Fish Physiol Biochem (2018) 44:1253–1264
https://doi.org/10.1007/s10695-018-0518-9

Effects of dietary microencapsulated *Cymbopogon flexuosus* essential oil on reproductive-related parameters in male *Rhamdia quelen*

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Received: 23 January 2018 / Accepted: 8 May 2018 / Published online: 18 May 2018

Abstract In aquaculture, nutrition and supplemented diets have been shown to affect broodstock reproductive performance. In this study, we investigated the effects of dietary supplementation with *Cymbopogon flexuosus* essential oil (CFEO) microcapsules on reproductive-related parameters in silver catfish (*Rhamdia quelen*) male broodfish. Adult male broodstocks were separated into three groups according to the concentrations of supplemented CFEO (0.0 = control; 1.0 or 3.0 mL per kg of diet). After 20 days under experimental conditions, the animals were euthanized and the gonads were harvested for gonadosomatic index, sperm analysis, oxidative stress, and histopathology; testosterone levels were measured in the plasma; gene expression of *prl*, *smtl*, *pomca*, and *pomcb* was assessed in the pituitary gland by real-time PCR. The results showed no alterations on reproductive parameters in *R. quelen* males treated with *Cymbopogon flexuosus* essential oil compared to the control-diet animals. In conclusion, CFEO microcapsules supplied for 20 days in the concentrations of 1.00 or 3.00 mL per kilogram of diet did not affect the reproduction criteria evaluated in this study in male silver catfish.

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Keywords Broodstock · Silver catfish · Supplemented diet · Gonad · Reproduction

Introduction

Silver catfish *Rhamdia quelen*, member of the Siluriformes order, is widely distributed throughout South America (Gomes et al. 2000). This species has aroused great interest for fish farming in the southern region of Brazil, mostly due to its resistance to handling procedures, prolificacy and growth potential, good feed conversion ratio, meat flavor and texture, absence of intramuscular spines, and good acceptance of artificial diets (Gomes et al. 2000; Canton et al. 2007; Parra et al. 2008; Baldisserotto 2009).

Fish nutrition is closely related to their reproductive performance (Bombardelli et al. 2010), and studies relating these factors are of great importance. Dietary compounds can affect the endocrine system and consequently reproductive parameters, namely, semen quality (Watanabe and Vassallo-Agius 2003), fecundity (Tyler and Sumpter 1996), and the quality of gametes, embryos, and larvae (Izquierdo et al. 2001). Studies have shown the strong potential of certain plants and their extracts as additives in fish feed, not only as dietary supplements, but also as growth promoters (Zeppenfeld et al. 2014), antioxidants (Saccol et al. 2013), antibacterial (Galina et al. 2009; Reverter et al. 2014), and delayer of gonadal maturation (Kareem et al. 2016).

Cymbopogon flexuosus is popularly known as lemongrass and is widely used in traditional medicine (Carlini et al. 1986). *C. flexuosus* essential oil (CFEO) is widely used in aromatherapy as pain reliever, in skincare and cosmetic products. It is also used in pesticide and food industries due to its excellent biocompatibility and inherent antibacterial, antifungal, insecticidal, antiseptic, and antiinflammatory properties (Torres 1996). This oil is characterized by the presence of monoterpenes such as citral (a racemic mixture of geranial and neral), geraniol, citronellol, citronellal, linalool, elemol, 1,8-cineole, limonene, geraniol, methyl heptenone, geranyl acetate, and geranyl formate (Ganjewala et al. 2008; Ganjewala 2009; Ganjewala and Luthra 2010). In addition, CFEO citral chemotype has recently been reported in *R. quelen* to act as anesthetic and sedative when added to the water (Dos Santos et al. 2016) and as growth promoter when supplied by the diet (Baldisserotto et al. 2015). However, the effects of CFEO on fish reproduction are yet unknown.

The use of essential oils can be used for avoid oxidative damage. Ngoula et al. (2017) showed that oral administration of essential oil of guava (*Psidium guajava*) mitigated the deterioration of sperm of guinea pig (*Cavia porcellus*). In this sense, the use of natural compounds with oxidant propriety may assist in the prevention of oxidative stress. These compounds act as antioxidants and inhibitors of lipid peroxidation. Oxidative stress is a state related to increased cell damage triggered by oxygen and oxygen-derived free radicals known as reactive oxygen species (ROS). Due to the high levels of polyunsaturated fatty acids in plasma membranes, spermatozoa of both mammals (Lenzi et al. 2002) and fish (Pustowka et al. 2000) are particularly susceptible to oxidative damage.

Despite the established relationship between nutrition and reproduction in fish (Izquierdo et al. 2001), there is little information available to date regarding Neotropical species, such as silver catfish. Recently, Rampelotto et al. (2018) demonstrated that the addition CFEO to silver catfish diet for 20 days improved protein deposition and carcass yield. Therefore, the aim of this study was to evaluate the effects of feed supplementation with CFEO on testis histology and gonadosomatic index, sperm analysis, hormonal indicators, and oxidative stress biomarkers of silver catfish male broodstocks. In addition, we aim to test the microencapsulated form of CFEO, since generally, essential oils are very unstable in the presence of light, heat, oxygen, and moisture. Moreover, CFEO presents low miscibility with water and high volatilization rates, becoming unstable in pharmaceutical formulations (Boukhatem et al. 2014). Besides, we hypothesize that the dietary addition of CFEO (citral chemotype) affects reproductive parameters in silver catfish males.

Materials and methods

Essential oil (CFEO)

CFEO (leaves) was donated by Bio Natural Essenciais (Três Passos, Rio Grande do Sul, Brazil). The composition of CFEO was determined using an Agilent 6890 gas chromatograph coupled to a mass spectrometry detector (GC-MS) Agilent 5973 with a HP5-MS column (5% phenyl, 95% methylsiloxane, 30 m × 0.25 μM ID × 0.25 mm) as described previously (Silva et al. 2012).

The CFEO constituents were identified by comparison with the Kovats retention index and mass spectra with a mass spectrum library. Ninety-three percent of EO compounds were identified, and the major constituents (> 1.5%) were geranial (45.7%), neral (32.1%), Z-verbenol (2.4%), citronellol (2.0%), Z-geraniol (2.0%), and caryophyllene (1.7%).

To prevent essential oil degradation before fish intestinal absorption, it was microencapsulated by complex conservation following the method described by Alvim and Grosso (2010), before incorporation into fish feed. The amount of CFEO contained in the microcapsules was quantified by gas chromatography-mass spectrometry after hydrodistillation using a Clevenger apparatus.

Experimental diets

Three diets were formulated: 0.0 (control), 1.0, and 3.0 mL of microencapsulated CFEO per kilogram of diet. Diets had similar composition except for the presence of CFEO (Table 1). The ingredients were manually homogenized, pelletized (6 mm), and then dried at 45 °C in an airflow oven for 24 h. After drying, feeds were stored at − 18 °C.

Table 1 Formulation and proximate composition of diets

	Experimental diets (mL EO per kg of diet)		
Ingredients of experimental diet	0.0	1.0	3.0
Meat and bones meal (g kg^{-1})	350	350	350
Soybean meal (g kg^{-1})	300	300	300
Broken corn (g kg^{-1})	150	150	150
Rice bran (g kg^{-1})	120	120	120
Canola oil (g kg^{-1})	30	30	30
Vitamin and mineral premix (g kg^{-1})	30	30	30
Sodium chloride (g kg^{-1})	10	10	10
Dicalcium phosphate (g kg^{-1})	10	10	10
Microcapsules containing CFEO (g kg^{-1})	0	4.3	13
Composition (%)			
Moisture	5.73	5.55	5.70
Protein	34.15	33.86	34.85
Fat	8.34	8.98	8.93
Ash	21.71	22.58	21.65
Crude fiber	3.13	3.01	3.27
NFE	27.28	26.21	25.40
CFEO	0	0.1	0.3

CFEO, *Cymbopogon flexuosus* essential oil; *NFE*, nitrogen-free extract

Moisture, ash, and crude protein of experimental diets were analyzed following the methods of Association of Official Analytical Chemists (AOAC 2005). Fat content was determined by gravimetric method (Bligh; Dyer 1959). Nitrogen-free extract (NFE) was determined by difference [NFE = 100 − (% moisture + % protein + % lipid + % crude fiber + % total ash)].

Animals

The study was conducted in the Laboratory of Fisheries of the Department of Animal Science, Universidade Federal de Santa Maria, Brazil. The experiment was carried out with a total of 90 male adult silver catfish (*R. quelen*) (initial weight = 403.1 ± 8.5 g) randomized into 15 tanks (270 L) with six fish per tank. Five independent replicates (five tanks) were conducted for each treatment. The tanks were connected to a water recirculating system with two biological filters, one activated carbon filter and a water reservoir with a capacity for 2000 L. Carbon filter was included to avoid cross-contamination of the water with CFEO released from fish food. No detectable amounts of CFEO components were found in the water after filter treatment (gas chromatography analysis, data not shown).

The animals were fed with the experimental diets twice a day (8:00 and 16:00 h) to apparent for 20 days. The daily average intake satiety of CFEO along the experimental period is amounted to 0, 0.43, and 1.68 mg kg^{-1} of fish and for control 1.0 and 3.0 mL of CFEO per kilogram of diet treatments, respectively. During experimental period, water quality was maintained as follows: 21.3 ± 0.3 °C; 8.5 ± 0.5 mg dissolved oxygen L^{-1}; 0.32 ± 0.03 mg $NH_3 + NH_4$ L^{-1}; and 0.17 ± 0.03 mg NO_2 L^{-1}.

At the end of the experimental period, fish were fasted for 24 h, euthanized by hypothermia followed by spinal cord section, and then gonads, semen, blood, and pituitary gland were harvested. Part of gonadal tissue was preserved in Beltsville thawing solution (BTS) and another part was fixed in buffered 4% paraformaldehyde for histopathological analysis. Blood was centrifuged at 1000×*g* for 5 min and plasma was stored at − 20 °C until analyses. Pituitary gland was stored in tubes containing TRIzol® reagent at − 80 °C until RNA extraction. All procedures were approved by the Ethical

Committee on Animal Use from Federal University of Santa Maria (protocol number 120/2014).

Gonadosomatic index

The gonadosomatic index (GSI) was calculated as GSI = [testis weight (g)/body weight of the animal (g)] × 100.

Mitochondrial functionality

The sperm mitochondrial functionality was evaluated according to He and Woods (2004) with adaptations (Varela Junior et al. 2012), using rhodamine 123 probe, which accumulates only in functional mitochondria. Five microliters of sample was incubated with 20 μL of rhodamine 123 solution (13 μM) at 20 °C for 10 min. After incubation, cells were counted using an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil) at × 400 magnification. Mitochondria were considered functional when sperm presented positive rhodamine 123 staining (green fluorescence) and nonfunctional when sperm presented no fluorescence. Results are expressed as the percentage of sperm with functional mitochondria compared with the total spermatozoa.

Sperm membrane integrity

The membrane integrity of the sperm was examined following the methodology of Harrison and Vickers (1990). For that goal, 5 μL of sample was diluted in 20 μL of saline solution with 1.7 mM formaldehyde, 20 μM carboxyfluorescein diacetate (CFDA), and 7.3 μM propidium iodide (PI). Fluorescence was verified at × 400 magnification using an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil). When the spermatozoa membrane was intact, CFDA accumulation occurred. Upon CFDA hydrolysis, carboxyfluorescein was produced and green fluorescence was generated. Spermatozoa with damaged membrane incorporated PI and emitted a red or red and green fluorescence. The percentage of sperm viability was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red or red, and green).

DNA integrity

Sperm DNA integrity was evaluated using the method of acridine orange described by Varela Junior et al. (2012), as the metachromatic colorant acridine orange in reaction to DNA emits green fluorescence and in case of reaction with a single-stranded DNA emits orange or red fluorescence, identifying DNA breakage. Sperm sample (45 μL) was diluted in 50 μL TNE buffer (0.01 M Tris-HCl; 0.15 M NaCl; 0.001 M EDTA; pH 7.2). After 30 s, 200 μL of Triton solution 1× was added, and 30 s later, 50 μL of acridine orange was added (2 mg mL^{-1} in deionized H_2O). The evaluation was performed after 5 min, avoiding exceeding 1 min of slide exposure. The sperm presenting green fluorescence were considered with intact DNA, and those presenting red or orange fluorescence were considered with denatured DNA. The rate of DNA integrity was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm analyzed (green, red, or orange).

Sperm kinetic analysis

The sperm was activated and recorded by computer-assisted semen analysis with AndroVision–Minitube, Germany (CASA) (Dziewulska, Rzemieniecki, Czerniawski and Domagala 2011). For the evaluation, five fields with at least 500 cells were captured 15–20 s after activation; capture of the fields was repeated 2–5 times. The parameters evaluated were total sperm motility (TM) (%), progressive motility (PM) (%), motility period (MP) (s), average path distance (DAP) (μm), curvilinear distance (DCL) (μm), straight line distance (DSL) (μm), average path velocity (VAP) (μm/s), curvilinear velocity (VCL) (μm/s), rectilinear velocity (VSL) (μm/s), straightness (STR) (VSL/VAP%), linearity (LIN) (VSL/VCL%), wobble (WOB), amplitude of lateral head displacement (ALH) (μm), and beat-cross frequency (BCF) (Hz). The motility period was evaluated at the time of activation to stop the forward movement of sperm (Varela Junior et al. 2015).

Testosterone levels in plasma

Total testosterone levels were measured with commercially available RIA kits DPC-total testosterone RIA test (DPC Med Lab Produtos Hospitalares LTDA., SP, Brazil). Parallelism of the dilution curves of the plasma

samples with the standard curve was demonstrated in all assays, with correlation coefficients ranging from 0.959 to 0.999. The inter- and intra-assay coefficients of variation varied from 9 to 12% and 6 to 9%, respectively.

RNA extraction, cDNA synthesis, and real-time PCR

Total RNA from pituitary tissue was extracted using TRIzol® as per instructions of the manufacturer. Quantification of RNA was performed using a NanoDrop spectrophotometer, and the RNA purity was assessed by the 260/280 nm absorbance ratio (Thermo Scientific). RNA was treated with 0.1 U DNase Amplification Grade (Invitrogen) for 15 min at 27 °C, followed by DNase inactivation with 1 μL of EDTA at 65 °C for 10 min. Double-stranded complementary DNA (cDNA) was synthetized from 500 ng of total RNA with random hexamer primers using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Quantitative polymerase chain reactions (qPCRs) were conducted in a CFX384 thermocycler (Bio-Rad) using BRYT Green® dye and Taq DNA polymerase from GoTaq® qPCR Master Mix (Promega Corporation), with 5 ng of cDNA in 10 μL. A common thermal cycling program (initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min) was used to amplify each transcript. Melting curve analyses were performed to verify product identity. The mRNA expression of prolactin (*prl*), somatolactin (*smtl*), and proopiomelanocortin (*pomca* and *pomcb*) was analyzed. The sequences used to design the primers were according to Baldisserotto et al. (2014) using the Primer Express software 3.3 (Applied Biosystems). Primers were validated by standard curves. Reactions with a coefficient of determination (R^2) higher than 0.98 and efficiency from 85 to 110% were considered optimized. Samples were run in duplicate and the results are expressed relative to *actb* and *rps18* levels. Primer sequences are as follows: *actb* F-GCAATGCCAGGGTACATGGT, *actb* R-CCACCTTCAACTCCATCATGAA; *rps18* F-AACCAGACAAATCGCTCCAC, *rps18* R-CCTGCGGCTTAATTTGACTC; *prl* F-ACCAGAGACAGGAGCTCGTTCT, *prl* R-AGCTCATGAGACCGTCCATGT; *smtl* F-CGAGGCCAGGACTTTGTTTG, *smtl* R-GACGCGCACAAGGTTTGAT; *pomca* F-ATGAAGCTCCAGAGTCCGTTC, *pomca* R-GATTCTTCCTCCACTCCGTTG; *pomcb* F-AGTCCACACCACCTTCTCCAT, *pomcb* R-TGCTCTTGGCATCTGTGTTCT. Data were then normalized to a calibrator sample using ΔΔCq method as previously described by Pfaffl (2001).

Biomarkers of oxidative stress

The gonadal tissue was homogenized in medium consisting 140 mM KCl and 30 mM sodium phosphate buffer (pH 7.4), and the supernatant fraction obtained was frozen at − 80 °C for future measurements. Lipid peroxidation was estimated by a TBARS assay according to Ohkawa, Ohishi, and Yagi (1979) performed by an MDA reaction with TBA, which was optically measured. Results were expressed as nmol mg protein^{-1}. Protein carbonyl measurement was assayed by the method described by Yan et al. (1995) and the carbonyl content was measured at 370 nm. Total superoxide dismutase (SOD) activity was determined as the inhibition rate of autocatalytic adenochrome generation at 480 nm. Enzyme activity was expressed as SOD units mg protein^{-1}. One SOD unit was defined as the amount of enzyme needed for 50% inhibition of adenochrome formation, as described by Misra and Fridovich (1972). Catalase (CAT) activity was assayed using ultraviolet spectrophotometry (Nelson and Kiesow 1972). Change of H_2O_2 absorbance in 60 s was measured by spectrophotometry at 240 nm and was reported as μmol mg protein^{-1}. Glutathione S-transferases (GST) activity was measured in the liver and muscle according to Habig, Pabst, and Jakoby (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The formation of DNPH was monitored by the increase in absorbance at 340 nm against blank. The activity was expressed as μmol GS-DNB min^{-1} mg protein^{-1}.

Protein was determined by the Coomassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm (Bradford 1976).

Testis histology

The histopathology was assessed by analysis of histological sections of the testis. After 24 h immersed in buffered paraformaldehyde, alcohol baths dehydrated the gonads in crescent concentrations, followed by the processes of diaphonization (xylol), impregnation, and inclusion in Paraplast Xtra (P3808—Sigma, Brazil), according to Carson and Hladik (2009) in automated

tissue processor (Leica ASP200S). After inclusion, the material was sliced in a motorized rotary microtome (Leica RM2255) with thickness of 5 μm. The slides were stained with hematoxylin and eosin (Carson and Hladik 2009). After the staining process, the material was visualized in an optic microscope under × 400 magnification.

Statistical analysis

Variables were analyzed for normality by the Shapiro-Wilk test followed by analysis of variance (one-away ANOVA) and Tukey's test. All analyses were done by Statistix® software 9.0.

Results

Testis histology and gonadosomatic index

Diet containing CFEO did not affect testis histology (Fig. 1) when compared to the control group. The gonadosomatic index was similar in the different treatments: 0.0 mL CFEO per kilogram of diet = 3.10 ± 0.19, 1.0 mL CFEO per kilogram of diet = 2.48 ± 0.22, and 3.0 mL CFEO per kilogram of diet = 3.31 ± 0.3.

Analysis of cell organelles and sperm kinetics

Analysis of cell organelles (membrane integrity, mitochondrial functionality, and DNA fragmentation) and parameters of sperm kinetics (TM, PM, MP, DAP, DCL, DSL, VAP, VCL, VSL, STR, LIN, WOB, ALH, and BCF) did not present any significant effect of the diets (Table 2).

mRNA expression

The relative expression of the genes encoding prolactin (*prl*), somatolactin (*smtl*), and proopiomelanocortin (*pomca* and *pomcb*) was not affected by dietary CFEO supplementation (Fig. 2).

Testosterone levels in plasma

Plasma testosterone levels did not show any difference among groups and amounted to nanograms per deciliter: 0.0 mL CFEO per kilogram of diet = 1306.6 ± 791.5, 1.0 mL CFEO per kilogram of diet = 1824.0 ± 738.7, and 3.0 mL CFEO per kilogram of diet = 1289.6 ± 596.5.

Biomarkers of oxidative stress

Protein carbonyl, LPO (measured as TBARS), SOD, CAT, and GST did not differ between groups (Table 3), demonstrating that diets supplemented with CFEO did not affect the antioxidant status and did not damage protein or lipids in the testis of silver catfish.

Discussion

Citral, an isomeric mixture of geranial and neral, is a naturally occurring aliphatic aldehyde of the monoterpene series. The results of the present study regarding CFEO composition corroborate previous findings of

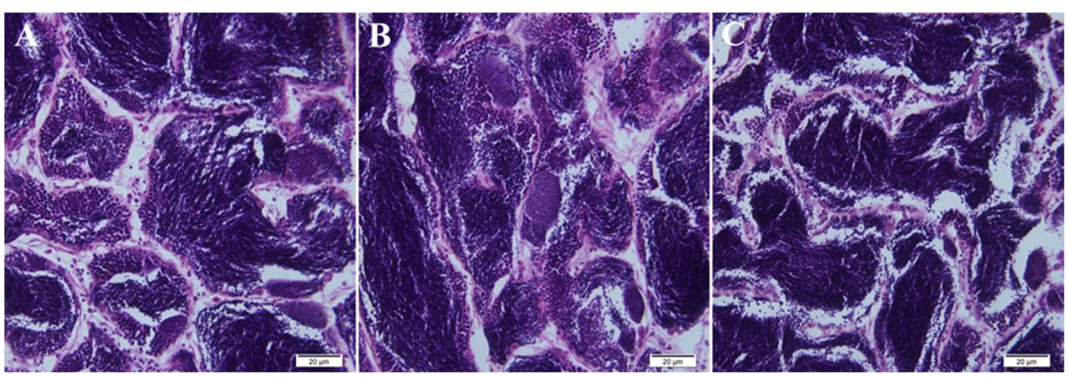

Fig. 1 Photomicrograph of gonads of silver catfish (*Rhamdia quelen*) fed with diets containing different concentrations of microencapsulated essential oil (EOCF) of *Cymbopogon flexuosus* ($n = 9$). **a** 0.0 mL EOCF per kilogram of diet (control), **b** 1.0 mL EOCF per kilogram of diet, and **c** 3.0 mL EOCF per kilogram of diet. Slides were stained with hematoxylin and eosin (Carson and Hladik 2009). Bars = 20 μm

Table 2 Sperm kinetic analysis and the analysis of cell organelles of silver catfish (*Rhamdia quelen*) fed with diets containing different concentrations of microencapsulated *Cymbopogon flexuosus* essential oil (CFEO). Data are expressed as mean and standard error of the mean

	Treatments (mL CFEO per kg of diet)		
	0.0	1.0	3.0
Membrane integrity (%)	88.7 ± 1.6	86.6 ± 2.9	91.4 ± 2.3
Mitochondrial functionality (%)	88.0 ± 3.4	93.9 ± 1.1	93.0 ± 1.6
DNA integrity (%)	97.1 ± 1.1	99.4 ± 0.6	95.6 ± 2.6
TM (%)	40.8 ± 2.3	46.0 ± 2.4	43.9 ± 2.2
PM (%)	29.1 ± 2.0	35.4 ± 2.4	32.2 ± 2.2
MP (s)	197.2 ± 42.4	159.4 ± 31.5	145.0 ± 16.8
DAP (μm)	14.9 ± 0.4	15.0 ± 0.3	15.2 ± 0.5
DSL (μm)	18.8 ± 0.4	19.2 ± 0.4	19.0 ± 0.5
DCL (μm)	11.7 ± 0.4	11.4 ± 0.3	12.0 ± 0.6
VAP (μm/s)	33.9 ± 0.8	35.0 ± 0.8	34.7 ± 1.0
VSL (μm/s)	43.0 ± 1.0	44.8 ± 1.1	43.3 ± 1.1
VCL (μm/s)	26.6 ± 0.8	26.4 ± 0.7	27.2 ± 1.1
STR	0.8 ± 0.01	0.7 ± 0.01	0.8 ± 0.01
LIN	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.01
WOB	0.8 ± 0.01	0.8 ± 0.01	0.8 ± 0.01
ALH (μm)	1.9 ± 0.12	2.2 ± 0.09	1.8 ± 0.10
BCF (Hz)	24.1 ± 0.5	23.5 ± 0.4	23.9 ± 0.5

TM total motility, *PM* progressive motility, *MP* motility period, *DAP* average path distance, *DCL* curvilinear distance, *DSL* straight line distance, *VAP* average path velocity, *VCL* curvilinear velocity, *VSL* rectilinear velocity, *STR* straightness, *LIN* linearity, *WOB* wobble, *ALH* amplitude of lateral head displacement, *BCF* beat-cross frequency

Data represent the mean ± SEM ($n = 9$). No significant difference between groups was observed (one-way ANOVA, $p > 0.05$)

citral as the main component of CFEO (Adukwu et al. 2016; Gupta et al. 2016). According to regulatory agencies in the USA (Federal Regulatory Code 21 CFR part 182.20, US Food and Drug Administration) and Europe (Commission Implementing Regulation No. 872/2012), geranial and citral can be considered safe for human consumption as food additives (Daniel et al. 2016).

The diet may influence several reproductive parameters in some fish species (Duray et al., 1994). Vegetable extracts have demonstrated promising results as feed additives (Galina et al. 2009; Saccol et al. 2013; Zeppenfeld et al. 2014); however, studies regarding their effects on reproductive parameters are still scarce. Our results have shown that diets supplemented with CFEO were neither beneficial nor prejudicial to the analyzed parameters as gonadal histology, oxidative stress, semen parameters, hormonal levels, and pituitary gene expression.

The GSI is used to monitor the gametogenesis progression in teleost fishes (Barcellos, Wassermann, Scott, Woehl, Quevedo, Ittzes, Krieger and Lulhier 2001). Convict cichlid (*Cichlasoma nigrofasciatum*) fed a diet supplemented with 0.15 g essential oil of fennel fruits (*Foeniculum vulgare*) per kilogram of diet for 40 days presented an increase in GSI (Sotoudeh 2016). In contrast, Nile tilapia (*Oreochromis niloticus*) presented a decrease in GSI after 90 days of diet with extracts of *Azadirachta indica* leaves and *Carica papaya* seeds at 2 g per kilogram of diet (Kareem et al. 2016). Silver catfish males fed with CFEO did not present any alteration in this parameter. On the other hand, Rampelotto et al. (2018) demonstrated that the supplementation with CFEO (1 mL per kg of diet) decreased the gonadosomatic index in silver catfish (considering both sexes together). Thus, probably, the GSI reduction observed by Rampelotto et al. (2018) is related to females.

Sperm mitochondria are organelles that play a fundamental role in cell metabolism and in ATP production through oxidative phosphorylation (Devenish et al. 2008), participating in the flagellar movement activation during sperm motility (Guthrie and Welch 2012).

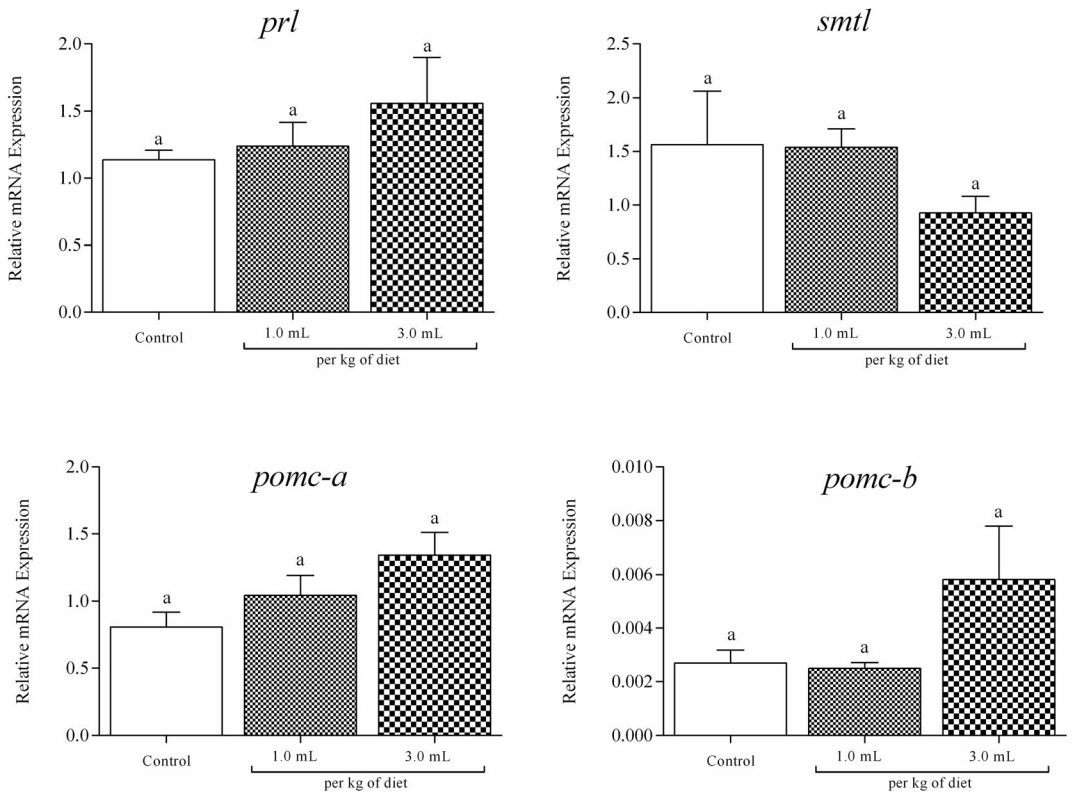

Fig. 2 Expression of prolactin (*prl*), somatolactin (*smtl*), and proopiomelanocortin (*pomca* and *pomcb*) in the pituitary tissue of silver catfish fed with different concentrations of dietary microencapsulated of essential oil of *Cymbopogon flexuosus*. Data represent the mean ± SEM ($n = 6$). No significant differences between groups were observed (one-way ANOVA, $p > 0.05$)

Oxidative stress may be produced by a variety of reactive oxygen species (ROS), including the superoxide (SO) anion, hydrogen peroxide (HP), and nitric oxide (Devenish et al. 2008). Supplementing *R. quelen* diet with microencapsulated CFEO did not significantly affect either sperm membrane integrity or mitochondrial functionality, proposing that this supplementation did not increase ROS in the testicles of silver catfish and all the animals presented release of sperm during manipulation.

Table 3 Biomarkers of oxidative stress in the testes of silver catfish (*Rhamdia quelen*) fed with diets containing different concentrations of microencapsulated *Cymbopogon flexuosus* essential oil (CFEO)

	Treatments (mL CFEO per kg of diet)		
	0.0	1.0	3.0
Protein carbonyl	23.55 ± 0.91	24.11 ± 2.56	28.47 ± 2.35
TBARS	2.31 ± 0.02	2.26 ± 0.06	2.11 ± 0.06
SOD	11.07 ± 1.97	9.96 ± 0.73	9.22 ± 1.61
CAT	1.01 ± 0.06	0.98 ± 0.09	1.09 ± 0.12
GST	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01

TBARS thiobarbituric acid reactive substances (nmol MDA mg protein^{-1}), protein carbonyl (nmol carbonyl mg protein^{-1}), *SOD* superoxide dismutase (SOD units mg protein^{-1}), *CAT* catalase (μmol mg protein^{-1}), and *GST* glutathione-S-transferase (μmol GS-DNB min^{-1} mg protein^{-1})

Data represent the mean ± SEM ($n = 8$). No significant differences between groups were observed (one-way ANOVA, $p > 0.05$)

Sperm DNA integrity is an important parameter in the prognosis of infertility and in the outcome of assisted reproductive procedures (Shamsi et al. 2011), directly influencing paternal genetic contribution to offspring (Figueroa et al. 2013; Santos et al. 2013). DNA integrity was not altered in the sperm of fish fed with both doses of CFEO compared to the control group. Generally, motility is the standard parameter to evaluate the sperm quality (Billard et al. 1995; Alavi and Cosson 2005). The motility values resulting from experimental conditions revealed no deleterious effects of CFEO on sperm quality. In contrast, studies conducted by Kowalska et al. (2016) showed that broodstock males of medaka (*Oryzias latipes*) fed diets supplemented with resveratrol at 0.08 g kg^{-1} BW day^{-1} exhibited negative effect in several sperm motility parameters (VCL, VAP, BCF, ALH, and LIN). On the other hand, by adding nutrients and probiotics to the diet, some studies showed a positive effect in motility and fertilization, such as occurred with goldfish (*Carassius auratus*) fed with 0.15 g kg^{-1} of astaxanthin (Tizkar et al. 2015) and *O. niloticus* fed with probiotic Hydroyeast Aquaculture® at 15 g kg^{-1} (Mehrim et al. 2015), improving their reproductive efficiency.

Many hormones are intimately related to reproduction, and their levels or expression can influence the broodstock and consequently their offspring. In this study, plasma testosterone levels presented no differences in silver catfish fed with CFEO compared to the control group. Testosterone is a good indicator of male fertility as the primary steroid hormone widely used to evaluate spermatogenesis (Kime 1993; Barannikova et al. 2004), and testosterone levels may be influenced by the diet (Izquierdo et al. 2001). Previous studies have shown an increase in testosterone levels of rainbow trout fed with gossypol, a natural compound of cottonseeds (Dabrowski et al. 2000; Dabrowski et al. 2001).

In teleosts, prolactin acts in reproduction, growth, development, osmoregulation, behavior, and metabolism (Power 2005). The prolactin-producing cells are found in early development, soon after hatching (Laiz-Carrion et al. 2003), growth, and differentiation of embryos and larvae in fishes (Naito et al. 1993). Despite the limited evidences on the physiological functions of somatolactin in reproduction, this hormone appears to stimulate gonadal steroidogenesis in vitro in coho salmon (*Oncorhynchus kisutch*) (Planas et al. 1992) and to regulate stress adaptation associated with hatching (Villaplana et al. 1997). Thus, a downregulation of *prl* or *smtl* in broodstocks may negatively influence the offspring, impairing the outbreak and development. In this regard, neither prolactin (*prl*) nor somatolactin (*smtl*) gene expression in the pituitary gland was altered by the presence of CFEO in the diet of silver catfish broodstock.

Proopiomelanocortin (*pomc*)-derived peptides have been associated to a wide range of physiological processes in vertebrates, including body pigmentation, steroidogenesis, reproduction, immune response, food intake, and energy homeostasis (Heijnen et al. 1987; Tsatmali et al. 2000; Takahashi and Kawauchi 2006). In teleosts, *pomc* has been related to appetite control, growth, and stress response (Zhang et al. 2012; Volkoff 2016) and consequently affecting reproductive parameters. In the present study, CFEO-supplemented diet did not regulate the transcripts of *pomca* and *pomcb* in the pituitary gland in silver catfish after 20 days of treatment. Grass carp (*Ctenopharyngodon idellus*) fed with restricted rations presented a significant downregulation in the expression of *pomc* gene (Gong et al. 2017). Our gene expression results of *pomc* in silver catfish reinforce the hypothesis that dietary supplementation with CFEO does not affect reproductive parameters.

Antioxidants are substances capable of scavenging free radicals and they can improve fish reproduction, whereas small amounts of reactive oxygen species (ROS) produced from mitochondrial oxidative phosphorylation may impair fertility (Mansour et al. 2003; Helfenstein et al. 2010; Losdat et al. 2011). Essential oils of *Cymbopogon* genus were shown to be effective as antioxidants (Cheel et al. 2005; Vázquez-Brione and Guerrero-Beltrán 2015), particularly *Cymbopogon citratus* and its major compound citral (Guimarães et al. 2011). However, the antioxidant properties of *C. flexuosus* have not yet been evaluated. In the present study, in the gonads of silver catfish fed with microencapsulated CFEO, antioxidant enzymes (SOD, CAT, and GST) did not differ between the dietary treatment groups. It was also supposed that CFEO did not damage lipids or proteins, corroborated by the absence of tissue lesion in histopathological analysis. Male convict cichlids (*Amatitlania nigrofasciata*) fed a diet supplemented with carotenoids presented significant differences in gonadal carotenoid content among treatment groups, suggesting that dietary carotenoids were indeed sequestered in the gonads, consequently, playing a limited role in protecting sperm from oxidative damage (Sullivan et al. 2014). The results presented here show that CFEO does not change testes oxidative status.

Previous studies have suggested beneficial effects of citral-based essential oils as feed additives for silver catfish. The addition of 2.0 mL essential oil of *Aloysia triphylla* per kilogram of diet, which contains citral as its major component, increased the growth of silver catfish after 60 days of treatment (Zeppenfeld et al. 2016). Additionally, this essential oil showed a potential protective effect against *Aeromonas hydrophila* infection (Dos Santos et al. 2017). Rampelotto et al. (2018) showed that dietary CFEO supplementation for 20 days did not induce any toxic effect in silver catfish and at 1 mL per kilogram diet improved carcass yield and protein deposition and therefore can be indicated for preslaughter supplementation for silver catfish. The present study indicated that dietary addition of citral-based CFEO had no negative effects on reproductive parameters, confirming the safety of this compound to feed reproductively mature silver catfish males.

In summary, our results indicate that microencapsulated CFEO used as dietary additive did not alter reproductive parameters, oxidative stress in the testicles, and gene expression in the pituitary gland of *R. quelen* males. Thus, the use of microencapsulated CFEO for 20 days can be used to improve protein deposition and carcass yield in *R. quelen* male broodfish without affecting reproductive-related parameters. In addition, we suggest new studies to better evaluate the effects of CFEO dietary supplementation during long-term conditions, in order to evaluate the effect of this essential oil throughout gonadal maturation in *R. quelen*.

Compliance with ethical standards All procedures were approved by the Ethical Committee on Animal Use from Federal University of Santa Maria (protocol number 120/2014).

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