual health*. Curr. Zool., 69: 393–400.

Harma A. 2003. *Automatic identification of bird species based on sinusoidal modeling of syllables.* [In:] *IEEE International Conference on Acoustics*, *Speech and Signal Processing*, pp. 545–548.

Heinz G.H., Gysel L.W. 1970. *Vocalization behavior of the Ring-necked Pheasant*. The Auk, 87(2): 279–295. https://www.jstor.org/stable/4083920, access: 5.06.2024.

Johnsgard P. 2017. *North American quails, partridges, and pheasants*. Lulu. com., pp. 97–104.

Kim I.S., Yang H.H. 2001. *Seasonal changes of testicular weight, sperm production, serum testosterone, and in vitro testosterone release in Korean ring-necked pheasants (Phasianus colchicus karpowi).* J. Vet. Med. Sci., 63(2):151–156. https://doi.org/10.1292/jvms.63.151.

Marler P., Peters S., Ball G.F., Dufty Jr. A.M., Wingfield J.C. 1988. *The role of sex steroids in the acquisition and production of birdsong*. Nature, 336(6201): 770–772.

Mateos C. Carranza J. 1999. *Effects of male dominance and courtship display on female choice in the ring-necked pheasant*. Behav. *Ecol. Sociobiol.* 45(3–4): 235–244. https://doi.org/10.1007/s002650050558.

Mateos C. 1998. *Sexual selection in the ring-necked pheasant: a review*. Ethol. Ecol. Evol. 10(4): 313–332. https://doi.org/10.1080/08927014.1998.9522846.

Neal L., Briggs F., Raich R., Fern X. Z. 2011. *Time-frequency segmentation of bird song in noisy acoustic environments.* [In:] *2011 IEEE International Conference on Acoustics, Speech and Signal Processing* (ICASSP): 2012–2015. https://doi.org/10.1109/ICASSP.2011.5946906.

SHIREN J., PING D., JIANHUA L., YANG Z. 1996. *The sound spectrographic analyses on the call of golden pheasant Chrysolophus pictus*. Zool. Res., 17(4): 403–409.

SLABBEKOORN H., SMITH T.B. 2002. *Bird song, ecology and speciation*. Philos. Trans. R. Soc. Lond. B Biol. Sci., 357(1420): 493–503. https://doi.org/10.1098/rstb.2001.1056.

SLABBEKOORN H., ELLERS J., SMITH T.B. 2002. *Birdsong and sound transmission: the benefits of reverberations*. The Condor, 104(3): 564–573.

VON SCHANTZ T., GRAHN M., GORANSSON G. 1994. *Intersexual selection and reproductive success in the pheasant Phasianus colchicus*. Am. Nat., 144(3): 510–527.

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 39(3): 151–165, Y. 2024

DOI: 10.31648/pjns.10258

EMAMECTIN BENZOATE EXPOSURE IMPAIRED GERM CELL MATURATION IN THE TESTES OF ADULT ALBINO RATS*

***Assia Kamel*[1], *Samira Akdader-Oudahmane*[2], *Zohra Hamouli-Said*[3]**

[1] ORCID: 0000-0001-8461-0639
[2] ORCID: 0000-0003-0959-9112
[1–3] Department of Biology and Physiology of Organisms
University of Sciences and Technology Houari Boumediene (USTHB), Bab Ezzouar, Algiers, Algeria
[2] Department of Biology
Faculty of Biological Sciences and Agricultural Sciences
University of Mouloud Mammeri, Tizi-Ouzou, Algeria

Key words: EMB, oxidative stress, toxicity, testis.

Abstract

Certain pesticides represent a substantial risk to reproductive function, and their possible reprotoxic effects have been the focus of numerous research. Emamectin Benzoate (EMB), a biopesticide widely used in agriculture, household items, and veterinary health, can cause tissue damage in the male reproductive system due to oxidative toxicity. The purpose of this study was to investigate the effects of Emamectin Benzoate toxicity in male Wistar rats. Fourteen rats were divided into two groups of seven each. For 15 days, the EMB group received a daily dose of EMB corresponding to 20 mg/kg by gavage. Distilled water was given to the control group. Oxidative stress, DNA fragmentation, histopathology and germ cell degeneration of testis were investigated. Emamectin administration induced oxidative stress in testicle evidenced by elevated levels of MDA, protein carbonyl and percentage of DNA fragmentation with depletion of the defense system (CAT and SOD activities). Emamectin produced several histopathological changes in the testis, these results suggesting that the subchronic exposure to emamectine benzoate affects the redox state which contribute to testicular impairment.

Introduction

Pesticides, also known as plant protection products, are a class of chemicals that are commonly used in agriculture to preserve crops and

Address: Assia Kamel, University of Sciences and Technology Houari Boumediene (USTHB), Bab Ezzouar, Algiers, Algeria, e-mail: assia.kamel_fsb@usthb.edu.dz

* This work was supported by scientific research project of Ministry of Higher Education and Research (Grant number 200220183-Prfu).

prevent hazardous organisms in order to increase the quantity and quality of food produced (PARRON et al. 2011). As a result, characterizing the hazards presented by these pollutants has become a major ecotoxicological task. Pesticides are now thought to be very hazardous to both human health and the environment. Because of their extensive usage, persistence, and bioaccumulation along the trophic chain, they are among the most harmful contaminants in the environment. EMB is a potent neurotoxic agent from the Avermectin family that is produced through the fermentation of *Streptomyces avermitilis*. B1a ($C_{49}H_{75}NO_{13}C_7H_6O_2$, 90%) and B1b ($C_{48}H_{73}NO_{13}C_7H_6O_2$, 10%) are EMB's active homologues. Radiolabeled emamectin B1a benzoate or MAB1a is rapidly absorbed, distributed and excreted following oral and intravenous administration. In rat, More than 98% of the total radioactivity was found in faeces, with <0.4% in urine and <1.6% in the tissues (MUSHTAQ et al 1996). Tissue distribution and bioaccumulation appeared minimal, the metabolism of MAB1a benzoate appears to involve primarily N-demethylation to AB1a. AB1a was the only metabolite detected in the faeces, liver, kidneys, muscle and fat, the amount of this metabolite represented about 1 to 2% of the radioactivity one day post -treatment, but increased to 19% of radioactivity on 7 post -treatment . The biotransformation of MAB1a results in formation of the metabolite AB1a, which is present in lower amounts compared with the parent compound in various tissues and is excreted mainly via faeces (WOLTERINK et al. 2012). EMB is a plant protection product that is anti-parasitic, antibacterial, and insecticidal. Significant amounts move through the soil, migrate, and are likely to wind up in agricultural commodities, particularly animal foodstuffs (WANG et al. 2006). Emamectin is structurally similar to abamectin and ivermectin. EMB acts as a disruptor of neurotransmitter activity (chloride channel activator) in the target organism and results in paralysis and death. Although the data about the effects of EMB on antioxidant status is insufficient, many studies have confirmed that EMB insecticides created oxidative stress in intoxicated animals (TEKELI et al. 2023, TEMIZ 2020). EMB caused elevation of malondialdehyde and reduction of antioxidant enzymes in rats, and/also it has been reported that emamectin induced single and double-strand DNA breaks in human liver cells (ZHANG and ZHAO 2017) and chromatin condensation with nuclear fragmentation in leukemia K562 and Molt-4 cells in vitro (YEN et al. 2017). Studies have shown that workers who have been exposed to pesticides for a long time may experience oxidative damage to their various organs and decreased sperm quality (LATCHOUMYCANDANE and MATHUR 2002). EMB exposure, whether chronic or subchronic, causes reproductive and fertility problems (AITKEN et al. 2022). This study aims

to evaluate the redox state, DNA fragmentation and testicular toxicity induced after subchronic exposure to EMB in albinos Wistar rats.

Materials and Methods

Chemicals

Commercial formulation of emamectin benzoate was PROMED 5_{SG} (CAS No: 12052064), containing 5% emamectin benzoate, A pure reference standard of emamectin benzoate (Medmac, Agrimatco, Algeria) was purchased from the Ministry of Agriculture and Rural Development (Direction de la Protection des Végétaux et Contrôles Techniques DPVCT, Algeria). Tris base, EDTA, Triton x-100, NaCl and diphenylamine, thiobarbituric acid (TBA), 5, 5′-dithio-bis (2-nitrobenzoic) acid (DTNB) were obtained from commercial sources (Sigma Aldrich, France).

Animals

All experimental procedures were approved by the institutional animal care and use committee of the University of Houari Boumedienne in Algeria (USTHB) and were carried out in accordance with to the ethical approval number: 981-1 law of August 22, 1998, of the National Administration of Algerian Higher Education and Scientific Research. 14 adults male Wistar rat weighing (133 g ±38 g) was obtained from IPA (Algiers Pasteur Institute). The supplied rats were placed in cages. Maintained under controlled temperature conditions (21–23°C) and 12–12 h photoperiodic cycle, All the animals were fed with standard pellets (ONAB: Office National des Aliments de Bétail, Alger, Algeria) and water *ad libitum*.

Experimental design

Healthy adult rats (n = 7) were randomly divided into 2 groups (n = 7 per group) as follows: group 1 control (C) – received distilled water (placebo); group 2 (EMB) – received by oral gavage 20 mg/kg/day of EMB (equivalent to 1/3.75 th oral LD50) (WOLTERINK et al. 2012) for 2 weeks. EMB was prepared in distilled water. Animals were carefully observed during the 15 days of treatment and attention was paid to signs that would indicate neurotoxicity, including tremor, salivation, and diarrhea. Rats were sacrificed at the end of the experiment by decapitation. The testicle was quickly removed and immediately weighed and stored for biochemical analysis or fixed for histopathological studies.

Determining pro-oxidant/anti-oxidant status

Preparation of testicular homogenates. After decapitation, the testes were immediately excised and frozen at −20°C. Each testis was placed in a cold buffer containing 30 mmol/L monosodium hydrogen phosphate (NaH_2PO_4; pH 7.4), 0.1% Triton X-100, 0.12 mol/L NaCl and 0.3 mmol/L PMSF and was homogenized with a Staufen homogenizer. The homogenates were centrifuged at 1,000 g for 5 min at 4°C. The protein concentrations were determined using the Bradford method (BRADFORD 1976).

Assessment of oxidative markers. The level of lipid peroxidation was determined by measuring malondialdehyde using the BIRD and DRAPER (1984) TBARS (Thiols Barbituric Acid Reactive Species) test. The testicular protein carbonyl content (PCC) was determined by the method described by LEVINE et al. (1990).

Evaluation of enzymatic and non-enzymatic antioxidants. The method of MISRA and FRIDOVICH (1972) was used to determine testicular superoxide dismutase (SOD) activity by measuring the inhibition of epinephrine auto-oxidation at pH 10.2. Testicular catalase (CAT) activity was obtained using the method of AEBI (1984) by measuring the decrease in hydrogen peroxide (H_2O_2) concentration at 240 nm. Reduced glutathione (GSH) was assayed using 5, 5′-dithio-bis (2-nitrobenzoic) acid (DTNB). The absorbance of DTNB was measured at 412 nm in a microplate reader (BIO-TEK) because it forms a yellow complex with GSH.

DNA fragmentation assay. DNA fragmentation in the testicular homogenate was quantified by the diphenylamine reaction procedure described by PERANDONES et al. (1993). Slices of the testes were homogenized (1:9/w:v) in cold hypotonic lysis buffer containing (10 mM Tris base, 1 mM EDTA and 0.2% Triton X-100). The homogenates were centrifuged at 3,000 · g for 15 min. the supernatant and the pellet were used for quantification of fragmented DNA. The developed blue color was quantified at 578-nm. Percentage of DNA fragmentation in each sample was expressed by the formula: % DNA fragmentation = (O.D Supernatant / O.D Supernatant + O.D Pellet) ·100.

Histological/Histochemical study. The testis was embedded in paraffin after being fixed in 10% neutral buffered formalin and dehydrated in a graded series of ethanol. Each block was cut into four-micron-thick sections and stained using the following methodology: Hematoxylin and eosin (HE) stains were used to demonstrate the general histological fea-

tures, while the periodic acid-Schiff (PAS) technique allowed to show mucopolysaccharides in the testis and observed under Optika B-500 TPL "TS-View" light microscope.

Germ cell degeneration. Testis specimens were fixed with 4% glutaraldehyde, buffered with 0.2 M phosphate buffer (PB), pH 7.4 and post-fixed with 1% osmium tetroxide in the same buffer. They were dehydrated in an increased series of ethanol followed by propylene oxide and embedded in Epon 812. Semi-thin sections (0.5 μm) were cut on an ultra-microtome (LKB BROMMA) and stained with toluidine blue. Toluidine blue staining was performed for the identification of germ cell degeneration. Germ cell degeneration was expressed as a percentage of tubular cross-sections containing degenerating cells. 20 tubular cross-sections per animal were scored. All counts were performed at 1000× magnification in the light microscope (Optika B-500 TPL "TS-View).

Statistical analysis

All values are expressed as mean SEM (mean standard error of the mean) by Student's *t*-test. All statistical tests are performed using Graph pad prism Software version 5.

Results

Evaluation of body and testis weights

Body weight gain (or lost) (%) was calculated as follows: Final body weight – initial body weight · 100/initial body weight. Table 1 shows that body weight increased by 12%; ($p > 0.05$) after EMB treatment when compared with the control group. Relative paired testes weight was 1.06 ±0.03 g/100 g BW in the control group, decreased non significantly by 2% after EMB treatment (Table 1).

Table 1
Percentage body weight gain and relative testis weight, in rats from control and EMB-treated groups. Data are expressed as the means ±SEM of 7 animals for each group. Values are significantly different ($p < 0.05$).

Body weight gain [g]	Control	EMB
Initial body weight	133.51 ±11.13	146.89 ±8.37
Experimentation body weight	219.55 ±11.25	246.15 ±10.87
Body weight after treatment [%]	64%	67%
Relative testis weight [g/100 g BW]	1.06 ±003	1.04 ±0.05

Assessment of oxidant/antioxidant biomarkers

Administration of emamectin to rats at a dose level equivalent to 1/37.5 th oral LD_{50} for 2 weeks caused significant increase ($p < 0.05$) of MDA level (Figure 1*a*) and protein carbonyl content (PCC) ($p \leq 0.05$) (Figure 1*b*) while reduced activities of CAT (Figure 1c) and SOD (Figure 1*d*). There was no significant difference ($p > 0.05$) in testicular GSH concentration between EMB-exposed and control animals (Figure 1e).

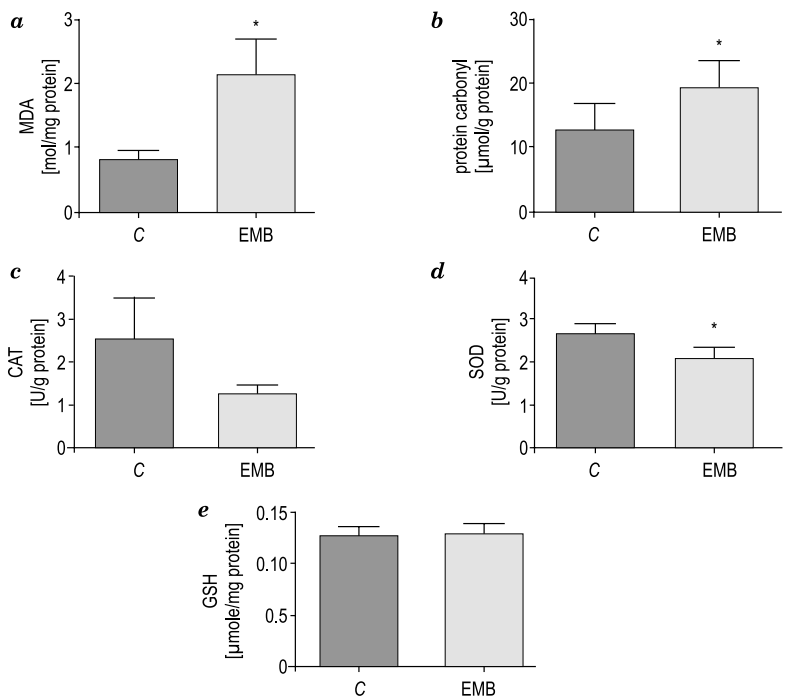

Fig. 1. Effects of EMB on oxidative stress parameters (oxidant /antioxidant): *a* – MDA level [nmol/g protein]; *b* – protein carbonyl [µmol/g protein]; *c* – CAT activity [U/g protein]; *d* – SOD activity [U/g protein]; *e* – GSH [µmol/mg protein] in the testis of rat. Values expressed as the means ±SEM of seven animals for each group; *$p < 0.05$; Values are significantly different

DNA fragmentation assay findings

The percentage of DNA fragmentation of emamectin-treated rats significantly increased in comparison to the control group. The estimated mean values were 17.57 ±2.05% in control group and 27.71 ±6.3% in EMB group (50% higher than control, ($p \leq 0.05$) indicating that the testes are vulnerable to the toxic insult of emamectin (Figure 2*a*).

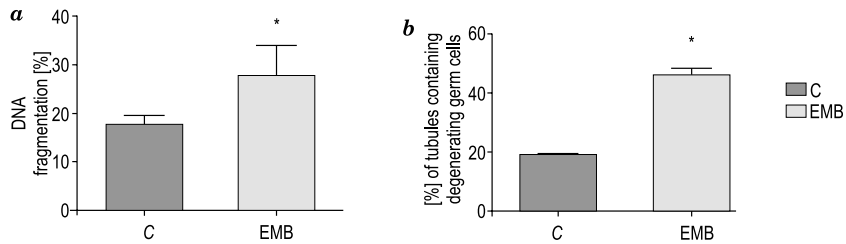

Fig. 2. Percentages of DNA fragmentation and tubules containing degenerating germ cell in the testes of rats from control and EMB-treated groups: *a* – DNA fragmentation [%]; *b* – % of tubules containing germ cell degenerating. Values expressed as the means ±SEM of seven animals for each group; *$p < 0.05$; values are significantly different

Germ cell degeneration finding

We observed that in treated rats, 46% of seminiferous tubule cross-sections containing degenerating germ cell. The incidence of tubules containing degenerating germ cells was 19.05 ±0.27 % in the control group, increased to 46.20 ±2.3% in EMB group (2.4-fold of control, $p < 0.05$) (Figure 2*b*). Tubular cross-sections containing degenerating cells, identified as having strongly condensed darkly stained nuclei in toluidine-blue-stained sections (Figure 3*b*), in addition to the basal germ cells showing loss of their cytoplasm with very dark nuclei characterized by pyknotic nuclei (Figure 3*b*), thus the nuclei of acrosomal caps of late spermatids are strongly stained and were seen in the basal level of the seminiferous epithelium, indicating failure of sperm relase (Figure 3*c*).

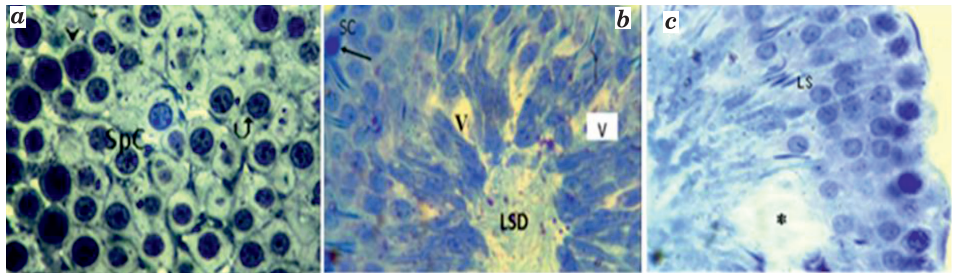

Fig. 3. A semithin section of a testis of a rat: *a* – control group showing normal tissue architecture; SpC – spermatogenic cell, primary spermatocytes (arrowhead), spermatids (curved arrow); *b*, *c* – EMB – treated group showing vacuolations (V); depletion, extensive vacuolization (V) of the germinal epithelium; sertoli cells (SC) resting on the basement membrane; LSD – low spermatozoa density; failure of late spermatid release (LS); lacunar spaces (*), pyknotic nuclei of basal germ cells (arrows). Toluidine blue ×1000
Source: own study, photo by Assia Kamel, Samira Akdader-Oudahmane and Zohra Hamouli-Said

Histopathological study

Hematoxylin and eosin

The microscopic analysis of cross sections of controls rats testes reveal a normal structural appearance of seminiferous tubules surrounded by a basal lamina and separated by interstitial tissue containing Leydig cells as well as blood vessels in between (Figure 4*a*). Each tubule was lined by germinal epithelium, which is a complex stratified epithelium containing germ cells at various stages of the spermatogenetic cycle, with cell types represented by spermatogonia, Sertoli cell, spermatocytes, round spermatids, late spermatids and variable numbers of mature sperms were present in the lumen of the tubules (Figure 4*b*, *c*).

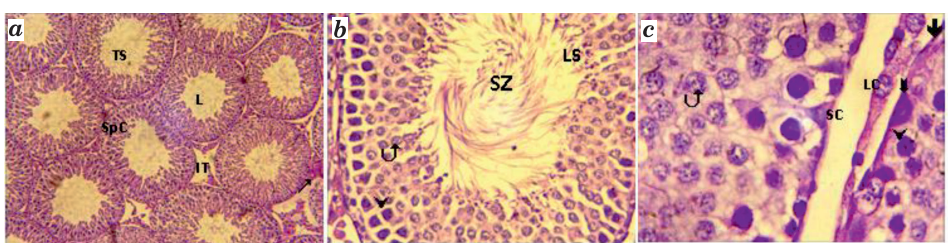

Fig. 4. Cross sections of the testis of the control group, showing: *a* – rounded seminiferous tubules (Ts) lined by spermatogenic cells (Spc) and separated by the interstitial tissue (IT) with blood vessels in between (arrow). H&E ×100; *b* – the seminiferous tubule containing germ cell, including spermatogonia (bifid arrow), primary spermatocytes (arrowhead), and spermatids (curved arrow), spermatozoa (Sz). H&E ×400; *c* – spermatogonia (bifid arrow), primary spermatocytes (arrowheads), spermatids (curved arrow), sertoli cells (SC), leydig cells (LC) and myoid cells (thick arrow). H&E ×1000
Source: own study, photo by Assia Kamel, Samira Akdader-Oudahmane and Zohra Hamouli-Said

Testis of rats from emamectin treated group showed disturbances in the normal architecture of the testis. Some of the seminiferous tubules showed abnormal shapes with indentation and contour irregularity, severe dilated congested blood vessels and mononuclear cells infiltration were also seen between the tubules (Figure 5*a*, *c*). In addition, reduced size of tubular lumen and degenerated cytoplasm of germ cell, karyolysis and pyknosis of some nuclei were also seen (Figure 5*a*, *d*, *e*) Regarding the interstitial tissue, it was more spaced, containing many vacuoles (Figure 5*d*) multifocal vacuolation of Sertoli cell cytoplasm were also observed, and the tubules were filled with dark acidophilic cells with fragmented nuclei (Figure 5*d*, e) Preserved Sertoli cells with pyramidal elongated nuclei and prominent nucleoli were also seen (Figure 5*e*) we denoted also reduction in the quantity of spermatozoa in the lumen of the seminiferous tubules (Figure 5*c*).

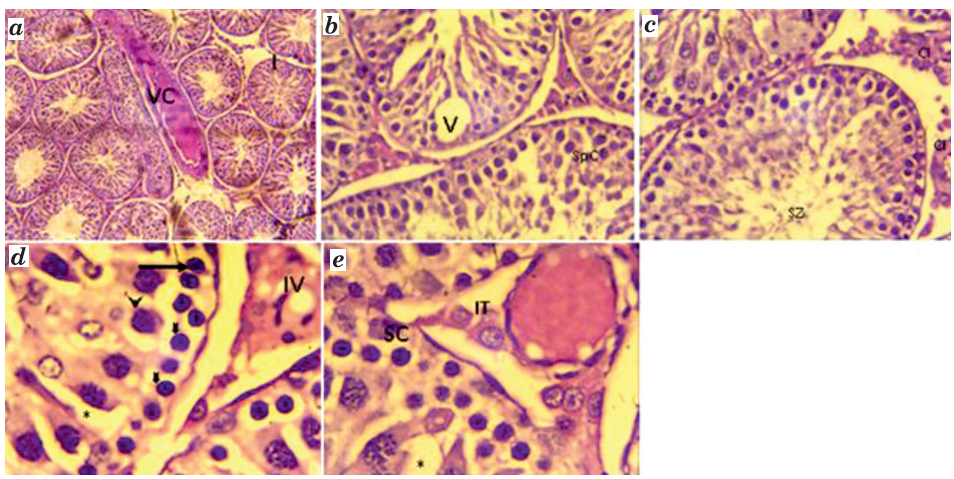

Fig. 5. Cross sections of a rat testis of EMB – treated group, showing: *a* – Irregular contour tubules (I), and vascular congestion (VC), with decreasing size of tubular lumen (L). H&E ×100; *b*, *c* – loss of spermatogenic cells (Spc), vacuolization (V), cell infiltration (CI) and reduction in spermatozoa count (SZ). H&E ×400; *d*, *e* – lacunar spaces (*), basal germ cells with very dark nuclei (arrow), vacuolated spermatogonia (bifid arrows) and vacuolated primary spermatocytes (arrowhead); vacuolated interstitial tissue (IV), sertoli cell (SC); interstitial tissue (IT). H&E ×1000
Source: own study, photo by Assia Kamel, Samira Akdader-Oudahmane and Zohra Hamouli-Said

Periodic Acid-Schiff's (PAS)

The control group showed an apparent strong PAS-positive reaction in the basement membrane of the seminiferous tubules, acrosomal caps of spermatids and spermatozoa, as well as in the interstitial tissue in between the seminiferous tubules (Figure 6*a*) whereas primary and secondary

spermatocytes showed negative affinity. These findings suggested that spermatid and spermatozoa contained more glycogen and carbohydrates. Sections of the EMB group showed a depletion of carbohydrate content, which was concentrated as a faint coloration at the basal lamina and interstitial tissue, an apparent weak PAS-positive reaction in Sertoli cell (Figure 6*b*).

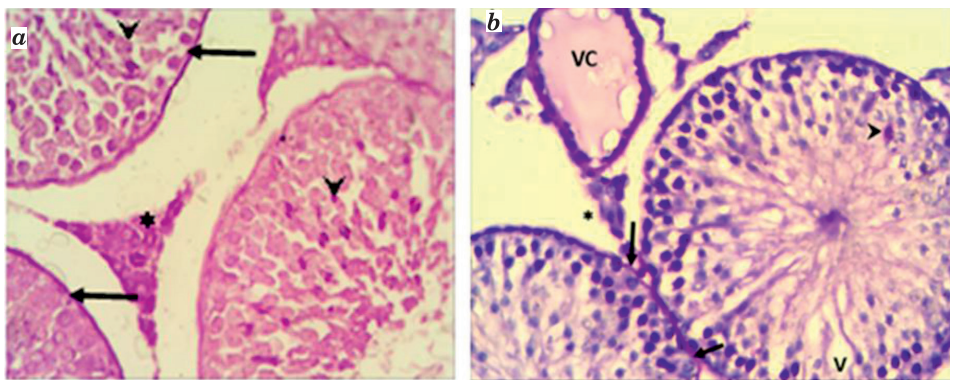

Fig. 6. Cross sections of rat testis from control and EMB – treated groups showing the PAS reaction: *a–b* basal lamina (arrow), acrosomal cap (arrowhead), and interstitial tissue (*); vascular congestion (VC); vacuole (V). PAS ×400; *a* – control, *b* – EMB
Source: own study, photo by Assia Kamel, Samira Akdader-Oudahmane and Zohra Hamouli-Said

Discussion

Emamectin benzoate is a macrocyclic lactone insecticide derived from the avermectin series of natural. The accumulation of avermectin in the environment makes avermectin a most important molecules for toxicological studies (ZHU et al. 2013, MA et al. 2014). The present investigation demonstrated that subchronic administration of emamectin to male Wistar rats for 2 weeks caused some moderate symptoms such as reduced activity, increasing weakness and hair loss.

Although both EMB-treated and control rats exhibited body weight gain during the experimentation period, EMB exposure increased body weight after 2 weeks of treatment. Our findings agree with those of KHALDOUN-OULARBI et al. (2014) who reported that emamectin administration (5 mg/kg BW) for 14 days to rats had a significant increase in body weight. Duration of exposure to EMB and dose may influence the body weight. The stress caused by the pesticide, according to AGBOHESSI et al. (2014), could explain the differences in body weight. Stress affects growth by influencing feeding behavior and food conversion efficiency.

Our results indicate that 15 days treatment with EMB decreased the relative testicular weight. Our findings coincide with the findings of DAWOUD et al. (2021) who showed a decrease in liver, testis, spleen and heart weights in rats treated with EMB (5 mg/kg BW diet) for 8 weeks.

The tissue loss seen in our study, which was characterized by the presence of degenerative lesions, could explain the decrease in relative testicular weight. Furthermore, subchronic administration of emamectin to Wistar male rats for 2 weeks caused significant elevation of malondialdehyde (MDA) and protein carbonyl levels in testis while the activities of CAT and SOD were reduced, which indicated that emamectin could induce oxidative stress. Our findings agree with the findings of TEKELI et al. (2023) who found that EMB dramatically raised MDA concentrations in the testis. Similar findings were reported that emamectin benzoate increased TBARS levels at 28 days in rat liver (EL-SHEIKH and GALAL 2015). These findings also showed consistent results with previous studies in the avermectin group emamectin benzoate and abamectin pesticides made by other authors (ABOUZEID et al. 2017, ZHANG et al. 2020). This could lead to the creation of hypermetabolic state by enhancing mitochondrial respiration, leading to an excess generation of reactive oxygen species (ROS) and the induction of oxidative stress. Indeed, lipid peroxides promote damage to testicular cell membranes, and the testes have been considered susceptible to oxidative damage because of the high content of polyunsaturated fatty acids. On the other hand, the testes possess an elaborate range of enzymatic (SOD, CAT, γGT, G6PD) and nonenzymatic antioxidant defenses (e.g., GSH) to detoxify ROS. The activities of SOD and CAT are related to metabolism of superoxide radicals and hydrogen peroxide. The increase in testicular level of protein carbonyl may be an indication of protein oxidation by ROS. ROS have been shown to alter or inactivate proteins in both reversible and irreversible ways (MANAWADI and KALIWAL, 2010). Inhibition of antioxidant enzymes (CAT, SOD) indicate that cellular defense mechanisms in intoxicated animals failed to protect against the overproduction of ROS (ZHANG and ZHAO 2017, MOHAMED and ABDELRAHMAN 2019).

Our present study revealed that subchronic administration of emamectin to rats caused significant increase in DNA fragmentation and germ cell degeneration in testis of intoxicated animals. DNA is an important germ plasm in organisms, and the damage of DNA can induce cell apoptosis or death (HONG et al. 2017). Apoptosis is responsible for the cytotoxic effect induced by many chemicals characterized by fragmentation of nuclear DNA into internucleosomal fragments (ZHANG et al. 2020, ZHANG and ZHAO 2017). EMB causes both apoptosis and the breakage of both sin-

gle- and double-strand DNA on Tn5B1-4 cells, and it also causes the death of QSG7701 cells, which may occur via mitochondrial-mediated intrinsic apoptotic pathways (LUAN et al. 2017). The apoptosis induced by emamectin may be attributed to ROS generation which in turn causes activation of the mitochondrial-dependent intrinsic pathway resulting in disruption of mitochondrial function and subsequent mitochondrial membrane potential collapse and release of cytochrome-c.

In the current study, the EMB – treated group revealed irregularities in the outline of the seminiferous tubules. These irregularities may be due to tubular shrinkage of the degenerated seminiferous tubules (MOHAMED and ABDELRAHMAN 2019). Moreover, dilated congested blood vessels appeared between the tubules This finding was attributed to EMB toxicity. Thus, vacuolated cytoplasm of Sertoli cells and degenerated cytoplasm of germ cell were detected. These findings were in accordance with previous studies (TAKELI et al. 2023, KHALDOUN et al. 2015). These pathological manifestations could be the result of the increased oxidative damage induced by Avermectin. ROS generated by EMB can damage membrane components of the cell and lead to the leakage of cytoplasmic enzymes, correlated with other alterations at molecular level, involving the DNA fragmentation. Additionally, basal insertion of sperm in between spermatogenic cells may be due to rapid disruption of the junctions between Sertoli and germ cells (ABARIKWU et al. 2010). Some authors attributed this to the change in the proportion of myoid cells and collagen fibers, which may hinder the proper spermatozoa release into the lumen (EL-SHAFAI et al. 2011).

In this work, some seminiferous tubules contained only a few spermatozoa in their lumen. These findings are in agreement with those of KHALDUN et al. (2015). These results can be explained by ROS formation leading to to defect in the process of spermatogenesis, with a decreased number of mature sperm. There seems to be good evidence to suggest a causative chain of events involving, EMB toxicity, oxidative stress, sperm DNA damage, and impaired sperm function. The results of this study affirm the toxicity of Promed ® on laboratory animals. Biochemical and histopathological biomarkers showed the effects of EMB on sperm maturation. However, it is necessary to study the potential detoxification of pesticides by certain medicinal plants and test the phototherapeutic property on the toxic effects of EMB. Study design and short study periods are important limitations. This could have led to an overestimation of the effect. Future research should reconfirm these findings by conducting larger-scale studies.

Conclusion

From this study, it can be concluded that EMB caused structural changes in the testes of adult albino rats and imbalance of the enzymatic antioxidant system associated with massive production of ROS, which cause DNA fragmentation, germ cell degeneration and induced multiple sperm abnormalities, suggesting male infertility. Subchronic exposure of male Wistar rats to emamectin benzoate impairs testicular redox system, which is considered the key molecular mechanism.

Acknowledgements. The authors thank the Algerian Ministry of Agriculture and Rural Development (Direction de la Protection des Végétaux et des Contrôles Techniques, DPVCT) for providing the emamectin benzoate standard.

Accepted for print: 9.09.2024

References

ABARIKWU S.O., ADESIYAN A.C., OYELOJA T.O., OYEYEMI M.O., FAROMBI E.O. 2010. *Changes in sperm characteristics and induction of oxidative stress in the testis and epididymis of experimental rats by a herbicide, atrazine*. Arch. Environ. Contam. Toxicol., 58: 874–882. https://doi.org/10.1007/s00244-009-9371-2.

ABOU-ZEID S.M, ABUBAKR H. O., MOHAMED M. A., EL-BAHRAWY A. 2017. *Ameliorative effect of pumpkin seed oil against emamectin induced toxicity in mic*e. Biomed. Pharmacother., 98: 242. https://doi.org/10.1016/j.biopha.2017.12.040.

AEBI H. 1984. *Catalase in vitro*. Methods. Enzymol., 105: 121–126. https://doi.org/10.1016/s0076-6879(84)05016-3.

AGBOHESSI T.P., TOKO I.I., N'TCHA I., GEAY F., MANDIKI S.N.M., KESTEMONT P. 2014. *Exposure to agricultural pesticides impairs growth, feed utilization and energy budget in African Catfish Clarias gariepinus (Burchell, 1822) fingerlings*. Int. Aquat. Res., 6: 229–243. https://doi.org/10.1007/s40071-014-0083-5.

AITKEN R.J., BROMFIELD E.G., GIBB Z. 2022. *Oxidative stress and reproductive function: The impact of oxidative stress on reproduction: A focus on gametogenesis and fertilization.* Reproduction., 164(6): F79-F94. https://doi.org/10.1530/REP-22-0126.

BIRD R., DRAPER H. 1984. *Comparative studies on different methods of malonaldehyde determination*. Methods. Enzymol., 105: 299–305. https://doi.org/10.1016/s0076-6879(84)05038-2.

BRADFORD M. 1976. *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein- dye binding*. Anal. Biochem., 72: 248–254. https://doi.org/10.1006/abio.1976.9999.

DAWOUD S.F., AL-AKRA T.M., ZEDAN A.M. 2021. *Hepatoprotective effects of chitosan and chitosan nanoparticles against biochemical, genetic, and histological disorders induced by the toxicity of emamectin benzoate.* Rep. Biochem. Mol Biol., 10(3): 506. https://doi.org/10.52547/rbmb.10.3.506.

EL-SHAFAI A., ZOHDY N., EL- MULLA K., HASSAN M., MORAD N. 2011. *Light and electron microscopic study of the toxic effect of prolonged lead exposure on the seminiferous tubules of albino rats and the possible protective effect of Ascorbic Acid*. Food. Chem. Toxicol., 49: 734–743. https://doi.org/10.1016/j.fct.2010.11.033.

El-Sheikh S.A., Galal A.A. 2015. *Toxic effects of sub-chronic exposure of male albino rats to emamectin benzoate and possible ameliorative role of Foeniculum vulgare essential oil*. Environ Toxicol. Pharmacol., 39: 1177–1188. https://doi.org/10.1016/j.etap.2015.04.008.

Hong Y.X., Yang G., Yan Y., Huang F., Zuo Y., Shen Y., Ding., Cheng Y. 2017. *Effects of glyphosate on immune responses and haemocyte DNA damage of Chinese mitten crab, Eriocheirsinensis*. Fish Shellfish. Immunol., 71: 19–27. https://doi.org/ 10.1016/j.fsi.2017.09.062.

Khaldoun O.H., Allorgec D., Richevalc C., Lhermittec M., Djenase N. 2015. *Emamectin benzoate (Proclaim®) mediates biochemical changes and histopathological damage in the kidney of male Wistar rats. (Rattus norvegicus)*. Toxicol Anal. Clin., 27(2): 72–80. https://doi.org/10.1016/j.toxac.2014.11.002.

Khaldoun O.H. 2014. *Biochemical and histopathological changes in the kidney and adrenal gland of rats following repeated exposure to Lambda-Cyhalothrin*. J. Xenobiot., 4(1): 2240. https://doi.org/10.4081/xeno.2240.

Latchoumycandane C., Mathur P. 2002. *Induction of oxidative stress in the rat testis after short-term exposure to the organochlorine pesticide methoxychlor*. Arch. Toxicol., 76: 692–698. https://doi.org/10.1007/s00204-002-0388-9.

Levine R.L., Garland D., Oliver C.N., Amici A., Climent I., Lenz A.G., Stadtman E.R. 1990. *Determination of carbonyl content in oxidatively modified proteins*. Methods. Enzymol.,186: 464–478. https://doi.org/10.1016/0076-6879(90)86141-h.

Luan S.X., Yun W., Rao C., Xiao Z., Xu J., Lang., Huang Q. 2017. *Emamectin benzoate induces ROS-mediated DNA damage and apoptosis in Trichoplusia Tn5B1-4 cells*. Chem. Biol. Interact., 273: 90–98. https://doi.org/10.1016/j.cbi.2017.06.004.

Ma J., Zhou C., Li Y., Li X. 2014. *Biochemical responses to the toxicity of the biocide abamectin on the freshwater snail Physa acuta*. Ecotoxicol. Environ. Saf., 101: 31–35. https://doi.org/10.1016/j.ecoenv.2013.12.009.

Manawadi S., Kaliwal B. B. 2010. *Methomyl induced alteration in mice hepatic-oxidative status. Int J Biotechnol*. Appl. International., 2(2): 11–19. http://dx.doi.org/10.9735/0975-2943.2.2.11-19

Misra H.P., Fridovich I. 1972. *The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase*. J. Biol. Chem., 247(10): 3170–3175.

Mohamed D.A., Abdelrahman S.A. 2019. *The possible protective role of zinc oxide nanoparticles (ZnOnPs) on testicular and epididymal structure and sperm parameters in nicotine-treated adult rats (a histological and biochemical study)*. Cell Tissue. Res., 375: 543–558. http://dx.doi.org/10.1007/s00441-018-2909-8.

Mushtaq M., Syintsakos L., Krieter P., Colletti A., Arison B,. Crouch L,. Wislocki P. 1996. *Absorption, tissue distribution, excretion, and metabolism of 3H and 14C-labeled emamectin benzoate in rats*, J. Agric. Food. Chem., 44: 3342–3349.

Parrón T., Requena M., Hernández A.F., Alarcón R. 2011. *Association between environmental exposure to pesticides and neurodegenerative diseases*. Toxicol. Appl. Pharmacol., 256(3): 379–385. https://doi.org/10.1016/j.taap.2011.05.006.

Perandones C.E., Illera V.A., Peckham D., Stunz L.L., Ashman R.F.1993. *Regulation of apoptosis in vitro in mature murine spleen T cells*. J. Immunol., 151: 3521–3529.

Tekeli M.Y., Eraslan G., Bayram L.Ç., Aslan C., Çalimli S. 2023.*The protective effects of baicalin and chrysin against emamectin benzoate-induced toxicity in Wistar albino rats*. Environ. Sci. Pollut. Res. Int., 30(18): 53997–54021. https://doi.org/10.1007/s11356-023-26110-5.

Temiz Ö. 2020. *Biopesticide emamectin benzoate in the liver of male mice: evaluation of oxidative toxicity with stress protein, DNA oxidation and apoptosis biomarkers*. Environ. Sci. Pollut. Res. Int ., 27(18):23199-23205. https://doi.org/10.1007/s11356-020-08923-w.

Wang X., Piao X., Chen J., Hu J., Xu F. Tao. 2006. *Organochlorine pesticides in soil profiles from Tianjin. China*. Chemosphere., 64(9):1514–1520 https://doi.org/10.1016/j.2005.12.052.

Wolterink G., Van Kesteren P., McGregor D. 2012. *Emamectin benzoate. Pesticide residues in food*. 2011, p. 211.

Yun W., Rao C., Xiao C., Huang Q. 2017. *Apoptosis of leukemia K562 and Molt-4 cells induced by emamectin benzoate involving mitochondrial membrane potential loss and intracellular Ca2+ modulation*., Environ. Toxicol. Pharmacol., 2: 280–287. https://doi.org/10.1016/j.etap.2017.04.013.

ZHANG Z., ZHAO X., QIN X. 2017. *Potential genotoxic and cytotoxicity of emamectin benzoate in human normal liver cells. Oncotarge*t., 8(47): 82185. https://doi.org/10.18632/oncotarget.18988.

ZHANG Y., KONG C., CHI H., LI J., XING J., WANG F., ZHAI, Q. 2020. *Effect of a beta-cypermethrin and emamectin benzoate pesticide mixture on reproductive toxicity in male mice in a greenhouse environment.* Toxicol. Mech. Methods., 30(2): 100–106. https://doi.org/10.1080/15376516.2019.1669241.

ZHU W.J., LI M., LIU C., QU J.P., MIN Y.H., XU S. W., LI S. 2013. *Avermectin induced liver injury in pigeon: mechanisms of apoptosis and oxidative stress.* Ecotoxicol. Environ. Saf., 98, 74–81. https://doi.org/10.1016/j.ecoenv.09.021.

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 39(3): 167–185, Y. 2024

DOI: 10.31648/pjns.10023

EFFECT OF EXTENDER AND DILUTION RATIO ON THE SPERM MOTILITY, VIABILITY, AND EGGS FERTILITY OF *CLARIAS BATRACHUS*, LINNAEUS 1758 (PISCES: CLARIIDAE)

***Luvi S. Handayani*[1], *Siti Maulida*[2], *Sri R. Rahayu*[3], *Nanda M. Razi*[4], *Mehmet Kocabas*[5], *Filiz K. Kocabas*[6], *Kartini Eriani*[7], *Nur Fadli*[8], *Moh N. Siti-Azizah*[9], *Kasi Marimuthu*[10], *Zainal A. Muchlisin*[11]**

[1] ORCID: 0000-0002-1329-5376
[2] ORCID: 0000-0002-8417-2541
[5] ORCID: 0000-0002-7934-6500
[6] ORCID: 0000-0001-8334-5802
[7] ORCID: 0000-0002-9063-5944
[8] ORCID: 0000-0001-6845-1383
[9] ORCID: 0000-0002-9890-9612
[10] ORCID: 0000-0002-0825-6583
[11] ORCID: 0000-0002-0858-1853

[1–4, 11] Graduate School of Mathematics and Applied Sciences
Universitas Syiah Kuala, Banda Aceh, Indonesia
[5] Department of Wildlife Ecology and Management
Faculty of Forestry
Karadeniz Technical University Trabzon, Turkey
[6, 11] Fisheries Faculty
Munzur University, Tunceli, Turkey
[7] Department of Biology, Faculty of Mathematics and Natural Sciences
Universitas Syiah Kuala, Banda Aceh, Indonesia
[8] Department of Aquaculture, Faculty of Marine and Fisheries
Universita Syiah Kuala, Banda Aceh, Indonesia
[9] Institute of Marine Biotechnology and Climate Adaptation
University Malaysia Terengganu, Malaysia
[10] Department of Environmental Sciences
Tezpur Central University, Tezpur 784028, India

Key words: short-term storage sperm, sperm quality parameters, fish breeding, *Clarias batrachus*.

Address: Zainal A. Muchlisin, Graduate School of Mathematics and Applied Sciences Universitas Syiah Kuala, Banda Aceh, Indonesia, e-mail: muchlisinza@usk.ac.id

Abstract

The study aims to determine the type of extender and the best dilution ratio for short preservation walking catfish *Clarias batrachus* sperm at a refrigeration temperature of 4°C. The study employed a completely randomized design with two factors, each consisting of 6 treatments and 3 replications. The experiments were divided into 2 stages: the first stage tested several types of extenders, namely tap water, Ringer's solution, physiological solution, Alsever's solution, urea solution, and glucose base solution, all at the same dilution level of 1:20 (sperm: extender, v/v). The second stage examined various levels of sperm dilution ratios, namely 1:10, 1:20, 1:30, 1:40, 1:50, and 1:60 (sperm: extender, v/v). The samples were stored at a refrigerated temperature of 4°C. Furthermore, motility and viability were monitored at 24-hour intervals for next 6 days, and on the final day of the experiment. The ANOVA test showed that the type of extender and dilution ratio significantly affected the motility and viability of the spermatozoa and percentage of fertilization ($P < 0.05$). Generally, the quality of sperm decreased after preservation for 144 hours at 4°C, but diluted with Ringer solution at a ratio of 1:40 (sperm: extender, v/v), still maintains good parameters. Therefore, it is recommended that Ringer's solution at a dilution ratio of 1:40 is suitable for preserving walking catfish *C. batrachus* sperm at a refrigerated temperature of 4°C.

Introduction

Walking catfish *Clarias batrachus* (LINNAEUS 1758), is a freshwater fish successfully cultivated in Indonesia and several other countries, such as Malaysia, Thailand, India, Bangladesh, Singapore, Philippines, Myanmar, and Sri Lanka (ADAN 2000, KHAN et al. 2000, DAS 2002, ARGUNGU et al. 2013, HARDI et al. 2018). Several reasons limit the cultivation of this local catfish by fish farmers, including difficulty in obtaining good quality larvae due to underdeveloped breeding technology and the relatively slow growth compared to the African catfish, resulting in increased production costs (CANOLA 2010, SAPTADJAJA et al. 2020).

The availability of high-quality broodstock is essential in breeding programs resulted the good quality of larvae (MUCHLISIN et al. 2006). The spawning season of catfish occurs once a year in the wild, namely at the beginning of the rainy season (JOTHILAKSHMANAN and MARX 2013). In addition to the unsynchronized gonad maturation between male and female, there were some limitations (RAJAKUMAR and SENTHILKUMARAN 2014). The male broodfish has to be sacrificed during catfish-induced breeding to collect the sperm. Furthermore, the catfish sperm is usually very thick and limited in volume (MARIMUTHU et al. 2019, ENZELINE et al. 2022), indicating it needs a diluent or extender. The extender increases the volume of sperm fluid, reducing its density and allowing sperm to survive longer. It is needed for short and long-term sperm preservation by refrigeration (non-freezing) and freezing (cryopreservation) techniques.

The suitability of the extender for fish sperm needs to be studied because every sperm cell of a particular fish species has different pH, density, and electrolyte composition characteristics, causing it to respond differently to types of extenders (MUCHLISIN and SITI-AZIZAH 2010, MARIMUTHU et al. 2019, MAULIDA et al. 2022). Therefore, the extender should have the same or similar osmotic pressure as the sperm (isotonic) to keep the sperm cells immotile during the storage process (TAKEI et al. 2015, YANG et al. 2017). Sperm diluents should also provide spermatozoa with nutrients for aerobic and anaerobic metabolic processes (BAROZHA 2015), contain lipoprotein or lecithin to protect against temperature shock, and maintain pH stability (VERA-MUNOZ et al. 2009, DE SOUZA ANDRADE et al. 2014, BERNÁTH et al. 2022). Using inappropriate extenders and concentrations can have adverse effects on sperm physiology, such as osmotic shock (CUEVAS-URIBE and TIERSCH 2011). Several extenders commonly used in artificial fish breeding include Ringer's solution tested in depik *Rasbora tawarensis* sperm (ERIANI et al. 2021), artificial seminal plasma (ASP) in grouper *Epinephelus bruneus* (LIM and LE 2013) and seurukan fish *Osteochilus vittatus* (ADAMI et al. 2016), as well as glucose base for sperm of the climbing perch *Anabas testudineus* (MAULIDA et al. 2022).

Studies on the suitable type of extender and dilution ratio for the artificial breeding of local catfish *C. batrachus* have not been investigated. Furthermore, the sperm dilution ratio should be determined because fish sperm is usually highly dense. A high sperm density inhibits the activity of spermatozoa as they complete together to penetrate the micropile of the egg for fertilization, which occurs at a low rate (ALAVI and COSSON 2006, BERNÁTH et al. 2022). It can also affect the physiological processes and sperm cell respiration during storage, reducing its quality (DZYUBA et al. 2019, FIGUEROA et al. 2019).

The dilution ratio in fish sperm preservation has been studied. For example, dilution ratio of 1:100 and 1:60 (sperm: extender) produces better results in sperm-striped trumpeter *Latris lineata* (RITAR and CAMPET 2000). MUCHLISIN et al. (2004) reported that 1:20 is suitable for the African catfish *C. gariepinus* and baung fish *Mystus nemurus*. These previous studies indicate that the suitability of the dilution ratio depends on the species. However, the suitability of walking fish sperm has not been reported. Therefore, this study aims to determine the suitability of the extender type and its dilution ratio for walking catfish *C. batrachus* sperm.

Materials and Methods

Experimental design

This study employed a completely randomized design with 6 treatments of extenders and 3 replications. It consisted of two experiments: (*a*) testing six types of extenders to determine the best quality of walking catfish sperm, and (*b*) testing six levels of dilution ratio using the extender from the experiment (*a*) to determine the best one. The extenders tested were tap water, Ringer's solution, physiological solution, Alsever's solution, urea solution, and glucose base solution at the same dilution ratio (1:20, sperm:extender, v/v). After identifying the best type of extender, six levels of dilution ratios, namely 1:10, 1:20, 1:30, 1:40, 1:50, 1:60 (sperm: extender, v/v) were tested.

Broodfish and sperm collection

A total of 60 male and 40 female broodstocks with lengths and body weights of approximately 25–30 cm and 300–500 g, respectively, were obtained from fishermen in South Aceh, Nagan Raya, and Aceh Besar (Figure 1). They were weaned and acclimatized for 14 days in a broodfish pond at the Fish Breeding and Hatchery Laboratory, Faculty of Marine Affairs and Fisheries, Universitas Syiah Kuala. During adaptation, the broodfish were fed on a commercial diet with a crude protein content of more than 30% twice daily (8 a.m. and 5 p.m.) ad libitum.

After 2 weeks, five mature male broodstocks weighing 300–500 g each were selected from the broodstock pond. They were injected with Ovaprim (Syndel A024, Canada) at a dose of 0.5 ml/kg body weight with a single injection at 5 p.m., after which the fish were kept in a 150 L container for 10 hours. The sperm of male catfish cannot be collected using stripping techniques. Thus, the male was sacrified by dissecting the abdominal area of the fish, and the testes were removed from the body cavity and then chopped to sequize the sperm. Subsequently, the sperm were collected using a syringe separately for each male. The sperm were then placed in styrofoam with crushed ice (4°C). The fresh sperm was analyzed both macroscopically and microscopically. Finally, those with motility > 60% were pooled in a tube for use in the experiment.

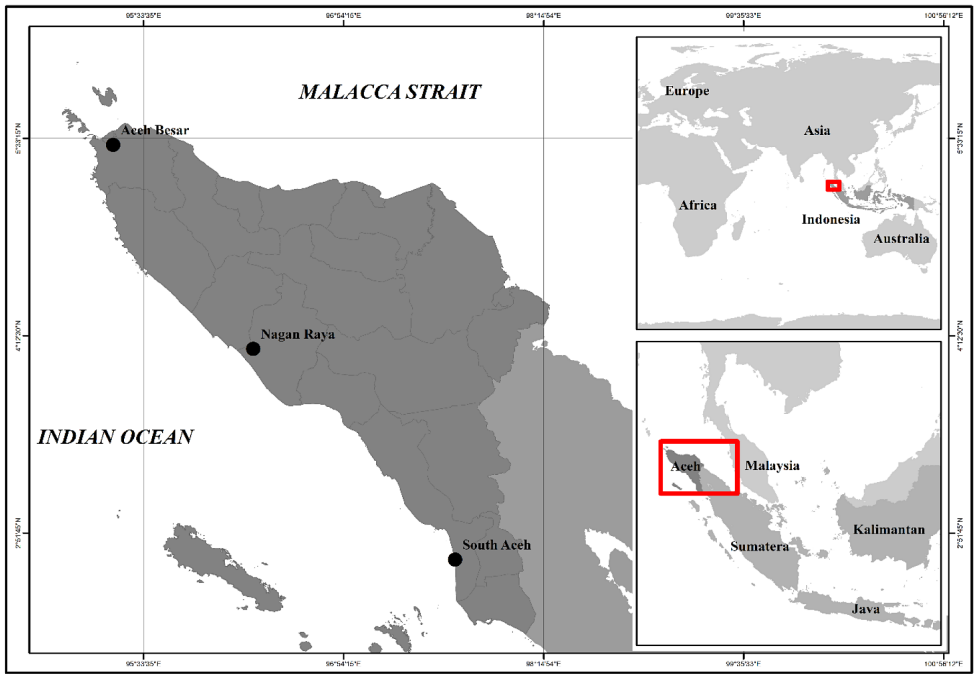

Fig. 1. The map of Aceh province Indonesia showing the location of broodfish origin at South Aceh, Nagan Raya and Aceh Besar Regencies

Extender preparation

Ringer's solution was formulated according to MUCHLISIN et al. (2004). Meanwhile, Alsever's, urea, and glucose base solutions were prepared following the standard procedures proposed by HOSSEN et al. (2017). The composition of the materials used for each extender is presented in Table 1.

Table 1

Chemical composition of the used extenders

Materials	Extender					
	tap water	ringer	physiological	alsever's	urea	glucose base
NaCl [g]	–	0.75	0.798	0.4	0.3	0.725
KCl [g]	–	0.02	–	–	–	0.04
$CaCl_2$ [g]	–	0.02	–	–	–	–
$NaHCO_3$ [g]	–	0.02	0.02	–	–	0.080
Glukosa [g]	–	0.5	0.5	–	–	0.20
$Na_3C_6H_5O_7$ [g]	–	–	–	0.8	–	–
Urea [g]	–	–	–	–	0.4	–
pH	7.2	8.1	7	9.0	8.7	8.3

Sperm preservation

In the first experiment, sperm was diluted with each extender at the same diluent ratio of 1:20 (sperm: extender, v/v). A total of 6 Erlenmeyer tubes (vol. 50 ml) were filled with 0.5 ml of sperm and mixed with 10 ml of the extender. The diluted sperm was divided into 18 cryotubes (vol. 1.25 ml).

In the second experiment, 6 Erlenmeyer tubes (vol. 50 ml) were filled with 0.5 ml of sperm. Additionally, 5 ml, 10 ml, 15 ml, 20 ml, 25 ml, and 30 ml of the best extender from experiment (a) were added for treatments A (1:10), B (1:20), C (1:30), D (1:40), E (1:50), and F (1:60), respectively. The sperm diluted with the tested extender was then distributed into cryotubes (vol. 1.25 ml), with each treatment performed with 3 replications. The cryotubes were refrigerated at 4°C, and sperm motility and viability were measured every 24-hour intervals for the next 6 days.

Macroscopic and microscopic analysis

The quality of fresh post-preserved sperm was analyzed macroscopically and microscopically. Macroscopic evaluation included color, sperm pH was measured using a pH meter (Lutron pH-222, Taiwan), sperm consistency was assessed by measuring the flow rate via a pooling tube based on (MAULIDA et al. 2024), and fertility rate, while microscopic evaluation included sperm concentration used haemocytometer method, motility, and viability rates. The preserved sperm were observed for motility and viability for 144 hours at 24-hour intervals, and the fertility was assessed at the

end of the experiment. The visualization of motility and viability of sperm was conducted using a stereo microscope (Zeiss Primo Star, Primostar 1, Fix-K., Bi, SF20, Switzerland) connected to a CCD camera with 400× magnification.

A total of 10 µl of sperm was dropped onto a glass slide and covered with a cover glass, then one drop of tap water was added at the edge to activate the sperm cells. The motility rate was determined by examining at least 200 randomly selected sperm cells. Motile spermatozoa were identified as those moving agilely forward (MUCHLISIN et al. 2004). Sperm motility was recorded for two minutes in five fields of view at the four corners and the center of the slide using recording software (Optilab Viewer 3.0). The recorded data was saved and calculated later. Furthermore, the following describes the process of using the 0.2% eosin staining method to observe the viability rate. Ten microliters of sperm were dripped onto a glass slide, and 0.2% eosin dye was added in a ratio of 1:1 (v/v). They were mixed evenly, and a smear sample was prepared under a stereo microscope with 400× magnification. The viability percentage was calculated by observing a minimum of 200 sperm in every 5 field views. Viable sperm were characterized by transparent and round sperm heads, while dead sperm were characterized by opaque pink and irregularly shaped heads (MAULANA and JUNIOR 2014).

Fertilization

A total of two mature female broodstock (weighing 300–500 g) were selected from the pond. The fish were injected with 0.5 ml/kg body weight of Ovaprim and kept in 150 L container box until ovulation. Subsequently, gentle pressure the fish's abdomen was to the genital pore, and the eggs were polled in a 100 ml beaker and placed in styrofoam containing crushed ice cubes (4ºC).

Post-preservation sperm of 0.5 ml was mixed with 0.5 ml of eggs (1:1 v/v), and 2 drops of tap water were added for activation. The mixture was stirred using a soft chicken feather and left for 5 minutes to allow fertilization of the eggs. Subsequently, 100 eggs were randomly selected and incubated in a glass tank at 27°C. Fertilization success was calculated after 4 hours of mixing sperm and eggs. Fertilized and unfertilized eggs appear transparent and white cloudy, respectively (MUCHLISIN et al. 2015, MUTHMAINNAH et al. 2018). The following formula was used to calculate fertility rate: Fertility rate [%] = (total number of fertilized eggs/total number of incubated eggs) x100.

Data analysis

Data on pH, color consistency, and sperm concentration were tabulated and then analyzed descriptively. Meanwhile, motility, viability, and fertility data were examined for normality and subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS software (ver. 22.0) to determine the best treatment.

Results

According to the macroscopic analysis, the fresh sperm of the walking catfish was milky white at a pH of 6 and had high consistency. Meanwhile, the microscopic analysis showed a concentration of $76.25 \cdot 10^9$ cells mL^{-1} with motility, viability, and fertility of 85.16%, 83.16%, and 82.00%, respectively, as shown in Table 2.

The morphology of live sperm is transparent, while dead spermatozoa appear red because they absorb the eosin dye (Figure 2a and Figure 2b). The fertilized oocytes are transparent, with a vitelline membrane inside, while unmatured oocytes had a cloudy white color, as presented in Figure 2c and Figure 2d.

Table 2

The characteristics of the fresh sperm of *Clarias batrachus*

Parameter	Descriptions
Volume [ml/ fish]	0.35 ±0.13 ml
Color	milky-white
pH	6.0 ±0.5
Consistency	high consistency
Concentration [cells ml^{-1}]	$76.33 \pm 1.04 \cdot 10^9$
Motility [%]	85.16 ±0.28
Viability [%]	83.16 ±0.57
Fertility [%]	82.00 ±0.50

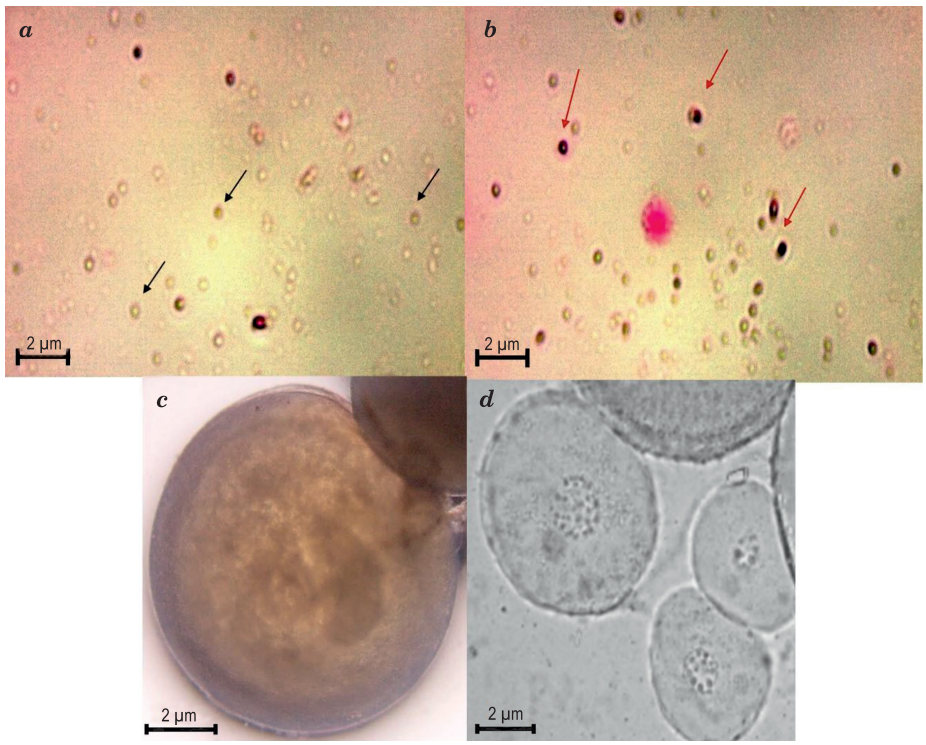

Fig. 2. (*a*, *b*) The appearance of the life sperm (black arrows) and dead sperm (red arrows), (*c*) fertilized oocytes, and (*d*) unmatured oocytes at 400× magnifications

The first experiment: evaluation of type of extenders

The results showed that the initial sperm motility and viability ranged from 79.16 to 83.50% and 75.00 to 80.50%, respectively. After 24 hours of preservation at 4ºC, the motility and viability decreased to 72.50% and 68.50% in tap water, 75.66% and 72.50% in Ringer's solution, 73.16% and 68.50% in physiological solution, 61.50% and 58.16% in Alsever's solution, 62.50% and 60% in urea solution, as well as 75.33% and 71.33% in the glucose base solution. Similar trends were also recorded in the 48 h to 144 h, as shown in Figure 3.

The ANOVA test on sperm quality after 144 hours of preservation at 4ºC showed that the extender significantly affected the motility, viability, and fertility of walking catfish *C. batrachus*. The highest percentages of motility, viability, and fertility, at 43.83%, 41.83%, and 44.16%, respectively, were obtained in Ringer's solution. These values were significantly different from other treatments, while the lowest motility of 14.5% was discovered in Alsever's solution, as shown in Table 3.

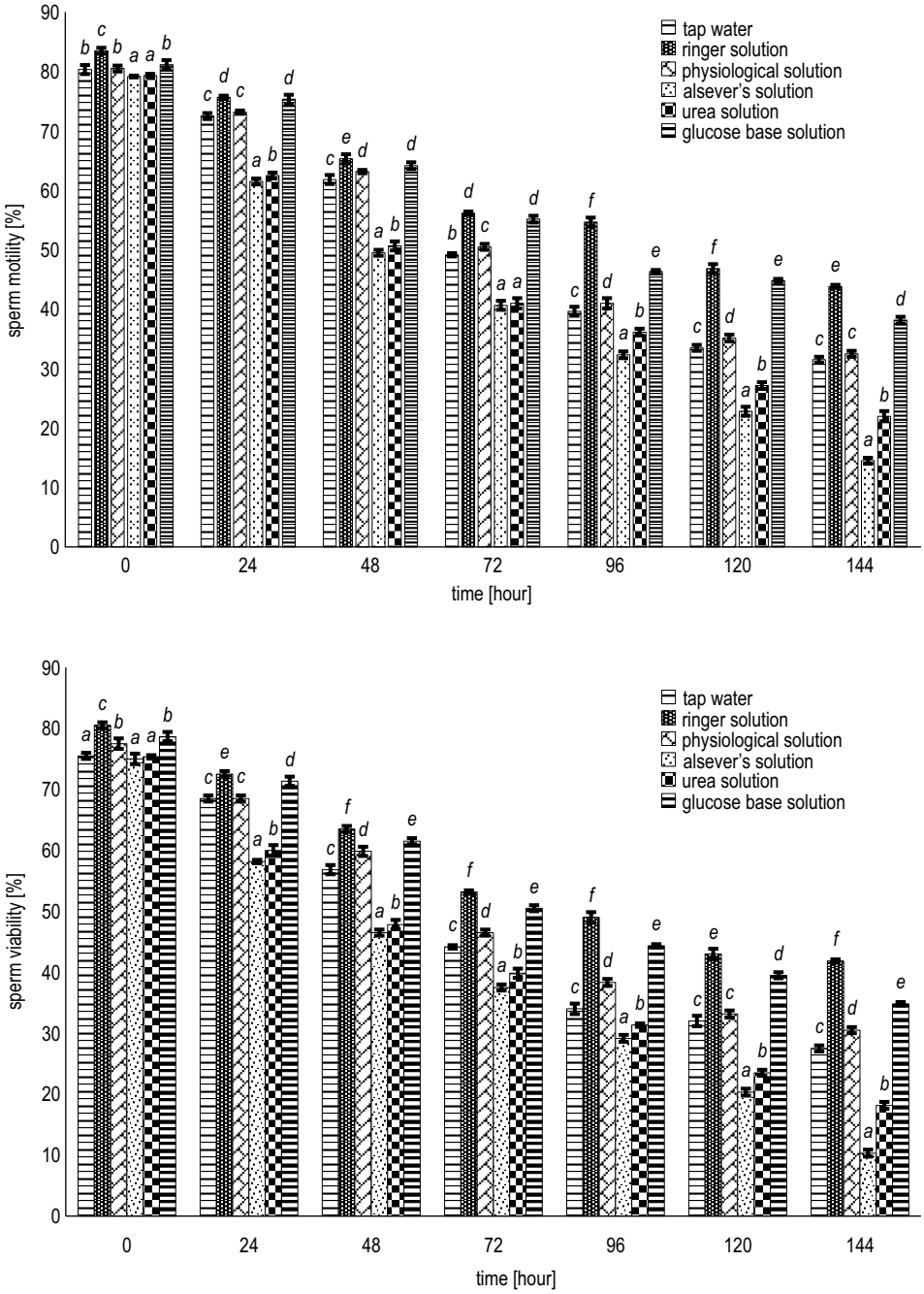

Fig. 3. The motility and viability trends of *Clarias batrachus* preserved in several types of extenders at a refrigerated temperature of 4°C

Table 3

The motility, viability and fertility of walking catfish *C. batrachus* sperm post preservation for 144 hours at 4°C according to extenders. The mean value ±SD in the columns with different superscripts is significantly different ($P < 0.05$)

Extender	Motility [%]	Viability [%]	Fertility [%]
Tap water	31.50 ±0.50^{c}	27.50 ±0.50^{c}	30.50 ±0.50^{c}
Ringer's solution	43.83 **±0.28**^{e}	41.83 **±0.28**^{f}	44.16 **±0.57**^{f}
Physiological solution	32.50 ±0.50^{c}	30.50 ±0.50^{d}	37.16 ±0.76^{d}
Alsever's solution	14.50 **±0.50**^{a}	10.33 **±0.57**^{a}	20.33 **±0.28**^{a}
Urea solution	22.00 ±0.86^{b}	18.16 ±0.57^{b}	24.16 ±0.28^{b}
Glucose base solution	38.16 ±0.57^{d}	34.83 ±0.28^{e}	41.50 ±0.50^{e}

The second experiment: evaluation of dilution ratio

The results showed that the initial sperm viability ranged from 72.83% to 78.83% and 73.83% to 79.50%. After 24 hours of preservation at 4°C, the motility and viability decreased to 70.00% and 57.83%, 76.16% and 70.50%, 73.83% and 60.00%, 77.50% and 71.33%, 75.33% and 66.50%, and 74.00% and 66.16% in 1:10, 1:20, 1:30, 1:40, 1:50, and 1:60 dilution ratios, respectively. The same trends were also recorded from 48 hours to 144 hours, as presented in Figure 4. The dilution ratio significantly affected the motility, viability, and fertility of walking catfish *C. batrachus*. The highest percentage were obtained at 1:40, with 42.83%, 40.16%, and 42.66% for motility, viability, and fertility, respectively. These values were significantly different from other treatments (Table 4).

Table 4

The parameters of walking catfish *C. batrachus* sperm post preservation for 144 hours at 4°C according to dilution. The mean value ±SD in the columns with different superscripts is significantly different ($P < 0.05$)

Dilution ratio (v/v)	Motility [%]	Viability [%]	Fertility [%]
1:10	12.66 **±0.28**^{a}	10.50 **±0.50**^{a}	18.33 **±0.28**^{a}
1:20	37.16 ±0.57^{e}	33.83 ±0.28^{e}	41.66 ±0.28^{e}
1:30	33.16 ±0.57^{c}	27.66 ±0.76^{c}	30.83 ±0.28^{c}
1:40	42.83 **±0.28**^{f}	40.16 **±0.28**^{f}	42.66 **±0.76**^{f}
1:50	35.33 ±0.28^{d}	30.50 ±0.50^{d}	38.50 ±0.50^{d}
1:60	22.83 ±0.76^{b}	19.16 ±0.57^{b}	24.00 ±0.86^{b}

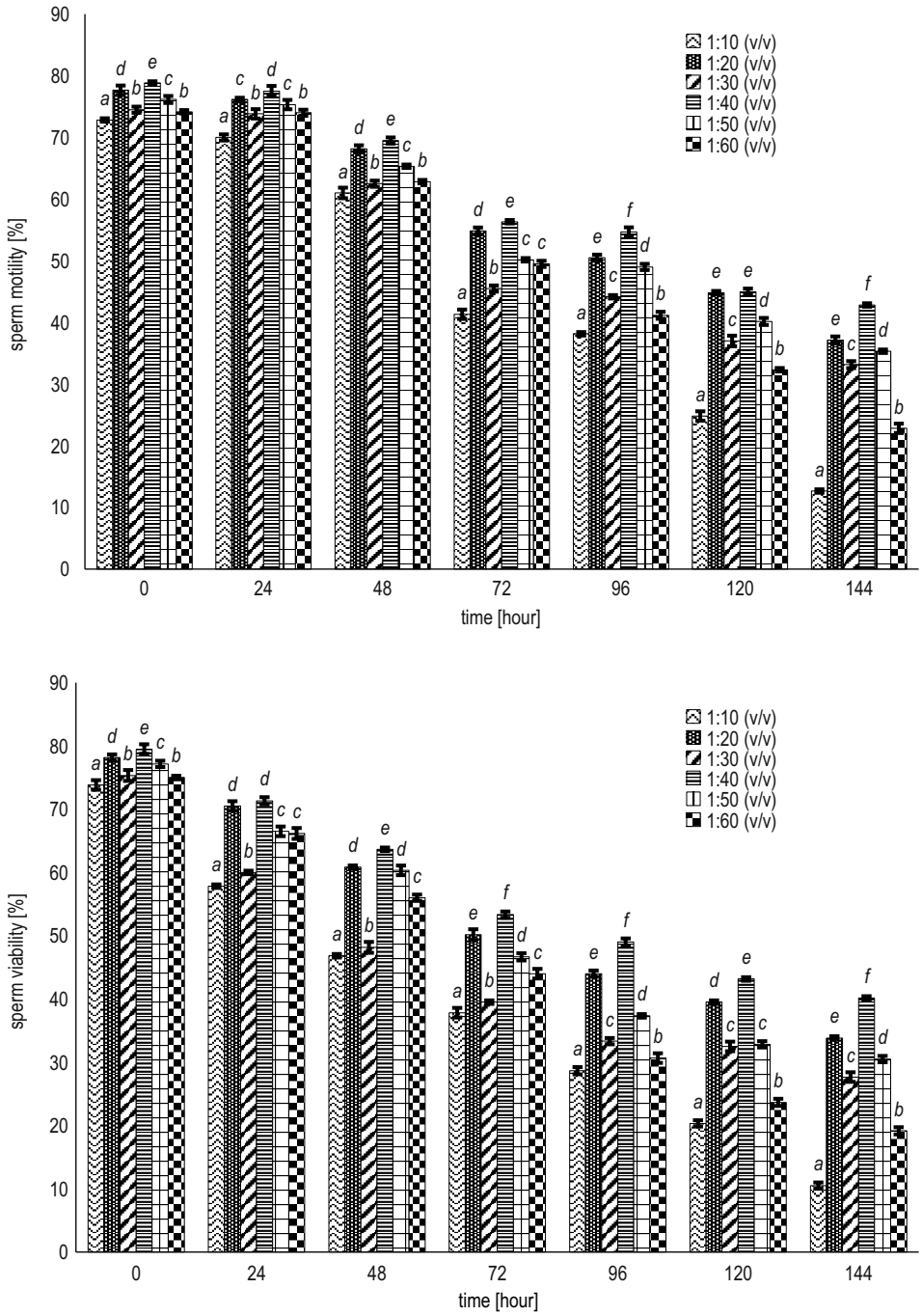

Fig. 4. The motility and viability trend of the *Clarias batrachus* sperm preserved at refrigerated temperature (4°C) for 144 hours according to dilution ratio

Discussion

The fresh sperm of the walking catfish *C. batrachus* appeared milky white at a pH of 6, exhibiting high consistency with initial motility, viability, and fertility of 85.16%, 83.16%, and 82.0%, respectively. Therefore, its quality is suitable for use in the preservation process. This aligns with the findings of MAULIDA et al. (2021) and MELO and GODINHO (2018), which stated that fresh fish sperm suitable for storage should have motility and viability above 70%. This study revealed a decreased in sperm quality after refrigeration preservation for 144 hours at 4°C in all treatments. However, sperm diluted with Ringer's solution could maintain motility, viability, and fertility better than other tested extenders. According to ALAVI et al. (2006), the quality of post-storage fish spermatozoa motility dependens on the osmolality of the dilution medium (extender), which is determined by its ion concentration. At high osmotic pressures, the mixing of sperm with extenders creates an imbalance in the plasma membrane due to variations in osmolality between the intracellular and extracellular fluids. This occurs because of water penetration, which then triggers sperm motility. Furthermore, Na^+ and Cl^- ions are the main electrolytes that play an essential role in maintaining the osmolality of the plasma and subsequently affect the level of viability and motility (ALAVI et al. 2004).

The best results were obtained from Ringer's solution due to its ionic composition and the concentration suitable for the sperm of walking catfish. Therefore, it has a more complete ionic composition than the other tested extenders, positively affecting sperm during preservation. The suitability of the solution for dilution has been reported in several fish. Examples include the sperm of bagrid catfish *Mystus nemurus* (MUCHLISIN and AZIZAH 2009), seurukan fish *Osteochillus vittatus* (MUTHMAINNAH et al. 2018), depik fish *Rasbora tawarensis* (MUCHLISIN et al. 2020), African catfish *C. gariepenus* (MAHFUDHAH et al. 2020), and naleh *Barbonymus* sp (MAULIDA et al. 2021). In addition to Ringer's solution, glucose base also had a good performance with slightly lower motility, viability, and fertility values. Previous studies, such as ABINAWANTO and LESTARI (2013) and HOSSEN et al. (2017), reported the suitability of the glucose base as an extender for several species of fish sperm. The cryopreservation of *Barbonymus gonionotus* sperm was conducted, where it was reported that the glucose base was suitable for storing this fish's sperm. Good performance was also experienced in the refrigeration storage of climbing perch *Anabas testudineus* sperm (MAULIDA et al. 2022). Furthermore, SAHIN et al. (2013) reported that glucose base is the best extender in refrigeration preservation of *Onchorynchus mykiss* sperm.

It was suspected that glucose compounds in the extender solution played an essential role in maintaining the sperm quality of fish. They were present in Ringer's solution, physiological solution, and glucose base with an average value of 0.5 to 0.20 g. However, there was no glucose in the urea and Alsever's solutions, as shown in Table 1. Several studies revealed that glucose plays an essential role in protecting spermatozoa from damage (Ciereszko et al. 2014, Domagała et al. 2014, Judycka et al. 2016, Judycka et al. 2018, Di Iorio et al. 2019). Sugar compounds act as extracellular cryoprotectants that function as external protection for sperm cells from temperature shock (Curcio et al. 2015, Behnamifar et al. 2021). This compound is often used in the frozen storage (cryopreservation) of fish sperm Muchlisin and Azizah 2009, Irawan et al. 2010, Muthmainnah et al. 2018). Glucose also plays an essential role in the non-freeze storage process (refrigeration), as recorded in this study. The quality of sperm diluted with the extenders containing sugar is better than urea solution and Alsever's solution without having compounds.

In Ringer's solution, Na^+, K^+, and Ca^{++} ions play a crucial role. These ions maintain the osmolality of the diluent to balance the sperm plasma and the structure and function of spermatozoa (Beirão et al. 2019, González-López et al. 2020). The osmotic pressure of a good extender is equal to and close to inside the sperm cell (isotonic), making the sperm immotile during the storage process. The fish sperm fluid (seminal plasma) contains Na^+, K^+, Zn^+, Ca^{++}, Mg^{++} ions, and energy substrates such as fructose, sorbitol, and glycerophosphocholine, as well as organic compounds including citric acid, amino acid peptides, proteins, lipids, hormones, and cytokines (Juyena and Stelletta 2012, Bustamante-González et al. 2016, Rashid et al. 2019). As explained above, the lowest motility, viability, and fertility values were identified in Alsever's solution. The chemical composition of this extender was 0.4 g NaCl and 0.8 sodium citrate ($Na_3C_6H_5O_7$). Therefore, the solution contains only Na^+ ions, hence, it does not effectively maintain sperm quality during storage. Shahriar et al. (2014) stated that combining Alsever's with 10% DMSO produced better cryopreservation of sperm from climbing perch *A. testudineus*. Therefore, it is necessary to combine Alsever's with a cryoprotectant to increase the effectiveness of this extender.

This study showed that egg fertility was directly proportional to sperm motility. This is consistent Cabrita et al. (2010), who stated that fertility is influenced by the motility and integrity of the sperm plasma membrane. The congruence between motility and fertility values was also reported Maulida et al. (2021) in *Barbonymus* sp. Several studies also demonstrated that despite sperm motility tending to be low, it can still provide

satisfactory fertility, as observed in seurukan fish *Osteochillus vittatus*, which can produce a fertility rate of 51.33% with sperm motility of 45.74%. This is because the density of the sperm is quite high while the number of fertilized eggs is relatively low. This means that the probability of eggs being fertilized is high. Furthermore, immotile sperm may fertilize the eggs because the eggs produce a gymnogamone I hormone as a sperm activator, which activates sperm motility (MERINO et al. 2024). In addition to sperm quality, the success of fertilization is also determined by the quality of the eggs used (BOZKURT and SECER 2005).

Based on the dilution ratio, the highest motility, viability, and fertility values were found at a dilution ratio of 1:40, while the lowest were recorded at 1:10. This could be due to a low dilution ratio, which leads to high sperm density, inhibiting the movement of spermatozoa to reach the eggs. According to ALAVI et al. (2007) and BERNÁTH et al. (2022), the chances of sperm fertilizing an egg are reduced by sperm density because they compete for entry into the micropyle. This leads to a low fertilization rate. The duration of sperm motility is significantly influenced by the dilution ratio because it occurs only within a very short period. The motility duration is only 1–2 minutes, with no movement observed after 5 minutes (COSSON 2004). Sperm motility can be negatively affected by excessively high sperm density (BOKOR et al. 2021). High sperm cell density can cause osmotic stress, reduce energy availability, and change the viscosity, which hinders sperm movement (DZYUBA et al. 2019, FIGUEROA et al. 2019). Therefore, information on the optimum dilution ratio is crucial. Improper dilution can disrupt sperm activation mechanisms and remove the protective effects of proteins in seminal fluid (ALAVI and COSSON 2005, KOCABAŞ et al. 2022). Therefore, an appropriate dilution ratio is necessary to maintain the optimal duration of sperm motility.

Conclusion

In conclusion, it can be observed that the extender and dilution ratio significantly affected sperm motility, viability, and fertility. Ringer's solution at a ratio of 1:40 resulted in better sperm quality than other types of extenders at all dilution levels. Therefore, it is recommended as a diluent for walking catfish *C. batrachus* sperm undergoing refrigeration preservation at 4°C.

Acknowledgments

The authors are grateful to the Ministry of Education, Culture, Research, and Technology of Indonesia for funding this study through the PMDSU research grant (Contract No: 93/UN11.2.1/PT.01.03/DPRM/2022).

Accepted for print 29.08.2024

References

ABINAWANTO S.R., LESTARI R. 2013. *Cryopreservation of java barb (Barbonymus gonionotus) using egg yolk as a cryoprotectant.* Global Veterinary, 10: 318–321.

ADAMI Y., FADLI N., NURFADILLAH N., ERIANI K., JALIL Z., MUCHLISIN Z.A. 2016. *A preliminary observation on the effect of sperm extenders on the fertilization and hatching rates of seurukan fish (Osteochilus vittatus) eggs.* Aquaculture, Aquarium, Conservation & Legislation, 9(2): 300–304.

ADAN R. 2000. *Catfish culture in Southeast Asia.* SEAFDEC Asian Aquaculture, 22(1): 16–17.

ALAVI S.M.H., COSSON J. 2005. *Sperm motility in fishes. I. Effects of temperature and pH: a review.* Cell Biology International, 29(2): 101–110.

ALAVI S.M.H., COSSON J. 2006. *Sperm motility in fishes (II) Effects of ions and osmolality: a review.* Cell Biology International, 30(1): 1–14.

ALAVI S.M.H., COSSON J., KAZEMI R. 2006. *Semen characteristics in Acipenser persicus in relation to sequential stripping.* Journal of Applied Ichthyology, 22: 400–405.

ALAVI S.M.H., COSSON J., KARAMI M., AMIRI B.M., AKHOUNDZADEH M.A. 2004. *Spermatozoa motility in the Persian sturgeon, Acipenser persicus: effects of pH, dilution rate, ions and osmolality.* Reproduction, 128(6): 819–828.

ALAVI S.M.H., RODINA M., POLICAR T., KOZAK P., PSENICKA M., LINHART O. 2007. *Semen of Perca fluviatilis* L.*: Sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility.* Theriogenology, 68(2): 276–283.

ARGUNGU L., CHRISTIANUS A., AMIN S., DAUD S., SIRAJ S., RAHMAN M.A. 2013. *Asian catfish Clarias batrachus (Linnaeus, 1758) getting critically endangered.* Asian Journal of Animal and Veterinary Advances, 8(2): 168–176.

BAROZHA D.L. 2015. *The effect of honey to motility and viability catfish (Pangasius pangasius) spermatozoa.* Jurnal Majority, 4(3).

BEHNAMIFAR A., BERNAL B., TORRES O., LUIS-CHINCOYA H., GGIL M., GARCÍA-CASADO P., RAHIMI S., WOELDERS H., SANTIAGO-MORENO J. 2021. *Research note: Evaluation of two methods for adding cryoprotectant to semen and effects of bovine serum albumin on quality characteristics of cryopreserved rooster spermatozoa.* Poultry Science, 100(6): 101093.

BEIRÃO J., BOULAIS M., GALLEGO V., O'BRIEN J.K., PEIXOTO S., ROBECK T.R., CABRITA E. 2019. *Sperm handling in aquatic animals for artificial reproduction.* Theriogenology, 133: 161–178.

BERNÁTH G., MILLA S., VÁRKONYI L., LEDORÉ Y., GRIFFITTS J.D., FONTAINE P., URBÁNYI B., BOKOR Z. 2022. *The effect of two different experimental rearing temperatures on the quality and the large-scale cryopreservation of Eurasian perch (Perca fluviatilis) sperm.* Theriogenology, 185: 127–133.

BOKOR Z., ŻARSKI D., PALIŃSKA-ŻARSKA K., KREJSZEFF S., KRÓL J., RADÓCZI J.I., HORVÁTH Á., VÁRKONYI L., URBÁNYI B., BERNÁTH G. 2021. *Standardization of sperm management for laboratory assessment of sperm quality and in vitro fertilization in Eurasian perch (Perca fluviatilis).* Aquaculture International, 29(5): 2021–2033.

BOZKURT Y., SECER S. 2005. *Effect of short-term preservation of mirror carp (Cyprinus carpio) semen on motility, fertilization, and hatching rates.* Israeli Journal of Aquaculture-Bamidgeh, 57: 20407.

Bustamante-González J.D., González-rentería M., Rodríguez Gutiérrez M., Cortés--García A., Ávalos-Rodríguez A. 2016. *Methodologies for spermatic evaluation in teleost*. International Journal of Aquatic Science, 7(2): 95–106.

Cabrita E., Sarasquete C., Martínez-Páramo S., Robles V., Beirão J., Pérez-Cerezales S., Herráez M. 2010. *Cryopreservation of fish sperm: applications and perspectives*. Journal of Applied Ichthyology, 26(5): 623–635.

Canola S.S.S.O. 2010. *Morphometric characteristics of Asian walking catfish Clarias batrachus (*L*., 1758) (Actinopterygii: Clariidae) from the laboratory culture*. Fisheries, 13(4): 01.

Ciereszko A., Dietrich G., Nynca J., Dobosz S., Zalewski T. 2014. *Cryopreservation of rainbow trout semen using a glucose-methanol extender*. Aquaculture, 420: 275–281.

Cuevas-Uribe R., Tiersch T.R. 2011. *Non-equilibrium vitrification: an introduction and review of studies done in fish*. Cryopreservation in Aquatic Species, 2nd edition. Tiersch TR, Green CC (eds.), Baton Rouge, LA: World Aquaculture Society, pp. 309–324.

Curcio M., Blanco-Fernández B., Costoya A., Concheiro A., Puoci F., Alvarez-Lorenzo C. 2015. *Glucose cryoprotectant affects glutathione-responsive antitumor drug release from polysaccharide nanoparticles*. European Journal of Pharmaceutics and Biopharmaceutics, 93: 281–292.

Das S. 2002. *Seed production of Magur (Clarias batrachus) using a rural model portable hatchery in Assam, India-A farmer proven technology*. Aquaculture Asia, 7(2): 19–21.

De Souza Andrade E., De Jesus Paula D.A., De Oliveira Felizardo V., Murgas L.D.S., Veras G.C. 2014. *Milt cryopreservation for rheophilic fish threatened by extinction in the Rio Grande, Brazil*. CryoLetters, 35(1): 8–14.

Di Iorio M., Esposito S., Rusco G., Roncarati A., Miranda M., Gibertoni P.P., Cerolini S., Iaffaldano N. 2019. *Semen cryopreservation for the Mediterranean brown trout of the Biferno River (Molise-Italy): comparative study on the effects of basic extenders and cryoprotectants*. Scientific Reports, 9(1): 1–9.

Domagała J., Dziewulska K., Czerniawski R. 2014. *Survival rate and growth in the wild of sea trout (Salmo trutta* L*.) fry obtained using frozen semen*. Fisheries & Aquatic Life, 22(4): 265–270.

Dzyuba V., Shelton W.L., Kholodnyy V., Boryshpolets S., Cosson J., Dzyuba B. 2019. *Fish sperm biology in relation to urogenital system structure*. Theriogenology, 132: 153–163.

Enzeline V., Nasrullah H., Sudrajat A.O., Zairin J.M., Alimuddin A., Widanarni W. 2022. *Spermatogenesis and sperm quality of male African catfish fed with Bacillus* sp. *NP5 probiotic supplemented diet*. Aquaculture, Aquarium, Conservation & Legislation, 15(1): 339–349.

Eriani K., Mustaqim M., Hasri I., Amalia R. 2021. *Effect of fetal bovine serum on the sperm quality of Depik (Rasbora tawarensis) after short–term cryopreservation*. Indian Journal of Animal Research, 1: 5.

Figueroa E., Lee-Estevez M., Valdebenito I., Watanabe I., Oliveira R.P.D.S., Romero J., Castillo R.L., Farías J.G. 2019. *Effects of cryopreservation on mitochondrial function and sperm quality in fish*. Aquaculture, 511: 634190.

González-López W.Á., Ramos-Júdez S., Giménez I., Duncan N.J. 2020. *Sperm contamination by urine in Senegalese sole (Solea senegalensis) and the use of extender solutions for short-term chilled storage*. Aquaculture, 516: 734649.

Hardi E.H., Nugroho R.A., Saptiani G., Sarinah R., Agriandini M., Mawardi M. 2018. *Identification of potentially pathogenic bacteria from tilapia (Oreochromis niloticus) and channel catfish (Clarias batrachus) culture in Samarinda, East Kalimantan, Indonesia*. Biodiversitas Journal of Biological Diversity, 19(2): 480–488.

Hossen B., Hossain M.S., Rashid I., Hasan M.N., Ethin R., Thapa H., Majumdar B.C. 2017. *Effect of extenders and storage periods on motility and fertilization rate of Silver Barb, Barbonymus gonionotus (Bleeker, 1850) semen*. International Journal of Agriculture Policy and Reseach, 5: 178–185.

Irawan H., Vuthiphandchai V., Nimrat S. 2010. *The effect of extenders, cryoprotectants and cryopreservation methods on common carp (Cyprinus carpio) sperm*. Animal Reproduction Science, 122(3–4): 236–243.

Jothilakshmanan N., Marx K.K. 2013. *Hybridization between Indian catfish,♀ Heteropneustes fossilis (Bloch) and Asian catfish, Clarias batrachus♂ (Linn.).* African Journal of Biotechnology, 12(9).

Judycka S., Cejko B., Dryl K., Dobosz S., Grudniewska J., Kowalski R. 2016. *The effect of supplementation of a trehalose-based extender with KCl on rainbow trout (Oncorhynchus mykiss) sperm freezability and post-thaw motility.* Aquaculture, 465: 303–310.

Judycka S., Nynca J., Liszewska E., Dobosz S., Grudniewska J., Ciereszko A. 2018. *Optimal sperm concentration in straws and final glucose concentration in extender are crucial for improving the cryopreservation protocol of salmonid spermatozoa.* Aquaculture, 486: 90–97.

Juyena N.S., Stelletta C. 2012. *Seminal plasma: an essential attribute to spermatozoa.* Journal of Andrology, 33(4): 536–551.

Khan M.R., Mollah M.A., Ahmed G.U. 2000. *Mass production of hybrid magur and its culture potential in Bangladesh.* Aquaculture Research, 31(5): 467–472.

Kocabaş M., Kocabaş F.K., Aksu Ö., Benzer F., Erişir M., Sahilli Y.Ç. 2022. *Aroclor 1254 impairs sperm quality, fertilization ability, and embryo development of rainbow trout (Oncorhynchus mykiss).* Environmental Science and Pollution Research, 29(56): 84581–84589.

Lim H.K., Le M.H. 2013. *Evaluation of extenders and cryoprotectants on motility and morphology of longtooth grouper (Epinephelus bruneus) sperm.* Theriogenology, 79(5): 867–871.

Mahfudhah R., Eriani K., Muchlisin Z.A. 2020. *The effect of glutathione on the quality of Aceh local catfish (Clarias gariepinus) spermatozoa after cryopreservation. Biosaintifika:* Journal of Biology & Biology Education, 12(1): 125–131.

Marimuthu K., Palaniandya H., Muchlisin Z.A. 2019. *Effect of different water pH on hatching and survival rates of African catfish Clarias gariepinus (Pisces: Clariidae).* Aceh Journal of Animal Science, 4(2): 80–88.

Maulana F., Junior M.Z. 2014. *Morphology, physiology, preservation of climbing perch Anabas testudineus Bloch 1792 sperm and the endurance to electric shock.* Jurnal Iktiologi Indonesia, 14(3): 211–223.

Maulida S., Eriani K., Fadli N., Nur F.M., Handayani L.S., Muchlisin Z.A. 2022. *The effect of extender on the quality of climbing perch (Anabas testudineus) sperm after refrigerated storage.* Aquaculture Research, 53(12): 4497–4504.

Maulida S., Eriani K., Nur F.M., Fadli N., Batubara A.S., Muhammadar A.A., Siti-Azizah M.N., Wilkes M., Muchlisin Z.A. 2021. *Effect of cryoprotectant on the motility, viability, fertilization, and DNA integrity of naleh fish Barbonymus* sp. *(Cyprinidae) sperm.* Brazilian Journal of Veterinary Research and Animal Science, 58: e168702–e168702.

Maulida S., Eriani K., Fadli N., Siti-Azizah M.N., Kocabas F.K., Kocabas M., Wilkes M., Handayani L.S., Rahayu S.R., Muchlisin Z.A. 2024. *Effect of type and concentration of antioxidant on sperm motility, viability, and DNA integrity of climbing perch Anabas testudineus Bloch, 1792 (Pisces: Anabantidae) post-cryopreservation.* Cryobiology, 114: 104851.

Melo F., Godinho H. 2018. *A protocol for cryopreservation of spermatozoa of the fish Brycon orthotaenia.* Animal Reproduction, 3(3): 380–385.

Merino O, Figueroa E., Valdebenito I., Risopatrón J., Merino M., Farías J.G. 2024. *Change in the swimming pattern of Salmo salar spermatozoa caused by the high temperature of the sperm motility activation medium*. Theriogenology, 219: 49–58.

Muchlisin Z.A., Siti-Azizah M.N. 2009. *Influence of cryoprotectants on abnormality and motility of baung (Mystus nemurus) spermatozoa after long-term cryopreservation.* Cryobiology, 58(2): 166–169.

Muchlisin Z.A., Hashim R., Chong A. 2004. *Preliminary study on the cryopreservation of tropical bagrid catfish (Mystus nemurus) spermatozoa; the effect of extender and cryoprotectant on the motility after short-term storage.* Theriogenology, 62(1–2): 25–34.

Muchlisin Z.A., Nadiah. W.N., Siti-Azizah M.N. 2015. *Exploration of natural cryoprotectants for cryopreservation of African catfish, Clarias gariepinus, Burchell 1822 (Pisces: Clariidae) spermatozoa.* Chezch Journal of Animal Sciences, 60(1): 10–15.

MUCHLISIN Z.A., SITI-AZIZAH M.N. 2010. *Preliminary study on the natural extenders for artificial breeding of African catfish Clarias gariepinus (Burchell, 1822).* Aquaculture, Aquarium, Conservation & Legislation Bioflux, 3(2): 119–124.

MUCHLISIN Z.A., HASHIM R., CHONG SHU CHIEN A. 2006. *Influence of dietary protein levels on growth and egg quality in broodstock female bagrid catfish (Mystus nemurus Cuv. & Val.).* Aquaculture Research, 37(4): 416–418.

MUCHLISIN Z.A., SARAH P.I., ALDILA D.F., ERIANI K., HASRI I., BATUBARA A.S., NUR F.M., MUSTAQIM M., MUTHMAINNAH C.R., ABINAWANTO A. 2020. *Effect of dimethyl sulfoxide (DMSO) and egg yolk on sperm motility, fertility and hatching rates of depik Rasbora tawarensis (Pisces: Cyprinidae) eggs after short-term cryopreservation.* Aquaculture Research, 51(4): 1700–1705.

MUTHMAINNAH C., ERIANI K., HASRI I., IRHAM M., BATUBARA A., MUCHLISIN Z.A. 2018. *Effect of glutathione on sperm quality after short-term cryopreservation in seurukan fish Osteochilus vittatus (Cyprinidae).* Theriogenology, 122: 30–34.

RAJAKUMAR A., SENTHILKUMARAN B. 2014. *Expression analysis of cyp11a1 during gonadal development, recrudescence and after hCG induction and sex steroid analog treatment in the catfish, Clarias batrachus.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 176: 42–47.

RASHID I., HOSSAIN M.S., SALAM M.A., RAFIQUZZAMAN S. 2019. *Evaluation of seminal plasma composition and spermatozoa quality parameters of silver barb, Barbonymus gonionotus Bleeker, 1850.* Fish Physiology and Biochemistry, 45(1): 105–114.

RITAR A., CAMPET M. 2000. *Sperm survival during short-term storage and after cryopreservation of semen from striped trumpeter (Latris lineata).* Theriogenology, 54(3): 467–480.

SAHIN T., KURTOGLU İ.Z., BALTA F. 2013. *Effect of different extenders and storage periods on motility and fertilization rate of rainbow trout (Oncorhynchus mykiss) semen.* Universal Journal of Agricultural Research, 1(3): 65–69.

SAPTADJAJA A.M., HASAN V., ARIEF M., ISRONI W. 2020. *First record of threatened asian catfish, Clarias batrachus (Linnaeus, 1758)(siluriformes, clariidae) from Kangean Island, Indonesia.* Ecology, Environment and Conservation, 26(3): 1055–1058.

SHAHRIAR K.M., SARDER M.R.I., TONNY U.S. 2014. *Sperm cryopreservation protocol of Anabas testudineus (Bloch 1792).* Lambert Academic Publishing, Bangladesh.

TAKEI G.L., MUKAI C., OKUNO M. 2015. *Regulation of salmonid fish sperm motility by osmotic shock-induced water influx across the plasma membrane.* Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 182: 84–92.

VERA-MUNOZ O., AMIRAT-BRIAND L., DIAZ T., VASQUEZ L., SCHMIDT E., DESHERCES S., ANTON M., BENCHARIF D., TAINTURIER D. 2009. *Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: Comparison to Triladyl® and Bioxcell®.* Theriogenology, 71(6): 895–900.

YANG S., HAN L., Huang R., LIUFU Y., MENG Z., LIN H. 2017. *Optimization of conditions for the cryopreservation of yellow catfish (Pelteobagrus fulvidraco) sperm*. Cryobiology, 76: 104–110.

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 39(3): 187–197, Y. 2024

DOI: 10.31648/pjns.9976

ROSE GERANIUM ESSENTIAL OIL RESTORATIVE POTENTIAL ON LEAD ACETATE INDUCED OVARY TOXICITY USING A MOUSE MODEL

***Massinissa Madouche*[1], *Nacera Boulila*[2], *Lynda Lakabi*[3], *Samira Akdader*[4], *Ferroudja Medjdoub-Bensaad*[5]**

[1] ORCID: 0009-0005-6187-4806
[2] ORCID: 0000-0001-7091-6581
[3] ORCID: 0000-0002-9747-4223
[4] ORCID: 0000-0003-0959-9112
[5] ORCID: 0000-0002-9396-3775
[1–5] PSEMRVC Laboratory
Faculty of Biological and Agronomical Sciences, Department of Biology
Mouloud Mammeri University, Tizi Ouzou, Algeria

Key words: ovary, geranium, essential oil, lead acetate, reproduction.

Abstract

Lead is known to cause tissue damage, folliculogenesis dysfunction, and increased follicular atresia. Rose geranium essential oil can be used for therapeutic purposes due to its anti-inflammatory and antioxidant properties. The present study aimed to evaluate the reparative potential of rose geranium (*Pelargonium graveolens* L'Hér) essential oil on the ovarian structure affected by low-dose lead exposure using a mice model. Adult BALB/c mice (n = 18) were divided into three groups: Group I, received physiological saline solution, while groups II and III were administered a single dose of lead acetate. Seven days after lead administration, group III received 300 µl/kg/day of rose geranium essential oil. Histological samples from the group (II) treated with 8 mg/kg of lead acetate showed altered ovarian structure. In contrast, those from group (III), treated with 300 µl/kg/day of geranium essential oil, showed improved ovarian structure. Group (II) showed the highest incidence of follicle atresia compared to the other two groups. Our research demonstrated that rose geranium essential oil had positive effect on the histological structure of the ovary, which had been altered by a low single dose of lead.

Introduction

Lead (Pb) is a heavy, soft, and silvery-white metal that can be a source of public health problems due to its widespread use in various applications (MASSANYI et al. 2020, UNDARYATI et al. 2020). This metal has a high level

Address: Massinissa Madouche, Mouloud Mammeri University, Tizi Ouzou, Algeria, e-mail: madouche.massinissa@fsbsa.ummto.dz

of toxicity that can occur without causing immediate symptoms upon exposure (UNDARYATI et al. 2020).

Lead is known to have adverse effects on the male and female reproductive systems. In women, it can cause reproductive system disorders, including infertility (KUMAR 2018). Experimental studies have reported that lead exposure can produce tissue alterations, edema, necrosis, and denudation of oocytes within ovarian follicles. In addition, it can cause dysfunction in folliculogenesis and increased follicular atresia at all stages of development (DHIR and DHAND 2010, DUMITRESCU et al. 2015, TAUPEAU et al. 2001, UCHEWA et al. 2019, WASEEM et al. 2014).

The effects of certain essential oils are being studied for their potential in the treatment of reproductive disorders. For example, spearmint (*Mentha crispata* L.) essential oil has been shown to reduce follicular atresia in rats with polycystic ovary syndrome (SADEGHI et al. 2017), while chamomile (*Matricaria chamomilla* L.) has been found to restore ovarian structure and increase the number of dominant follicles (ZANGENEH et al. 2010). Rose geranium *(Pelargonium graveolens* L'Hér), a plant that is primarily used by perfumers, belongs to the perennial Geraniaceae family, and its essential oil is known for its regenerative properties (BOUKHATEM et al. 2011). Geranium essential oil has a wide range of benefits. It is a natural relaxant and sedative for the nervous system, and a treatment for bronchitis, laryngitis, and menopausal problems. It is also effective in the treatment of eczema, athlete's foot, and respiratory tract health (RAJESWARA RAO 2013), and is additionally endowed with antimicrobial and antifungal properties (VERMA et al. 2016). This essential oil also exhibits anti-inflammatory and antioxidant properties, suggesting therapeutic potential (BOUKHATEM et al. 2013, SAID et al. 2022).

To our knowledge, no study has investigated the reparative effects of rose geranium essential oil on low-dose lead-induced ovarian toxicity. The aim of the present study was to evaluate the reparative potential of rose geranium essential oil on the ovarian structure affected by low-dose lead exposure, focusing on the overall structure and incidence of growing follicle atresia.

Materials and Methods

Mouse model and ethics statement

Eighteen 8-weeks-old BALB/c mice, with an average weight of 28.3 ±0.37 g, were used in this study. The animals were obtained from the small animal laboratory of the Institut Pasteur d'Algérie and housed in

the animal facility of the faculty. Mice had *ad libitum* access to water and food on a 12-hour light / 12-hour dark cycle. Throughout the experiment, temperature was maintained at 20 ±0.17°C, with humidity level of 60 ±0.04%. All procedures were conducted in compliance with Algerian legislation (Law number 95-322/195) concerning the protection of animals intended for experimentation and other scientific purposes. All animals were treated humanely and in accordance with the ARRIVE guidelines and approved by the faculty's scientific committee.

Chemicals and essential oil

Lead acetate trihydrate Pb $(CH_3COO)_2$ was obtained from the Faculty Chemical Store, while rose geranium essential oil (*Pelargonium graveolens* L'Hér. 1789) was extracted via steam distillation and provided by Yakouren Biocare, Algeria. Based on several studies, the main constituents of geranium essential oil were citronellol, geraniol, linalool, citronellyl formate, geranyl formate and isomenthone (VERMA et al. 2016, BOUKHRIS et al. 2013, RAJESWARA RAO 2013, BOUKHATEM et al. 2010).

Experimental design

After 1 week of acclimatization, the estrous cycle of mice was synchronized using Whitten effect (ZAKARIA and SUKARDI 2019), and the animals were randomly assigned to three groups. They were weighed daily to monitor weight trends, and all treatments were administered intraperitoneally. The experiment was conducted for 28 days as follow:

Group I: Received physiological saline solution from the 7^{th} to the 28^{th} day.

Group II: Received a single dose of lead acetate on the first day.

Group III: Received a single dose of lead acetate (8 mg/kg) on the first day, followed by a daily dose (300 µl/kg) of geranium essential oil starting from de the 7^{th} day. The dose of essential oil was chosen based on the study of BOUKHATEM et al. (2013).

Histological specimen preparation

At the end of the experimental procedure, all animals were euthanized, and their ovaries were extracted, degreased, weighed, and fixed in Bouin's fluid for 24 hours. Specimens were then embedded in paraffin blocks and, sections of 5 µm thickness were cut and stained with hematoxylin-eosin (CARSON and HLADIK 2009). Histological observations were performed using a Leica DM/LS light microscope at magnifications of ×100 or ×400.

Atretic growing follicle count

For each sample, one section was taken every 10 µm, resulting in 5 slides for each ovary. To evaluate atretic growing follicles, identification was conducted at ×100 or ×400 magnification. A primary follicle was noted if it contained an oocyte surrounded by a single layer of cuboid granulosa cells, while a secondary follicle was identified by the presence of, at least, two layers of cuboid granulosa cells surrounding the oocyte, without an antrum. An antral follicle was considered if it exhibited multiple layers of cuboidal granulosa cells with an antrum (KIM et al. 2022).

We evaluated growing follicle atresia based on the method described by USLU et al (2017). In summary, for primary and secondary follicles, we assessed the integrity of the oocyte and counted the number of pycnotic nuclei for each follicle. An antral follicle was considered atretic if it exhibited a fragmented oocyte (FO) or if it was deformed (FD). Atretic follicles were counted using a Leica DM/LS light microscope at ×400 magnification for primary and secondary follicles, and at ×100 magnification for antral follicles.

Statistical analysis

The total number of atretic follicles was counted and compared between the different groups. Additionally, for antral follicles, each category of fragmented oocytes (FO) and deformed follicles (FD) was counted and compared separately. Statistical analysis was conducted using analysis of variance (ANOVA) followed by Tukey's post hoc test (LAFAYE et al. 2014). The statistical software RStudio software version 2023.06.0-421 was used for data analysis.

Results

No mortality or behavioral changes were observed in any of the animals throughout the entire experimental period. Additionally, there were no changes in body weight or absolute and relative ovary weights in any of the groups.

Histological description

Histological examination of samples from control group (group I) revealed a normal ovarian structure without detectable alterations. Various stages of follicle development and corpus luteum were observed in the cortex, along with well-organized connective tissue and blood vessels in the stroma (Figure 1*a*). The oocytes appeared intact, and the granulosa cells were well-organized (Figure 1*b*). In contrast, samples from group (II), treated with 8 mg/kg of lead acetate, showed altered ovarian structure. Aeras of optical vacuum within the ovarian tissue were noted, along with advanced atresia of primary and secondary follicles, and diffuse edema. Denudation and deformation of the oocytes, as well as disorganization of the follicular cells at all stages of follicular development, were observed (Figure. 1*c*). Particularly affected were follicles, showing disorganized follicular layers and cells lacking adhesions (Figure 1*d*). However, in group (III), treated with 300 µl/kg of geranium essential oil, an improved ovarian structure was observed. Fewer areas of optical vacuum were noted under the optical microscope, and there was no edema (Figure 1*e*). Developing follicles appeared perfectly structured (Figure 1*f*).

Atretic growing follicle count

Primary and secondary follicle atresia was more pronounced in the group (II), treated with 8 mg/kg of lead acetate, than in the other two groups. There was a significant difference ($P < 0.01$) in the number of atretic primary follicles and a highly significant difference ($P < 0.001$) in the number of atretic secondary follicles (Table 1).

Group (II), treated with 8 mg/kg of lead acetate, exhibited the highest number of atretic antral follicles ($P < 0.05$). Specifically, the number of deformed antral follicles (DF) was higher in the group (II) treated with lead ($P < 0.05$) compared to the other two groups. However, the number of antral follicles with a fragmented oocyte (FO) remained unchanged across the different groups (Table 2).

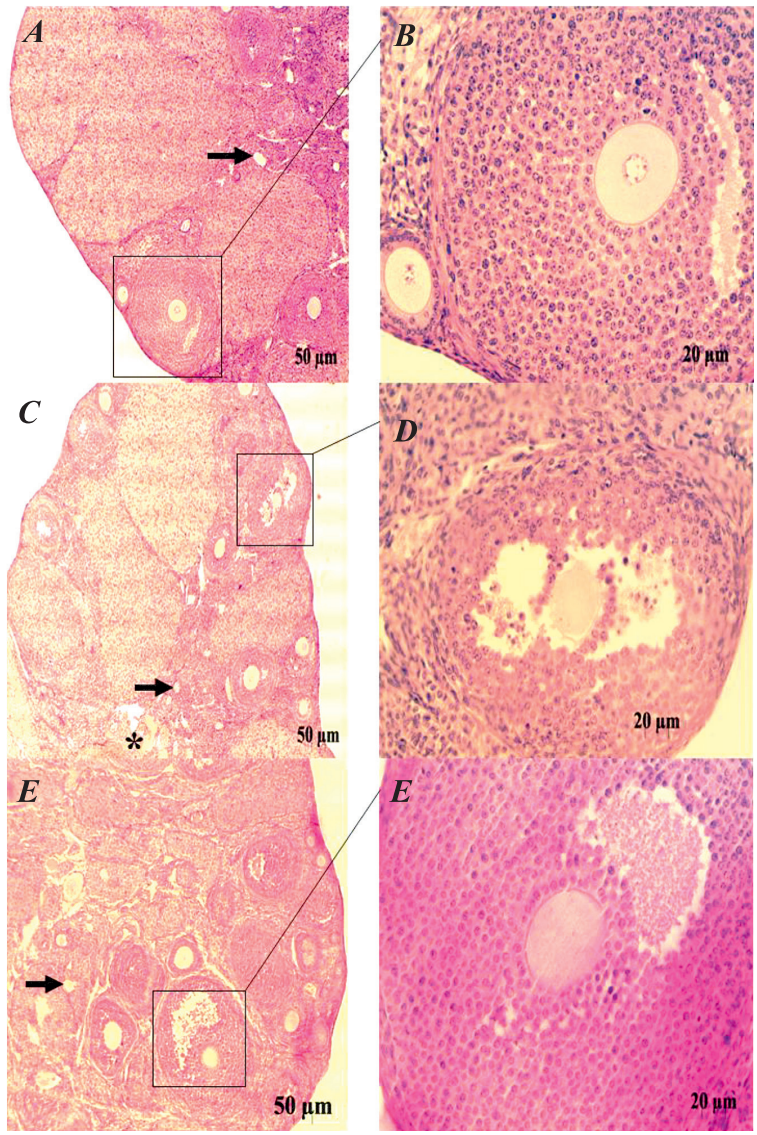

Fig. 1. A photomicrograph showing the ovarian histology from experimental groups stained with hematoxylin-eosin. *A* – control group ovary (I) at ×100 magnification. Unaltered tissue and follicles at different stages of development can be noted; *B* – detail of control ovary at ×400 magnification showing antral follicle with oocyte surrounded by perfectly arranged granulosa cells; *C* – ovary of group (II) treated with 8 mg/kg lead acetate at ×100 magnification. Diffuse edema is indicated by asterisk (*); *D* – antral follicle from group (II) at ×400 magnification exhibiting oocyte denudation and disorganized granulosa cells; *E* – ovary from group (III) treated with 300 µl/kg/day of geranium essential oil at ×100 magnification. Improved ovarian structure and follicles at different stages of development are observed; *F* – detail of antral follicle from group (III) at ×400 magnification showing improvement of follicular structure with no oocyte denudation, well-structured granulosa cells. Arrow indicates atresia of primary and secondary follicles in all groups

Table 1

Primary and secondary atretic follicle count

Groups	I	II	III
APF	6.83 ±2.23	20.7 ±5.68**	6.33 ±2.98
ASF	4.67 ± 0.51	7.68 ±0.51****	3.83 ±1.33

Explanations: The data are expressed as mean ± standard deviation. Groups (n = 6): control (I), 8 mg/kg of LA (II), 8 mg/kg of LA and 300 µl/kg essential oil (III). (APF) atretic primary follicles, (ASF) atretic secondary follicles. An asterisk (*) indicates a significant difference: group (I) and (III) versus group (II): $^{**}P < 0.01$, $^{****}P < 0.0001$

Table 2

Antral atretic follicle count

Groups	I	II	III
ATR	0.5 ±0.9	1.07 ±1.23*	0.36 ±0.72
FO	0.3 ±0.59	0.36 ±0.8	0.2 ±0.55
DF	0.2 ±0.48	0.7 ±0.98*	0.16 ±0.48

Explanations: the data are expressed as mean ± standard deviation. Groups (n = 6): control (I), 8 mg/kg of LA (II), 8 mg/kg of LA and 300 µl/kg essential oil (III). (ATR) total of atretic antral follicles, (FO) antral follicles with fragmented oocyte, (DF) deformed antral follicles. The asterisk (*) indicates a significant difference: group (I) and (III) versus group (II): $^{*}P < 0.05$

Discussion

Our results indicated that a low-dose administration of lead did not induce any change in body weight or ovarian weight in mice as reported by previous studies (TAUPEAU et al. 2001).

The literature reports that heavy metals have adverse effects on reproductive function, which can lead to infertility (BHARDWAJ et al. 2021, MASSANYI et al. 2020, RZYMSKI et al. 2015). Our histological observations showed that lead acetate induced an alteration in ovarian structure, particularly in the follicles, and are consistent with several studies demonstrating that this heavy metal causes damage to the ovary. In fact, at low doses, lead acetate causes hyperemia and degeneration of follicles at all stages of development, and vacuoles may be observed at several sites (DUMITRESCU et al. 2015, JUNAID and CHOWDHURI 1997).

Lead is a reprotoxic agent that accumulates in granulosa cells and induces morphological changes (JUNAID and CHOWDHURI 1997, VYLEGZHANINA et al. 1993). According to USLU et al. (2017), primary and secondary follicle atresia was assessed by the oocyte integrity and the number of pycnotic nuclei, while that of the antral follicles was determined by oocyte integrity and follicle shape. Our results showed thar follicular atresia was higher in the group treated with lead acetate, particularly in the second-

ary follicles, where the difference was highly significant (Table 1). Additionally, we noted that the number of deformed antral follicles (DF) was higher than in the other groups (Table 2). The majority of ovarian follicles fail to ovulate and degenerate through atresia (MCGEE and HSUEH 2000). However, several studies have reported that lead exposure can cause and exacerbate follicular atresia at all stages of development (QURESHI et al. 2010, TAUPEAU et al. 2001).

A large number of studies have focused on the effects of natural substances on health (AKINYEMI et al. 2018, BELLIK 2022, SAKHTEMAN et al. 2020). However, few studies have examined the effects of plants and their essential oils on female reproductive function (DOMARACHY et al. 2007, PARANDIN and YOUSOFVAND 2019, PARHIZKAR et al. 2016). To our knowledge, the effects of rose geranium essential oil on female reproductive function have never been studied, and very few studies have investigated the role of essential oils in restoring lead-damaged ovarian structure. Nevertheless, it is generally agreed that some of them have therapeutic properties. For example, research on the possible effect of essential oils on reproductive disorders in females indicates that administration of 300 mg/kg of spearmint essential oil to rats with polycystic ovary syndrome significantly reduced follicular atresia and restored ovarian structure (SADEGHI et al. 2017). In the context of fertility enhancement, administration of common fennel extract to healthy mice improved folliculogenesis and reduced the number of atretic follicles at all stages of development (KHAZAEI et al. 2011).

Follicular atresia is a natural event within the ovary, and apoptosis-induced follicular atresia can originate from granulosa cells or oocytes (XUAN and YIXUN 2003). As shown in our results, low-dose lead acetate administration altered the histological structure of the ovary and increased follicular atresia. However, structure and follicular degeneration were reduced by administering rose geranium essential oil. According to OUIES et al. (2020), lead acetate-induced follicular atresia in a pubertal and prepubertal rat model was reduced by *Nigella sativa* administration.

The main compounds of the Algerian geranium cultivar are citronellol and geraniol, natural monoterpenoids endowed with antioxidant and anti-inflammatory properties (ABE et al. 2004, BOUKHATEM et al. 2013, KOBAYASHI et al. 2016, MARUYAMA et al. 2006). The reduction in follicular atresia in group (III) is most probably due to the antioxidant properties of this essential oil. As reported by CAVAR and MAKSIMOVIC (2012), geranium essential oil can be a good source of antioxidants, and a study by BOUKHRIS et al. (2015) reported that the extract and essential oil of rose-scented geranium showed antioxidant activity. In fact, the results of the study

suggest that the major components of this oil can inhibit a range of free radicals. The administration of lead acetate has been observed to induce alterations in the antioxidant system, due to the activation of free radical oxidation (OSOWSKI et al. 2023). Therefore, it is imperative to investigate the antioxidant effects of this oil as a potential mechanism of action in attenuating ovarian toxicity induced by low doses of lead.

Conclusion

Our findings revealed that rose geranium (*Pelargonium graveolens* L'Hér) essential oil had a positive effect on the mouse ovary's histological structure, which was altered by a low single dose of lead. It was observed that there were no signs of toxicity, and growing follicle atresia was reduced. Therefore, this study pointed out rose geranium essential oil' restorative potential on lead acetate induced ovary toxicity in a mice model, while further research is needed to investigate the associated mechanisms.

Translated by Massinissa Madouche Accepted for print 5.09.2024

References

ABE S., MARUYAMA N., HAYAMA K., INOUYE S., OSHIMA H., YAMAGUCHI H. 2004. *Suppression of neutrophil recruitment in mice by geranium essential oil*. Mediators of Inflammation, 13(1): 21–24.

AKINYEMI A.J., FABOYA O.L., PAUL A.A., OLAYIDE I., FABOYA O.A., OLUWASOLA T.A. 2018. *Nephroprotective effect of essential oils from Ginger (Zingiber officinale) and Turmeric (Curcuma longa) rhizomes against cadmium-induced nephrotoxicity in rats*. Journal of Oleo Science, 67(10): 1339–1345.

BELLIK Y. 2022. *Protective effect of honey against aluminium-induced erythrocyte osmotic fragility and hemoglobin denaturation*. Polish Journal of Natural Sciences, 37(2): 205-218.

BHARDWAJ J.K., PALIWAL A., SARAF P. 2021. *Effects of heavy metals on reproduction owing to infertility*. Journal of Biochemical and Molecular Toxicology, 35(8): 1–21.

BOUKHATEM M.N., HAMAIDI M.S., SAIDI F., HAKIM Y. 2010. *Extraction, composition et propriétés physico-chimiques de l'huile essentielle du Géranium Rosat (Pelargonium graveolens* L*.) cultivé dans la plaine de Mitidja (Algérie)*. Nature et Technologie, 3: 37–45.

BOUKHATEM M.N., KAMELI A., AMINE FERHAT M., SAIDI F., MEKARNIA M. 2013. *Rose geranium essential oil as a source of new and safe anti-inflammatory drugs*. Libyan Journal of Medicine, 8(1): 22520.

BOUKHATEM M.N., SAIDI F., HAMAIDI M.S., HAKIM Y., MEKARNIA M. 2011. *Culture et exploitation industrielle du géranium rosat (Pelargonium graveolens) en Algérie : Etat des lieux et perspectives*. Phytothérapie, 9(5): 304–309.

BOUKHRIS M., SIMMONDS M.S.J., SAYADI S., BOUAZIZ M. 2013 *Chemical composition and biological activities of polar extracts and essential oil of rose-scented geranium, Pelargonium graveolens*. Phytotherapy Research, 27(8): 1206–1213.

Boukhris M., Hadrich F., Chtourou H., Dhouib A., Bouaziz M., Sayadi S. 2015 *Chemical composition, biological activities and DNA damage protective effect of Pelargonium graveolens L'Hér. essential oils at different phenological stages*. Industrial Crops and Products, 74: 600–606.

Carson L.F., Hladik C. 2009. *Histotechnology, a self-instructional Text* (3rd Edition). American Society for Clinical Pathology Press., pp.130–133.

Cavar S., Maksimovic M. 2012. *Antioxidant activity of essential oil and aqueous extract of Pelargonium graveolens L'Her*. Food Control, 23(1): 263–267.

Dhir V., Dhand P. 2010. *Toxicological approach in chronic exposure to lead on reproductive functions in female rats (Rattus norvegicus)*. Toxicology International, 17(1): 1–7.

Domaracky M., Rehak P., Juhas Š., Koppel J. 2007. *Effects of selected plant essential oils on the growth and development of mouse preimplantation embryos in vivo*. Physiological Research, 56: 97–104.

Dumitrescu E., Chiuriu V., Muselin F., Popescu R., Brezovan D., Cristina R.T. 2015. *Effects of long-term exposure of female rats to low levels of lead: Ovary and uterus histological architecture changes*. Turkish Journal of Biology, 39: 284–289.

Junaid M.D.K., Chowdhuri R. 1997. *Lead-induced changes in ovarian follicular development and maturation in mice*. Journal of Toxicology and Environmental Health, 50(1): 31–40.

Khazaei M., Khanahmadi M. 2011. *Study of foeniculum vulgare effect on folliculogenesis in female mice*. International Journal of Fertility and Sterility, 5(3): 122–127.

Kim S.J., Kim T.E., Jee B.C. 2022. *Impact of imatinib administration on the mouse ovarian follicle count and levels of intra-ovarian proteins related to follicular quality*. Clinical and Experimental Reproductive Medicine, 49(2): 93–100.

Kobayashi Y., Sato H., Yorita M., Nakayama H., Miyazato H., Sugimoto K., Jippo T. 2016. *Inhibitory effects of geranium essential oil and its major component, citronellol, on degranulation and cytokine production by mast cells*. Bioscience, Biotechnology, and Biochemistry, 80(6): 1172–1178.

Kumar S. 2018. *Occupational and environmental exposure to lead and reproductive health impairment: An overview*. Indian Journal of Occupational and Environmental Medicine, 22(3): 128.

Lafaye De Micheaux P., Drouilhet R., Liquet B. 2014. *Le logiciel R : Maîtriser le langage, effectuer des analyses (bio)statistiques (2e éd)*. Springer, pp. 541–551.

Maruyama N., Ishibashi H., Hu W., Morofuji S., Inouye S., Yamaguchi H., Abe S. 2006. *Suppression of carrageenan and collagen II-induced inflammation in mice by Geranium oil*. Mediators of Inflammation, 2006: 1–7.

Massanyi P., Massanyi M., Madeddu R., Stawarz R., Lukac N. 2020. *Effects of cadmium, lead, and mercury on the structure and function of reproductive organs*. Toxics, 8(4): 94.

Mcgee E.A., Hsueh A.J.W. 2000. *Initial and cyclic recruitment of ovarian follicles*. Endocrine Reviews, 21(2): 200–214.

Osowski A., Fedoniuk L., Bilyk Y, Fedchyshyn O., Sas M., Kramar S., Lomakina Y., Fik V., Chorniy S., Wojtkiewicz J. 2023. *Lead exposure assessment and its impact on the structural organization and morphological peculiarities of rat ovaries*. Toxics, 11(9): 769.

Ouies S.M., Ahmed S.R., Bassit A.S. 2020. *Effect of lead acetate on the ovarian growth in the pre-pubertal and pubertal periods in the offspring of albino rats and the possible protective role of Nigella Sativa oil*. Egyptian Journal of Histology, 44(3): 814–827.

Parandin R., Yousofvand N. 2019. *Foeniculum vulgare (Fennel) effects on puberty timing, reproductive function and behaviour in adult female mice following neonatal exposure*. Journal of Advances in Medical and Biomedical Research, 27(121): 16–22.

Parhizkar S., Latiff L.A., Parsa A. 2016. *Effect of Nigella sativa on reproductive system in experimental menopause rat model*. Avicenna Journal of Phytomedicine, 6(1): 95–103.

Qureshi N., Sharma R., Mogra S., Panwar K. 2010. *Amelioration of lead induced alterations in ovary of Swiss mice, by antioxidant vitamins*. Journal of Herbal Medicine and Toxicology, 4(1): 89–95.

RAJESWARA RAO B.R., 2013. *Biological and pharmacological activities of rose-scented geranium or rose-geranium*. In: Microbiology Applications. Har Krishan Bhalla & Sons, Dehradun, India, pp. 407–422.

RZYMSKI P., TOMCZYK K., RZYMSKI P., PONIEDZIALEK B., OPALA T., WILCZAK M. 2015. *Impact of heavy metals on the female reproductive system*. Annals of Agricultural and Environmental Medicine, 22(2): 259–264.

SADEGHI A.M., ALAEE S., BAGHERI M.J., BAHMANPOOR S. 2017. *Role of essential oil of Mentha Spicata (Spearmint) in addressing reverse hormonal and folliculogenesis disturbances in a polycystic ovarian syndrome in a rat model*. Advanced Pharmaceutical Bulletin, 7(4): 651–654.

SAID A.A., NASR Y., GALAL A.A.A., ABDELHAMID A.E., MOHAMED H.A., METWALLY M.M.M., SAID M.A., NASSAN M.A., DAHRAN N., MOHAMED A.A.R. 2022. *Concerns with male infertility induced by exposure to titanium nanoparticles and the supporting impact of Pelargonium graveolens essential oil : Morphometric records in male-wistar rats*. Life. 12: 639–651.

SAKHTEMAN A., PASDARAN A., AFIFI M., HAMEDI A. 2020. *An assay on the possible effect of essential oil constituents on receptors involved in women's hormonal health and reproductive system diseases*. Journal of Evidence-Based Integrative Medicine, 25: 1–9.

TAUPEAU C., POUPON J., NOME F., LEFEVRE B. 2001. *Lead accumulation in the mouse ovary after treatment-induced follicular atresia*. Reproductive Toxicology, 15(4): 385–391.

UCHEWA O.O., EZUGWORIE O.J. 2019. *Countering the effects of lead as an environmental toxicant on the microanatomy of female reproductive system of adult wistar rats using aqueous extract of Ficus vogelii*. Journal of Trace Elements in Medicine and Biology, 52: 192–198.

UNDARYATI Y.M., SUDJARWO S.A., I'THISOM R. 2020. *Literature review: Effect of lead toxicity on reproductive system*. Journal of Global Research in Public Health, 5(1): 1–8.

USLU B., DIOGUARDI C.C., HAYNES M., MIAO D.Q., KURUS M., HOFFMAN G., JOHNSON J. 2017. *Quantifying growing versus non-growing ovarian follicles in the mouse*. Journal of Ovarian Research, 10(3): 1–11.

VERMA R.S, CHANDRA P.R, CHAUHAN A. 2016. *Rose-scented geranium (Pelargonium* sp.*) oils*. In: *Essential oils in food preservation, flavor and safety*. Ed. Victor R. Preedy. Elsevier, pp. 697–704.

VYLEGZHANINA T.A., KUZNETSOVA T.E., MANEEVA O.A., NOVIKOV I.I., RYZHKOVSKAIA E.L. 1993. *Morphofunctional characteristics of the ovaries, thyroid gland and adrenal glands in experimental lead acetate poisoning*. Med. Tr. Prom. Ekol., (9–10): 6–8.

WASEEM N., BUTT S.A., HAMID S. 2014. *Amelioration of lead induced changes in ovary of mice, by garlic extract*. JPMA, 64(7): 798–801.

XUAN J., YIXUN U. 2003. *Follicular growth, differentiation and atresia*. Chinese Science Bulletin, 48(17): 1786–90.

ZAKARIA N.H., SUKARDI S. 2019. *Determination of the whiten effect based on vaginal cell characteristics, vulva appearance and behavior in grouped female mice*. Life Sciences, Medicine and Biomedicine, 3(8): 56. 1–6.

ZANGENEH Z. 2010. *Effects of Chamomile extract on biochemical and clinical parameters in a rat model of polycystic ovary syndrome*. Journal of Reproduction and Infertility, 11(3): 169–174.