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ORIGINAL ARTICLE

Effects of methyl farnesoate injection on spermatozoa number and reproductive indices in the narrow-clawed crayfish *Pontastacus leptodactylus*

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ABSTRACT

In this study, the effect of methyl farnesoate (MF) injection on haemolymph MF levels, spermatozoa production and the reproductive indices of gonadosomatic index (GSI), testicular index (TI) and vasosomatic index (VSI) were investigated in males of *Pontastacus leptodactylus* (Escholtz, 1823). Sixty male *P. leptodactylus* were used for the study. They were housed in a total of twelve tanks in their normal reproductive season in 2018 and were fed *ad libitum* with a pelleted food. Animals were injected once a week for five consecutive weeks, at doses of 250 (G2), 500 (G3) and 1000 (G4) ng MF g^{-1} of body weight. The doses of the injections, the durations used, and the frequency of administration were determined according to the literature. An increase was observed in gonadosomatic index and spermatozoal number of crayfish injected with 1000 ng of MF g^{-1} of body weight when compared to control (G1, no MF injection applied) and other experimental groups. In addition, the present study indicated that G4 crayfish had a higher GSI, TI and VSI than control crayfish. There was no difference in the level of hemolymph MF between control and MF injected crayfish. In conclusion, MF injection is effective for inducing increased gonadasomatic index and spermatozoal number in *P. leptodactylus.*

KEYWORDS

Decapoda, gonad, gonadosomatic index, male gamete, reproduction

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INTRODUCTION

In recent decades, astaciculture has gained importance because of high demands from international markets for crayfish (Kouba *et al.,* 2012a; 2012b). The narrow-clawed crayfish *Pontastacus leptodactylus* (Escholtz,

1823), a crustacean with a high commercial value, is widely stocked in Eastern Europe and the Middle East and is a species suitable for aquaculture (Harlıoğlu, 2004; Harlıoğlu and Harlıoğlu, 2004; Harlıoğlu and Farhadi, 2017). Basic knowledge of gamete biology may help enhance the quality of artificial reproduction in commercial decapod crustaceans such as crayfish (Yazicioglu *et al.,* 2016a; Farhadi and Harlıoğlu, 2019a).

The regulation of reproduction in decapods is controlled by a variety of different factors such as water temperature (Farhadi and Harlıoglu, 2018; Yazicioglu *et al.,* 2018), photoperiod (Harlıoğlu and Duran, 2010; Farhadi and Harlıoğlu, 2019b), biogenic amines (Farhadi *et al.,* 2020), gonad stimulating hormone, methyl farnesoate (MF), steroid hormones (Harlıoğlu *et al.,* 2018a), and neuropeptides (Swetha *et al.,* 2011; Harlıoğlu and Farhadi, 2017). Control of male reproduction is a key factor in crustacean aquaculture. For example, spermatozoa quality is one of the most influential factors and affects reproductive output in crustacean hatcheries (Wickins and Lee, 2002) and many factors affect spermatozoal production and quality in decapods including broodstock nutrition, water pollution, water temperature, captivity, hormones, neurotransmitters, stress, disease, male age, and size (Harlıoğlu *et al.,* 2018b).

Methyl farnesoate is a terpenoid hormone that is synthesized by the mandibular organ and is structurally similar to juvenile hormone III of insects. However, it differs in the presence of an epoxide moiety at the terminal end. MF is included in several hormonal processes in crustaceans. There are studies showing that MF induces gonadal development in some decapods (Alfaro *et al.,* 2008; Marsden *et al.,* 2008; Nagaraju and Borst, 2008; Zaleski and Tamone, 2014; Hemalatha *et al.,* 2016; Raghavan and Ayanath, 2018). For example, gonadal development of decapods induced by MF has been reported in the estuarine female crab *Neohelice granulata* (Dana, 1851) (see Medesani *et al.,* 2015) and freshwater crab *Oziothelphusa senex senex* (Fabricus, 1798) (see Reddy and Reddy, 2015) and Alfaro *et al.* (2008) found that MF has an effect on spermatophore quality in the white shrimp, *Litopenaeus vannamei* (Boone, 1931). Alnawafleh *et al.* (2014) reported that MF stimulates moulting and ovarian maturation in the Pacific White Shrimp *L. vannamei*. Similarly, it has been found that changes in MF levels affect testicular development (Nagaraju and Borst, 2008) and MF injection increases spermatozoal production in male decapods (Alfaro *et al.,* 2008). The molecular mechanism of MF in testicular development is not clear. Studies have shown that MF induces the expression of vitellogenin in female decapods such as the crucifix crab *Charybdis feriatus* (Linnaeus, 1758) (see Chan *et al.,* 2005) and *Portunus trituberculatus* (Miers, 1876) (see Liu *et al.,* 2016). Several studies have been carried out to decipher the biology and role of crayfish gametes in reproduction including the fine structure of crayfish immotile spermatozoa (Niksirat *et al.,* 2013a; Niksirat *et al.,* 2013b; Kouba *et al.,* 2015; Yazicioglu *et al.,* 2016b), spermatozoal capacitation (Niksirat *et al.,* 2014a; 2014b; Niksirat *et al.,* 2015a; 2016; Niksirat and Kouba, 2016), egg activation and attachment stalk formation (Niksirat *et al.,* 2015b), annual cycle of spermatozoan production (Farhadi and Harlıoğlu, 2019c), and artificial extrusion of the male gamete for reproduction (Farhadi *et al.,* 2019). However, the role of hormones such as MF on spermatozoa production in freshwater crayfish is still unknown.

This study aims to evaluate the effect of injection of MF at different doses on spermatozoal production and some associated reproductive parameters such as gonadosomatic index (GSI), testicular index (TI) and vasosomatic index (VSI) in male *P. leptodactylus*. Changes in the haemolymph MF levels after injection were also evaluated in our study.

Material and Methods

Crayfish and experimental design

Sixty adult male *P. leptodactylus* (mean body weight, 46.7 ± 3.4 g; mean carapace length 5.7 ± 0.2 cm) were obtained from the Keban Dam Lake (Elazığ, Turkey). Specimens were placed in a total of twelve outdoor concrete tanks (2 × 2 × 1 m) and exposed to a natural photoperiod in the crayfish reproduction unit of the Fisheries Faculty, Fırat University, Elazığ, Turkey. The crayfish were divided arbitrarily into four groups (15 individuals per group). In each tank, plastic pipes (20 cm in length and 7 cm in diameter) were supplied as shelters for the crayfish. Crayfish were daily fed (*ad libitum*) with a commercial pellet food

(Manufactured by Gürdal, Kahramanmaraş, Turkey, containing 35 % crude protein on a dry-weight basis and 3600 kcal/kg gross energy).

Water flow was 1.5 l/s per 1 m^2 in each tank. During the experiment, dissolved oxygen, pH, and water temperature was measured daily. Ammonia, iron, copper, alkalinity, hardness, calcium and water flow were measured. Methyl farnesoate (trans, trans MF, $C_{16}H_{26}O_2$) was purchased from Echelon (Echelon Biosciences Inc., Salt Lake City, USA) and dissolved in 10 ml of ethanol. This stock solution was kept at -70 °C. Methyl farnesoate injection was performed in three different doses. The stock solution was diluted in 0.85 % NaCl to reach the final concentration. All MF solutions were prepared weekly. Methyl farnesoate injection was not applied to the control group (G1). For G1, 0.02 ml ethanol was diluted to 0.05 ml with saline (physiological solution, 0.85 % salt water). The highest ethanol level was 40 % of the injection volume (20 µl ethanol + 30 µl physiological solution). Male crayfish were injected five times in the second abdominal muscle on days 1, 8, 15, 22, and 29 by means of 1-ml syringes (27 G needle) before mating.

The injection doses and the frequency of administration of the doses were determined according to Abdu *et al.* (2001), Alfaro *et al.* (2008) and Rodríguez *et al.* (2002):

G1: 0 ng MF body weight^{-1} (control)

G2: 250 ng MF body weight^{-1}

G3: 500 ng MF body weight^{-1}

G4: 1000 ng MF body weight^{-1}

Pontastacus leptodactylus usually starts mating and carries spermatozoa in the first week of January in the crayfish reproduction unit of the Fisheries Faculty (Harlıoğlu and Duran, 2010; Harlıoğlu *et al.,* 2013a; 2013b). Therefore, in the normal reproductive season, the first injection was performed on 1 December 2018 and the last injection was performed on 29 December 2018. The second, third and fourth injections were administered on the 8th, 15th, and 22nd days, in one-week intervals. Haemolymph samples were taken from crayfish one week after (4 January 2018) the last dose application based on the weekly dosing interval (1 week). In the present study, crayfish started to mate the first week of January 2019.

Haemolymph collection

To determine the MF levels in haemolymph, at least 1 ml haemolymph was collected from the heart of the crayfish using a sterile BD PrecisionGlideTM 1 ml syringe (26 G ½; 0.45 mm × 13 mm).

For MF analysis, haemolymph samples were transferred into tubes in 4 % NaCl and acetonitrile, keeping a (4:5) ratio of water to acetonitrile. Before extraction, 10 ng of the nonbiological isomer of MF (cis- trans MF), was added to each tube as an internal standard (Laufer *et al.,* 1998). Water and acetonitrile ratio were maintained at 4:5, v / v. 2 ml of n-hexane was added to this mixture to be homogenized and mixed thoroughly. The mixture was centrifuged at 750 g for 10 minutes and the upper hexane phase was transferred separately to a tube. This procedure was repeated three times and the hexane phases were dried under open nitrogen flow and the MF level was measured in a HPLC. A PDA (Photodiode Array) detector was used in the HPLC system. The wavelength was adjusted to 220 nm between 190-350 and analyzed by using a Nucleosil 100-5 (5 µm, 250 × 4.6 mm; Macherey-Nagel, Dueren, Germany) HPLC column (Borst *et al.,* 1987).

Reproductive parameters and spermatozoal counting

At the end of the experiment, the crayfish samples were stored at -20 °C until dissection. Their carapace length and weight were recorded. The whole reproductive system, vasa deferentia and testis, were also weighed. The gonadosomatic index (GSI, %) was calculated as reproductive system weight/body weight × 100, the testicular index (TI, %) was calculated as testis weight/body weight × 100, and the vasosomatic index (VSI, %) was calculated as vasa deferentia weight/body weight × 100) (Harlıoğlu *et al.,* 2013b).

For spermatozoal counting a modified protocol developed by Leung-Trujillo and Lawrence (1985) was employed. A 1 cm section of the distal vas deferens (DVD) was disaggregated in 1 ml of physiological solution for decapods (Harlıoğlu *et al.,* 2012). Then, spermatozoa were counted using a Neubauer camera. Spermatozoal count was expressed as spermatozoa/DVD section (Harlıoğlu *et al.,* 2012; Harlıoğlu *et al.,* 2013b; Farhadi *et al.,* 2018).

Statistical analyses

The normality and homoscedasticity of the data were examined using the Kolmogorov Smirnov and Levene's tests. Proportional data (GSI, TI, VSI) were normalized using squareroot arcsin transformation. Data were analyzed statistically using one-way analysis of variance (ANOVA) and Duncan's new multiple range test (SPSS 15.0). Significant differences were based on the $P < 0.05$ level.

Results

The quality of parameters of the water used in the experiment

Mean dissolved oxygen was 7.1 ± 0.2 mg/l; mean ammonia, iron and copper content were less than 0.001 mg/l (for each parameter); mean calcium was 40.3 ± 0.21 mg/l; mean alkalinity was 182.2 ± 1.21 mg $CaCO_3$/l; mean hardness was 34 ± 4 °fH; mean pH was 8.3 ± 0.22 (American Public Health Association, 1985). Mean water temperature was 1.58 ± 0.20 °C in December 2018 and 1.41 ± 0.12 in January 2019.

Determination of the effect of MF injection on haemolymph MF

Methyl farnesoate injection did not affect haemolymph MF (ANOVA, $F_{3,16} = 0.08$, $P = 0.97$) (Fig. 1). Methyl farnesoate levels in the haemolymph were 1.39, 1.41, 1. 38 and 1.39 (ng ml^{-1}) for G1, G2, G3 and G4 respectively (Fig. 1).

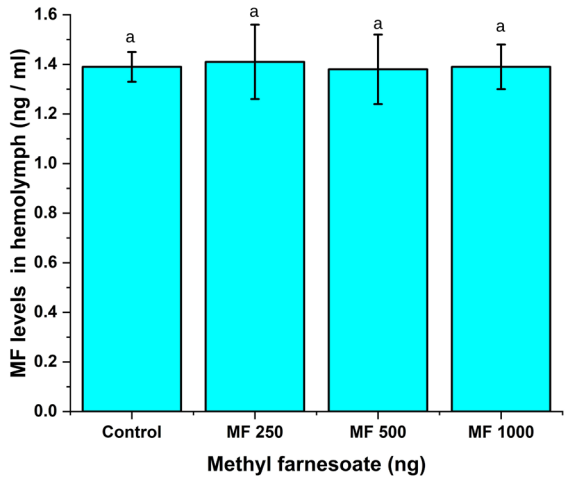

Figure 1. Effect of different dosages of MF on the MF concentration in the hemolymph of male *Pontastacus leptodactylus*. Letters indicate significant difference groupings ($P < 0.05$) (mean ± S.D; n = 15).

Effects of MF injection on GSI, TI, VSI, reproductive system, testis, and vasa deferentia weights

No mortality was observed in the present study. The highest weight of the reproductive system was found in the crayfish injected with G4 (1.54 g). Although there were no differences between G1 (1.07 g), G2 (1.05 g), and G3 (1.13 g), G4 showed a higher reproductive system weight than other groups (ANOVA, $F_{3,16} = 5.42$, $P = 0.009$) (Fig. 2). The results revealed that, GSI (%) was 2.27, 2.20, 2.40, and 3.38 for G1 (control), G2, G3 and G4, respectively. GSI was higher (ANOVA, $F_{3,16} = 10.11$, $P = 0.001$) in G4 than other treatments (Fig. 2). Testis weight (g) was 0.11, 0.12, 0.10 and 0.21 for G1, G2, G3 and G4, respectively. Testis weight was found to be higher in G4 than other treatments (ANOVA, $F_{3,16} = 14.81$, $P = 0.000$). The findings showed that the TI (%) value determined for the G4 group (0.46) was higher than the control group (0.24), G2 (0.25) and G3 groups (0.22) (ANOVA, $F_{3,16} = 12.96$, $P = 0.000$) (Fig. 3).

Vasa deferentia weight (g) was 0.89, 0.90, 0.96 and 1.25 for G1, G2, G3 and G4, respectively. Vasa deferentia weight was also found to be higher (ANOVA, $F_{3,16} = 3.10$, $P = 0.046$) in G4 compared to the other treatments. While the VSI (%) value was determined as 1.86, 1.89, 2.03 and 2.74 for G1, G2, G3 and G4, respectively. The VSI value was found to be higher (ANOVA, $F_{3,16} = 6.36$, $P = 0.005$) in G4 group than the control group and the other experimental groups (Fig. 4).

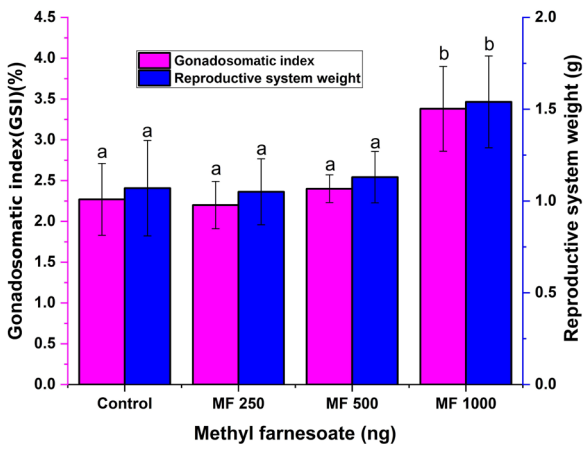

Figure 2. Effect of MF injection on reproductive system weight and GSI in male *Pontastacus leptodactylus*. Letters indicate significant difference groupings ($P < 0.05$) (mean ± S.D; n = 15).

Effect of MF injection on spermatozoal number

There was a no difference in spermatozoal number between the control (G1) (5.6 x10^6), G2 (5.80 x10^6) and G3 (7.0 x10^6) (Fig. 5). The highest number of spermatozoa were observed in G4 (8.20 x10^6) with no differences between G4 and G3. The lowest spermatozoal number (5.6 x10^6) was in the control group. Spermatozoal number was higher (ANOVA, $F_{3,16} = 3.33$, $P = 0.046$) in G4 than G1 and G2 treatments (Fig. 5).

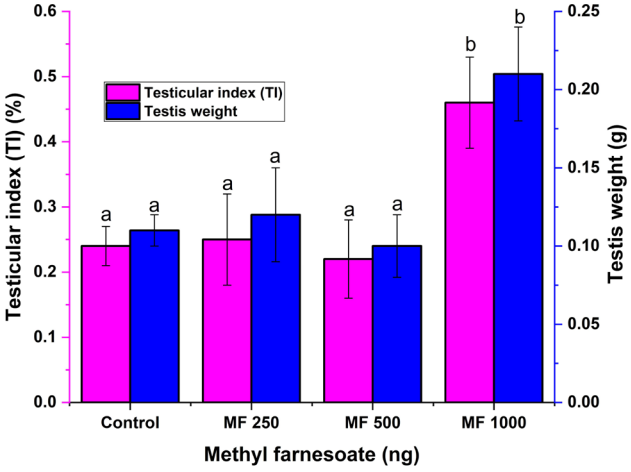

Figure 3. Effect of MF injection on testis weight and TI in male *Pontastacus leptodactylus*. Letters indicate significant difference groupings ($P < 0.05$) (mean ± S.D; n = 15).

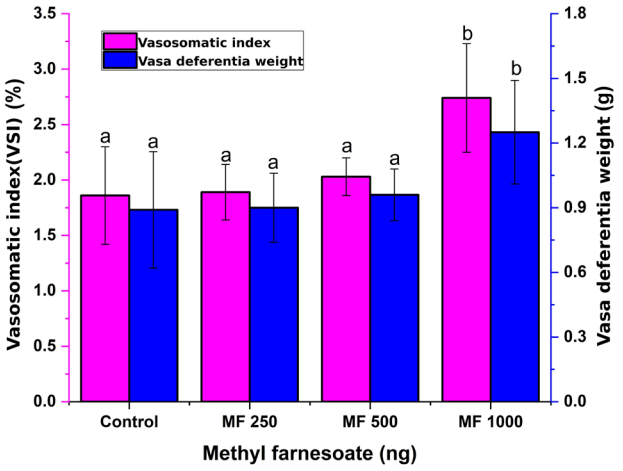

Figure 4. Effect of MF injection on vasa deferentia weight and VSI in male *P. leptodactylus*. Letters indicate significant difference groupings ($P < 0.05$) (mean ± S.D; n=15).

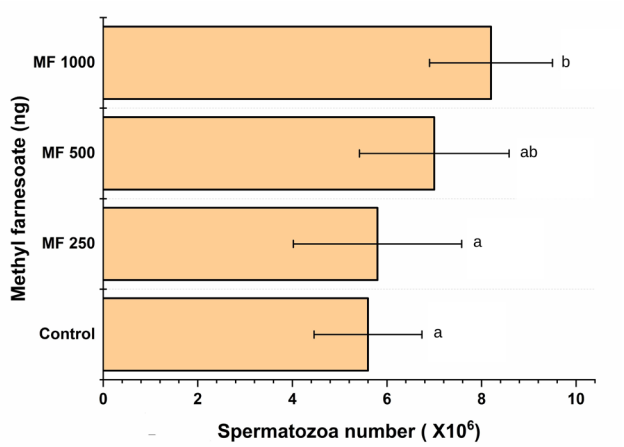

Figure 5. Effect of different dosages of MF on spermatozoal number (x 10^6) in male *Pontastacus leptodactylus*. Letters indicate significant difference groupings ($P < 0.05$) (mean ± S.D; n = 15).

Discussion

This study has shown that male *P. leptodctylus* injected with MF about six weeks prior to their reproductive period significantly increased spermatozoa production, reproductive system weight, GSI, TI and VSI. It was observed that after injection of 1000 ng MF body weight^{-1} GSI, TI and VSI of *P. leptodactylus* were significantly increased as compared to the control. Similar results have also been found by Kalavathy *et al.* (1999) on the effects of MF injection on the TI of freshwater crab *O. senex senex*. Kalavathy *et al.* (1999) injected animals with MF on days 1, 7 and 14 using 16 ng/crab and terminated the study on the 21st day. They found MF injection increased testicular weight, diameter, and size in this crab species, and also promoted the growth of testes by increasing the testicular index value, they concluded that MF was effective in stimulating testicular growth in freshwater crabs.

Nagaraju *et al.* (2004) found that MF injection at a 5 ng/freshwater shrimp dose significantly increased the TI of male *Macrobrachium malcolmsonii* (H. Milne Edwards, 1844). They determined a testicular index value of 0.33 in the control group that significantly increased to 0.52 as a result of MF injection. Based on these results, Nagaraju *et al.* (2004) argued that MF injection in *M. malcolmsonii* accelerated gonad development. Medesani *et al.* (2015) reported that

GSI in female crab, *N. granulata* was significantly higher in animals fed hormone enriched (17 – hydroxyprogesterone or MF) pellets compared to controls. Also, Marsden *et al.* (2008) reported that gonad size was increased in different decapod species following application of MF in vivo. In this study, it was determined that the GSI increased with MF injection in male crayfish. These results provide strong evidences that MF is involved in the control of reproduction in decapods.

Similarly, the testicular index of 0.24 in the control group significantly increased to 0.46 in T4 in the present study. It is thought this increase is due to the accelerating effect of MF on testicular development in decapods. Moreover, Nagaraju and Borst (2008) investigated the effects of environmental factors on testicular development of *Carcinus maenas* (Linnaeus, 1758) and found that environmental conditions such as salinity and water temperature affect the development of the testis by raising the MF value of the haemolymph.

Methyl farnesoate is a terpenoid hormone that has crucial roles in reproduction of both females and males (Laufer *et al.,* 1998). The level of this hormone was determined in different decapods in some studies, for example, the MF level of the crayfish *Procambarus clarkii* (Girard, 1852) haemolymph was found to be between 0.5 ng/ml and 3 ng/ml, (Laufer *et al.,* 1998) and 1.41 ng/ml (Laufer *et al.,* 2005). In addition, Borst and Tsukimura (1991) found that the MF value in the American lobster, *Homarus americanus* (Milne Edwards, 1837) hemolymph was 5 ng/ml, and Rotllant *et al.* (2001) determined that the MF of Norwegian lobster *Nephrops norvegicus* (Linnaeus, 1758) haemolymph in males and females was between 0.5 to 1 ng/ml. In the present study, the MF level of haemolymph was 1.38–1.41 ng/ml in *P. leptodactylus*.

In this study, the hemolymph MF analysis was determined one week after the last injection. The lack of any significant differences in the concentration of MF in the hemolymph of crayfish suggests that the injected MF dosages were converted to spermatozoal production and associated reproductive parameters at the end of the experiment.

Alfaro *et al.* (2008) found that MF injected into shrimp *L. vannamei* at a doze of 120 ng/g resulted in approximately 38 million spermatozoa, while the number of spermatozoa in the control was around 2 million. They also found that injection of juvenile hormone III into *L. vannamei* did not significantly increase the spermatozoal number compared to the control group. Therefore, Alfaro *et al.* (2008) concluded that MF is the reproductive hormone in *L. vannamei*, and that they cannot use juvenile hormone III instead of MF. Similarly, in this study, MF application increased spermatozoal count in crayfish.

The effects of hormones on the number of spermatozoa in decapods were investigated by other authors. For example, Fatihah *et al.* (2014) reported that the mean of sperm quantity in male mud spiny lobster, *Panulirus polyphagus* (Herbst, 1793) was increased with 17α-hydroxyprogesterone (17α-OHP) and 17α-hydroxypregnenolone (17α-OHPL) treated hormones. As a conclusion, Fatihah *et al.* (2014) recommended that higher doses of 17α-OHP and 17α-OHPL should be tried to observe whether there is any significant effect on sperm quality, quantity and accordingly higher hormone concentration.

Alfaro (1996) injected 17 α-Methyltestosterone into the shrimp *L. vannamei*. The number of spermatozoa in the control was 10.9 million, whereas it increased to 32.7 million in treated shrimp with 17 α-Methyltestosterone at a dose of 0.01 pg/g and reported it reached 31.7 million in the shrimp treated with 17 α-Methyltestosterone at a dose of 0.1 pg/g. In our study, a total of 5 MF injections were administered to crayfish once a week before mating. When compared to the control group, the number of spermatozoa obtained from 1000 ng MF body weight^{-1} treatment was higher.

Overlap has been reported between proteomic profiles of the freshwater crayfish eggs and spermatozoa (Niksirat *et al.,* 2014a; 2015a) and it would be interesting to run future experiments to explore the potential effects of MF elevation on the expression levels of similar proteins in male and female gametes.

This study shows that MF injection can notably increase some reproductive indices in male crayfish such as spermatozoal number, GSI, TI, and VSI of *P. leptodactylus*. The dose of 1000 ng MF body weight^{-1} treatment resulted in an increase in both the number of spermatozoa and the weight of the

reproductive system. The results of this study are consistent with the results of other studies on other decapod species (Nagaraju *et al.,* 2004; Alfaro *et al.*, 2008; Raghavan and Ayanath, 2018). Further research should be carried out to determine the effect of MF injection on reproductive endocrine metabolism, gamete composition (*e.g.*, protein and lipid profiles) and histology of different regions of the vas deference to assess changes in quantities and ratios of cell types in male reproductive tracts in crayfish.

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Additional information and declarations

Disclosure statement

The authors declare that there is no conflict of interest.

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