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Effects of Azomite enriched diet on gonadal steroid hormone levels and milt quality indices in *Oreochromis mossambicus*

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ABSTRACT

The effects of Azomite, a natural mineral of volcanic ash, on the sex steroid hormone (SSH) levels and milt quality indices (MQI); namely, the milt volume, motility of spermatozoa, its duration and sperm count, were evaluated in tilapia (*Oreochromis mossambicus*). Several reports have documented the positive effect of the Azomite supplementation on growth, survival, and immune response of aquatic organisms. In this study, a healthy brooder stock of male *O. mossambicus* was divided into four experimental groups, and fed with Azomite supplemented diets of varying concentrations: 0 g/kg body weight (Control), 2, 4, and 6 g/kg body weights. After the eight-week experimental trial feeding, the 4.0 g/kg Azomite supplemented diet presented greater SSH levels and MQI than the other diets and the control group. The quantity of milt enhanced with a significant ($p < 0.01$) increase in the 4 g/kg Azomite supplemented group compared to the control and other Azomite supplemented groups. Similarly, the maximum percentage of spermatocrit and sperm count was found in the 4 g/kg diet-fed group. Likewise, the highest percentage and duration of sperm motility, as well as sex-specific steroid hormones, such as testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (E_2) in the milt samples was found in 4 g/kg Azomite supplemented group. Dietary inclusion of 4 g/kg Azomite significant improved gonadal steroid hormone levels and milt quality indices in *Oreochromis mossambicus*.

1. Introduction

In teleost, the hormonal assay for sex steroids, such as testosterone and 11-ketotestosterone, are considered as indicators for monitoring the production and development of sperm cells. The motility of sperm cells is an important factor in sperm quality assessment. Other factors include sperm concentration, volume, seminal plasma pH, and osmolality; are the main sperm quality indicators measured in fish milt (Cejko, 2019).

Fish reproduction, similar to other animals, is under the direct influence of sex steroid hormones, which are essential for the formation of spermatozoa, maturation, and the physiology of gonads and reproduction. Also, the regulation of reproductive characteristics of fish depends on certain sex steroid hormones, like testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E_2). Whereas E_2 is the most important female hormone, a recent study has demonstrated that optimum estrogen level is "vital" for male reproductive behavior. For example, in the male teleost, E_2 regulates the formation of spermatogonia and functions of Sertoli cell (Musthafa et al., 2014).

According to Karimi et al. (2015), 17β-estradiol (E_2) and testosterone (T) are the main hormones responsible for spermatogenesis processes in aquatic organisms. On the other hand, these hormones were strongly connected in enhancing the concentration, duration, and motility of spermatozoa in Russian sturgeon, *Acipenser gueldenstaedtii* (Karimi et al., 2015). Seminal plasma plays a vital role in maintaining the quiescent state of sperm, by providing optimal conditions. This condition is achieved through equitable osmolality level, inorganic ion (electrolytes) concentrations, and organic constituents (metabolites) for the protection of spermatozoa (Lahnsteiner, 2003).

Previous studies have demonstrated that antioxidants, Zn, Selenium (Se), and other mineral compounds are necessary for reproductive processes (Mills, 1988; Ciereszko and Dabrowski, 1995, 2000; Baiomy

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et al., 2009). Trace metals, such as Magnesium, Selenium, Strontium, and Zinc have expressed positive and statistically significant correlations in sperm quality parameters (velocity and distance); whereas content of Copper and Mercury have produced negative results with progressive motility in common carp, *Cyprinus carpio* (Kovacik et al., 2018).

In this present study, Azomite, a naturally occurring volcanic mineral product, was selected as a functional feed additive in the basal diet. Azomite is an acronym for "A to Z of minerals, which also includes trace elements." Its chemical name is hydrated calcium sodium aluminosilicate ($NaK_2Ca_5Al_3Si_{21}O_{70}6H_2O$), and it is listed with the United States Code of Federal Regulations (21 CFR 582.2729) as an additive in livestock feed for anticaking and considered safe by the United States Food and Drug Administration (FDA). It comes under the protocols for use in animal feed by the Association of American Feed Control Officials (AAFCO) and is approved by Organic Materials Review Institute (OMRI), U.S.A.; as a natural mineral that can be used to enrich the diets in animal husbandry, aquaculture, and organic agriculture. It is proven in our recent findings that 4 and 6 g/kg Azomite supplemented feed improved survival rates, increased body weights, protein efficiency ratios, specific growth rates, feed utilization efficiency, and immunity for disease resistance in Mozambique tilapia against *A. hydrophila* (Musthafa et al., 2016).

In our previous studies, the 4 % and 6 % zeolite supplemented feed significantly increased body growth, immune response, and disease resistance in *C. striatus* against *A. invadans* (Jawahar et al., 2016). The feed, given at 4 % of body weith during the experimental period, consisted of a 1 % Azomite inclusion in the feed supplement containing 38.3 % crude protein. The *Pangasius hypothalamus* showed an increase in body weight at 90 days of the study period (Batool et al., 2018).

Azam et al. (2016) concluded that using Azomite as a feed at 0.75 % for 49 days can act as a growth enhancer in genetically male tilapia fingerlings. Furthermore, Tan et al. (2014) also used Azomite in the diet of white shrimp with dosages of 2 and 4 g/kg, which resulting in increases in growth performance and disease resistance.

Recently, Fodge and Fodge (2014) reported that Azomite enriched diet increased both digestive and immune enzymes *via* the enhancement of lysozyme (+ @40 %), superoxide dismutase (+ @15 %), phenoloxidase (+ > 90 %), and alkaline phosphatase (+25 %) in various tissues of Tilapia.

2. Material and methods

2.1. Preparation of experimental feed

The basal control fish feed consisted of a mix of 4 % rice bran, 53.4 % soybean meal, 26.6 % fish meal powder, 2 % corn flour, and 13 % wheat flour pulp. The mixture was then steamed for 15 min, and a 1 % vitamins and mineral premix and 40 ml/kg cod liver oil was added as the temperature lowered. The preparation of the Azomite (®V5 Organics, Chennai) incorporated diet, added to the control basal diet, varied at 2, 4, and 6 g kg^{-1} dry weight basis. The supplement mixtures were then steamed, cooled, and pelletized. The control and experimental feed were dried separately at 40 °C in an oven and packed in an airlock polythene bags for further use. The feed should be kept in a cool and dry place free from contamination (Table 1).

2.2. Experimental design

Mature adult male, *O. mossambicus* (mean weight 174.6 ± 3.7 g and length 13.72 ± 2 cm), were collected from aquaculture farm. The fishes were acclimatized in dechlorinated tap water for 21 days under lab conditions with a 12:12 light and dark photoperiod and fed 3 % of their body weight. The undigested waste particles, feces matter, and leftover feed were removed post feeding to prevent contamination and turbidity. Water (30 %) was changed every day. Temperature, pH, dissolved oxygen, and hardness ($CaCO_3$) were kept at 7.0 ± 0.2, 26.0 ± 1.0 °C, 6.0 ± 0.2 mg/l and 220 mg/L, respectively. The fishes were kept in four separate glass tanks, 25 each, in triplicate (4 × 25 × 3 = 300 fish); and were fed with (i) non-Azomite diet (control), (ii) 2 g/kg, (iii) 4 g/kg, and (iv) 6 g/kg Azomite diets.

Table 1
Formulation and proximate composition of experimental diets (% dry matter).

Diets (g/kg)	Control	2	4	6
Ingredients (g/kg)				
Dry fish meal [a]	450	450	450	450
Dehulled Soybean meal	200	200	200	200
Wheat	130	130	130	130
Rice	80	78	76	74
Maize	50	50	50	50
Fish oil [a]	40	40	40	40
Lecithin	20	20	20	20
Vitamin &Mineral [b]	20	20	20	20
Binder [c]	10	10	10	10
Azomite [d]	0	2	4	6
Proximate composition (g/kg)				
Moisture	76.2	73.0	72.0	74.9
Crude protein	405.6	407.0	407.3	410.5
Crude lipid	90.3	90.7	92.5	94.0
Crude fiber	22.0	23.5	25.5	30.6
Total ash	104.2	110.7	115.5	117.2

[a] Sardine fishmeal and fish oil. Bismifisheries, Mayiladuthurai, Tamil Nadu, India.
[b] Vitamin premix each kg contains: Vitamin A, 2,000,000 IU; Vitamin D, 400,000 IU; Vitamin E, 300 U; Vitamin K, 450 mg; Riboflavin, 800 mg; Panthothenic acid, 1 g; Nicotinamide, 4 g; Vitamin B12, 2.4 mg; Choline chloride, 60 g; Ca, 300 g; Mg, 11 g; I, 400 mg; Fe, 3 g; Zn, 6 g; Cu, 800 mg; Sarabhai Zydus Animal Health Ltd, Vadodara, Gujarat, India.
[c] Pegabind, BentoliAgri nutrition Asia pvt Ltd, Singapore.
[d] Azomite- V5 Organic Pvt. Ltd. Chennai.

2.3. Milt collection

The experiment was conducted over 8 weeks, and the milt was collected by gently pressing the abdomen region. The first drop of milt was wiped, and the external genital area was then wiped with distilled water and dried using tissue paper to prevent contamination with urine, feces, water, and blood (Musthafa et al., 2014). Milt was collected using 1 ml insulin syringes (± 0.01 ml, without needles) to analyze the total milt ejaculation per male. To maintain adequate aeration for the milt sample, approximately 30 % of the syringe space was left empty. After labeling, the samples were stored at 4 °C, protected from sunlight, and examined within 1 h of the time of collection (Magnotti et al., 2018; Zadmajid et al., 2018).

2.4. Spermatocrit

Approximately 90 % or 50 µl of fresh milt was collected in each microhematocrit capillary tubes (7.5 cm length and 0.12 cm internal diameter) and sealed. The tubes were centrifuged for 8 min at 3000 rpm and the percentage of each milt sample was estimated using light microscopy at 400 × magnification, immediately after diluted with deionized water as an activation solution (Zadmajid et al., 2018).

2.5. Sperm count

The sperm count was determined using a Burker cell hemocytometer. A small drop of diluted milt was placed on a clean glass slide and closed with the coverslip for microscopy. All experiments were carried out in triplicate. Within a few minutes, as the sperm became sedimented, the cells counts were observed at 100x magnification and calculated as spermatozoa × 10^6 per ml (Billard, 1977).

2.6. Sperm motility

The milt was diluted with deionized water (1:100) at room temperature (31 °C). Approximately 20 μl of the activated sperm cells were transferred to a microscope slide (25 × 75 mm), covered with a coverslip (22 × 22 mm, 0.95–1.05 mm thickness), and examined at 200× magnification. Assessment of sperm motility was carried out after 10 s of dilution and sperm movement was observed within 3 min. Observations were made by the same observer from different angles, and repeated at least three times for each milt sample. During the observation, only the sperms that were actively moving in a progressive straight-line and forward direction were considered as a good one. Whereas, simply vibrating or turning on their axes was considered as immotile and low-quality spermatozoa. The motility period was measured using a stopwatch from the time of sperm activation until a phase of immobility of all the spermatozoa. The percentage of sperm motility was calculated using the following formula:

$$\text{Percentage of motile sperm} = \frac{\text{No. of motile sperm counted}}{\text{No. of sperm counted}} \times 100$$

2.7. Hormone analysis

The selected sex-specific steroid hormones (SSH) were measured using appropriate RIA kits as per our previously published paper (Musthafa et al., 2014). Radio-immunoassay (RIA) was used to measure testosterone (T), 11-ketotestosterone (11-KT), and17β-estradiol (E2) in the serum and milt samples of the control and Azomite enriched tilapia, *O. mossambicus*. The concentrations of steroid hormones were determined using appropriate FRANSA radioimmuno assay kits (Catnos. Testoster-one: CM-TESTO; Estradiol17-b:SB-ESTR;CM-PROG supplied by FRANSA). Plasma was subsequently analyzed. All FRANSA RIA test kits made use of ^{125}I labeled hormones, which are intended for use with human samples. All readings of radioactivity were taken using a Beckman Gamma 8500 Microprocessor Counter.

2.8. Statistical analysis

The Pearson correlation coefficient test method was carried out to determine the statistical data at the level of 95 % using SPSS v. 16.0. Statistical significance was set at the level $p < 0.05$ and $p < 0.01$ with mean ± standard deviation (SD).

3. Results

The results of the SSH and MQI in *O. mossambicus* fed with Azomite supplementation diets are shown in Fig. 1, and Tables 2 and 3. The results indicated that SSH levels and MQI increased significantly when supplemented with 4 and 6 g/kg of Azomite mixed feed.

3.1. Milt volume

The quantity of milt enhanced with a significant ($p < 0.01$) increase in the 4 g/kg Azomite supplemented group, followed by a further ($p < 0.05$) increase in the 2 and 6 g/kg Azomite enriched diet groups. The sequential increment of milt volume was recorded as follows; 18.0 % increase in 2 g/kg; 40.0 % increase in 6 g/kg; and 48.6 % increase in 4 g/kg Azomite fed groups when compared to the control group.

3.2. Spermatocrit

The maximum (46.83 ± 1.57) percentage of spermatocrit was found in the 4 g/kg diet-fed group, followed by 6 g/kg (44.52 ± 3.34 %) and 2 g/kg (42.32 ± 2.18 %) Azomite supplemented diet-fed groups. A strong significant ($p < 0.01$) increment of spermatocrit was noticed at 4 and 6 g/kg Azomite supplemented groups; whereas a moderate significant ($p < 0.05$) increase was seen in the 2 g/kg Azomite enriched diet group, as compared to the control.

3.3. Sperm count

In this study, 4 and 6 g/kg Azomite supplemented groups exhibit strong significant ($p < 0.01$) enhancement of sperm count over both the control and 2 g/kg enriched diet groups.

3.4. Sperm motility & duration

A significant increase ($p < 0.01$) in the percentage and duration of sperm motility was found in 4 g/kg, followed by the 6 g/kg and 2 g/kg Azomite supplemented diet-fed groups compared to the control.

3.5. Sex-specific steroid hormones

The 4 g/kg Azomite supplemented group showed an increase ($p < 0.01$) in sex-specific steroid hormones, such as testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (E_2) in the milt samples, followed by the 6 g/kg and 2 g/kg Azomite supplemented groups when compared to the control group.

4. Discussion

The Azomite supplemented with the 4 g/kg diet group exhibited a 62 % of increase in milt volume over the 2 g/kg diet group; whereas, a 17 % milt volume difference was recorded between the 4 and 6 g/kg Azomite fed groups, and a 55 % milt volume difference was recorded between the 6 and 2 g/kg Azomite fed groups (Table 2). Milt volume in the present study showed a very strong positive correlation ($p < 0.05$) with the Azomite supplemented diets, which enhanced both milt volume and spermatozoa production. Similarly, a strong positive correlation was observed between 11-KT and duration of motility ($p < 0.05$). The 11-KT levels were positively correlated ($p < 0.01$) with other MQI; such as sperm count, milt volume, sperm motility, and duration of motility (Table 3). The quantity of milt and duration of the sperm motility increased more than 50 % with the 4 g/kg experimental diet.

Our results revealed that the spermatocrit percentage was highest in the 4 g/kg Azomite enriched diet group, which is 60.6 % greater than the 2 g/kg group, and 31.4 % greater than 6 g/kg group. In our findings, a higher osmolality rate was observed in milt fed the 4 and 6 g/kg supplements, compared to the 2 g/kg diet (Table 1). The sperm usually moved for a duration of 2 min in many of the freshwater fish, compared to 30 s in many other species (Morisawa and Suzuki, 1980; Perchec et al., 1993; Billard et al., 1995a). In our findings, the longest duration of sperm motility, 105 s, was attained in the 4 g/kg of Azomite enriched diet group.

The milt pH demonstrated in the present study showed no significant change ($p > 0.05$) in any of the Azomite treatment groups (Table 2). As the relationship between pH and fertilization rate followed a quadratic function, the pH of semen (from 8.0 to 8.2) presented a higher fertilization rate of about 80 %. Adversely, lower pH saline solutions (ranging from 7.8 to 8.0) inhibited motility, indicating optimal fertilization of the egg (Billard and Cosson, 1992).

In chum salmon (*Oncorhynchus keta*), an increase in external pH was determined to be responsible for the decrease in sperm mobility while traveling from the testis to the spermatic duct (Billard et al., 1995a; Billard et al., 1995b). Hence, seminal fluid pH may also affect the final maturation of spermatozoa. In the present study, the partial correlation coefficients revealed that the correlations between pH and fertilization rate, as well as the correlations between pH and sperm motility rate, were not codependent. Therefore, seminal fluid pH and sperm motility parameters were not used as independent indicators for semen fertilization capacity. Correlations between fertilization rate and osmolality were dependent on their correlation with pH (Table 3).

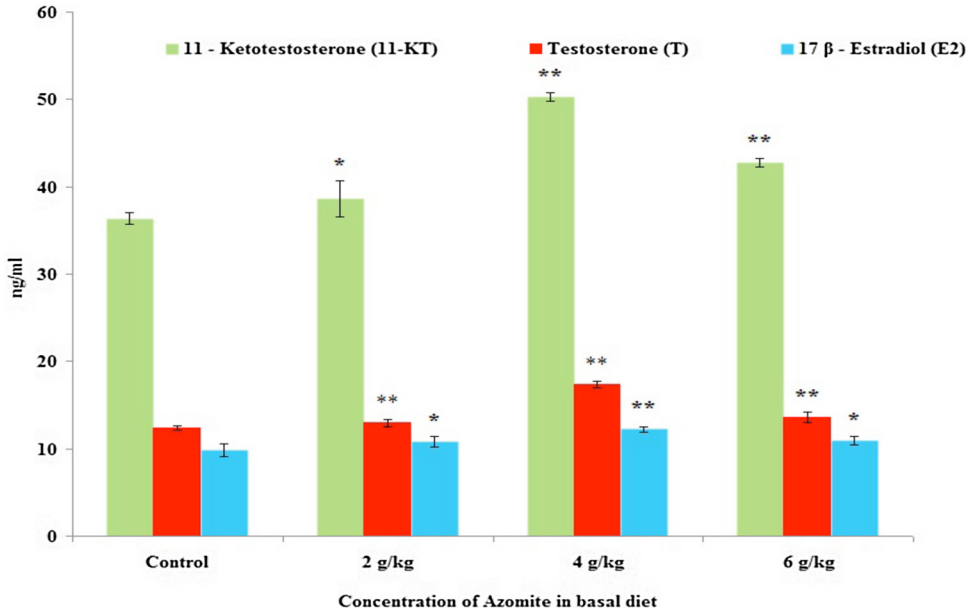

Fig. 1. Changes of testosterone (T), 11- ketotestosterone (11-KT), and 17β-estradiol (E_2) levels in the milt samples of *O. mossambicus* (n = 6) fed with the control and Azomite supplementation diets.

Based on elemental analysis, results of the Organic Materials Review Institute (OMRI), Eugene, USA, and U.S. Food and Drug Administration (FDA), revealed that Azomite contains high concentrations of Zn and Se. These metals serve as cofactors for more than 80 metalloenzymes involved in the transcription of DNA and the synthesis of protein, which are found to be the major components of germ cell maturity.

The present findings were analogous with boar fed dietary Selenium; which did not affect the sperm quality, volume, concentration, total sperm in one ejaculation, sperm motility, progressive motility, morphology, or antioxidant activity (Lovercamp et al., 2013). In rats, mice, chickens, pigs, sheep, and other cattle; an insufficiency of Se is associated with complications in reproduction and decreased sperm quality (Baiomy et al., 2009). Zn is essential for testicular development, differentiation, formation, and maturation of spermatozoa. Moreover, Zn-binding proteins are implicated in the genetic expression of steroid hormone receptors (Favier, 1992; Freedman, 1992), anti-apoptotic (Chimienti et al., 2003), and antioxidant (Zago and Oteiza, 2001) properties.

Several minerals and antioxidants improved male and female sex steroid hormone, as well as milt quality in fish and other animals. Adedara et al. (2019) investigated the reproductive function of male rats exposed to zinc and nickel over 45 days. The results showed significantly ($p < 0.05$) reduced levels of ROX species and LPO in the hypothalamus, epididymis, and gonads. Luteinizing hormone, follicle-stimulating hormone, serum, and intra-testicular testosterone, as well as sperm production and quality were also enhanced (Adedara et al., 2019). Zinc is an essential nutrient for living beings, as it plays a vital role in several biological activities. Sperm motility duration, seminal plasma quality, reproductive performance, and spermatocrit percentage were higher in broodstock male rainbow trout (*Oreochromis mykiss*) supplemented with 40 mg/kg of mineral zinc ($ZnSO_4$) over 16 weeks (Kazemi et al., 2020).

The study carried out by Krishnaiah et al. (2019) revealed that the feed supplemented with natural trace minerals, such as zinc and copper in a dose-dependent manner, caused intense sexual behavior, enhancement in sperm number per ejaculate, total motility, spermatozoa gene expression; and altered LH, testosterone, and T4 hormones in osmanabadi breed bucks (Krishnaiah et al., 2019). Zn and Se are metallic elements that influence beneficial health, as well as play important roles in spermatogenesis and male fertility. Se serves in a group of antioxidant compounds, called seleno-proteins, many of which prevent the oxidative degradation in the cell membranes.

Supplementation with Zn and Se in basal diets has been found to exert beneficial effects on spermatogenesis, as well as improve sperm quality in aquatic organisms (www.livestrong.com/article/463788-the-effects-of-selenium-zinc-on-spermatogenesis/). Sperm motility did not show a strong correlation with any other MQI and SSH in the present study, yet maintained a positive relationship with T and E_2. The highest sperm motility was noted during high levels of T and E_2 (Fig. 1). Overall, no negative correlation was found between SSH and MQI when fed the Azomite supplementation diets in the present study (Table 2). The current finding was undertaken to meet the expectations of farmers and hatcheries to improve the milt quality and fertilization rates in broodstock male tilapia.

Table 2
Changes milt quality indices (MQI) in *O. mossambicus* (n = 6) fed with control and Azomite supplementation diets.

Groups	Milt volume (ml)	Spermatocrit (%)	Sperm count (x 10^6)	Sperm motility (%)	Duration of motility (Seconds)	Osmolality (mOsm/kg)	pH
Control	0.37 ± 0.08	39.42 ± 0.49	18.18 ± 1.27	67.83 ± 6.55	55.50 ± 1.71	261.83 ± 2.19	7.62 ± 0.17
2 g/kg	0.45 ± 0.05*	42.32 ± 2.18*	20.03 ± 0.50*	71.67 ± 1.03*	62.30 ± 0.75*	265.50 ± 1.12*	7.85 ± 0.08
4 g/kg	0.72 ± 0.10**	46.83 ± 1.57**	27.80 ± 2.16**	74.17 ± 3.37**	105.00 ± 9.57**	289.17 ± 6.52**	7.93 ± 0.21
6 g/kg	0.62 ± 0.10*	44.52 ± 3.34**	24.33 ± 7.07**	72.83 ± 3.13*	76.00 ± 2.43*	276.83 ± 7.88**	7.62 ± 0.17

' ± ' Standard deviation.
* Significant at the 0.05 level.
** Significant at the 0.01 level.

Table 3
Spearman's correlation between sex steroid hormone levels and milt quality indices in *O. mossambicus* fed with control and Azomite supplementation diets.

Parameters	Milt volume (ml)	Sperm count (x 10^6)	Sperm motility (%)	Duration of motility (Seconds)	11-KT	T	E_2
Milt volume (ml)	–	–	–	–	–	–	–
Sperm count (x 10^6)	0.997**	–	–	–	–	–	–
Sperm motility (%)	0.919	0. .903	–	–	–	–	–
Duration of motility (Seconds)	0.951*	0.972*	0.841	–	–	–	–
11-KT	0.964*	0.981*	0.861	0.999**	–	–	–
T	0.991**	0.997**	0.922	0.979*	0.987*	–	–
E_2	0.930	0.944	0.922	0.967	0.970*	0.968*	–

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

5. Conclusion

To the best of our knowledge, this is the first study addressing the effects of Azomite supplementation diets on sex-specific steroid hormones and milt quality indices in tilapia, *O. mossambicus* under laboratory conditions. The present study revealed that the 4 and 6 g kg^{-1} Azomite supplementations enhanced the sex-specific steroid hormone levels and milt quality indices, due to the presence of trace minerals (Zn, Cu, Cr, and Se) which are essential for testicular development, differentiation, formation, and maturation of spermatozoa. This study suggests that the Azomite enriched diets elevate the milt quality and augment reproductive performances of *O. mossambicus* in place of synthetic hormones, such as HcG, LH_{RH}, and Ova-prime. Further studies are needed to confirm the effect of Azomite in other fish species to be used in the field of aquaculture.

Author statement

Habeeb Ashik Ahamed: Conduct experiment
Mohamed Jamal Mohamed: Contribution to the sample collection
Kantha Deivi Arunachalam: Analysis and interpretation
G. I. Darul Raiyaan: Literature search
Mohamed Saiyad Musthafa: Conception and design
S. Subeena Begum: Contribution to the manuscript preparation
Hien Van Doan: Final approval of the article

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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