



## Citral as food additive for common snook – zootechnical parameters and digestive enzymes

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**ABSTRACT:** Essential oils of plants whose main compound is citral showed beneficial effects when added to fish feed. The objective of the present study was to evaluate the dietary effect of the addition of citral on zootechnical parameters and digestive enzyme activities of *Centropomus undecimalis*. Juveniles were fed for 45 days with diets containing different amounts of citral (0.0 - control, 0.5, 1.0, and 2.0 mL per kg of diet). The water quality parameters were kept stable during the experiment and no mortality was observed. At the end of the experimental period, the treatment 0.5 mL citral per kg of diet had the lowest weight gain and specific growth rate, and the highest feed conversion, while the same parameters did not differ between the other treatments. Pepsin activity was higher in the stomach of fish fed with 0.5 mL citral per kg of diet and amylase activity was higher in the intestine of fish fed with 0.5 and 2.0 mL citral per kg of diet compared to the control group. Intestinal lipase activity was higher in all groups that were fed with citral compared to the control group. Chymotrypsin and trypsin activities showed no difference between groups. Consequently, dietary addition of citral at any of the levels tested is not recommended for common snook.

**Key words:** *Centropomus undecimalis*, amylase, pepsin, lipase, growth, marine fish farming.

## Citral como aditivo na ração para robalo flecha - parâmetros zootécnicos e enzimas digestivas

**RESUMO:** Óleos essenciais de plantas, cujo composto majoritário é o citral, mostraram efeitos benéficos quando adicionados à ração para peixes. O objetivo do presente estudo foi avaliar o efeito da adição de citral sobre os parâmetros zootécnicos e atividades das enzimas digestivas de *Centropomus undecimalis*. Os juvenis foram alimentados por 45 dias com dietas contendo diferentes quantidades de citral (0,0 - controle, 0,5, 1,0 e 2,0 mL por kg de ração). Os parâmetros de qualidade da água foram mantidos estáveis durante o experimento e nenhuma mortalidade foi observada. Ao final do período experimental, o tratamento 0,5 mL citral por kg de ração teve o menor ganho de peso e taxa de crescimento específico e a maior conversão alimentar, enquanto os mesmos parâmetros não diferiram entre os demais tratamentos. A atividade da pepsina foi maior no estômago de peixes alimentados com 0,5 mL de citral por kg de ração e a atividade de amilase foi maior no intestino de peixes alimentados com 0,5 e 2,0 mL citral por kg de ração comparado ao grupo controle. A atividade da lipase intestinal foi maior em todos os grupos que foram alimentados com citral, em comparação ao grupo controle. As atividades de quimotripsina e tripsina não mostraram diferença entre os grupos. Consequentemente, a adição de citral na ração em qualquer um dos níveis testados não é recomendada para robalo flecha.

**Palavras-chave:** *Centropomus undecimalis*, amilase, pepsina, lipase, crescimento, piscicultura marinha.

## INTRODUCTION

Common snook, *Centropomus undecimalis*, is a carnivore with several characteristics that qualifies it as a marine species

for intensive fish farming, since it has high market value, is well adapted to captivity, easily accepts inert foods and has a good feed conversion ratio (RHODY et al., 2014; TUCKER JR, 1987). In addition, it is an important fish for sport fishing in

the American continent (LOWERRE-BARBIERI; VOSE; WHITTINGTON, 2003).

The search for the maximum food efficiency has promoted the use of dietary additives to control harmful agents to the digestive process and thus improve the zootechnical indexes. Several essential oils (EOs) have been used as dietary additives for fish, improving growth and resistance to diseases (ZHENG et al., 2009; FERREIRA et al., 2014; SÖNMEZ et al., 2015; MOHAMADI SAEI et al., 2016; BRUM et al., 2017; ZEPPEFELD et al., 2016, 2017).

Citral is the main compound of *Aloysia triphylla* EO (essential oil), whose dietary addition improved growth and oxidative status of silver catfish, *Rhamdia quelen* (ZEPPEFELD et al., 2016, 2017). Dietary supplementation with the microencapsulated EO of *Cymbopogon flexuosus*, which also has citral as the main compound, increased carcass yield and protein deposition in silver catfish (RAMPELOTTO et al., 2018). Often the major EO component is primarily responsible for its biological activities, but dietary supplementation with citral did not improve oxidative parameters and innate immunity in common snook (MORI et al., 2019). However, the effect of citral on growth and digestive enzymes activity in this species was not studied, then the objective of the present study was to evaluate the efficacy of dietary supplementation with citral as a growth promoter of common snook and to analyze its effects on digestive enzymes activities.

## MATERIALS AND METHODS

### Animals and rearing conditions

The experiments were performed in the Marine Fish Culture Laboratory (LAPMAR) at

the Federal University of Santa Catarina (UFSC), Florianópolis, Santa Catarina, Brazil. Juvenile common snook *Centropomus undecimalis* (initial weight  $2.75 \pm 0.02$  g and total length  $7.75 \pm 0.01$  cm) were obtained by induced spawning of brood stocks as described by PASSINI et al. (2013) and maintained at the LAPMAR in salinity 35 ppt.

Juveniles were randomly distributed in four water recirculation systems with salinity  $31.12 \pm 2.31$  ppt and temperature of  $31.59 \pm 0.91$  °C as suggested by MICHELOTTI et al. (2018) (n=30 per tank). Each recirculation system consisted of three circular tanks (150 L). The water contained in these experimental units was removed through a central pipeline with a bag filter (50 µm), a biological filter, a foam fractionator and an ultraviolet sterilizer (60 w). After the treatment, the water returned to the experimental tanks.

The fish were acclimated for four days to the experimental feed. Remains of food and feces were removed daily through siphoning, and an average of 25% of the water was renewed.

### Water parameters

The water parameters were checked daily (temperature, pH and dissolved oxygen) or weekly (alkalinity, total hardness, ammonia and nitrite) throughout the experimental period, as described by MICHELOTTI et al. (2018). The water parameters remained stable during the experiment, with no significant difference between treatments (Table 1).

### Citral

Citral ( $\alpha$ -citral=60.15%,  $\beta$ -citral=39.85%) was obtained from Sigma-Aldrich® (St. Louis, Missouri, USA). The quantification of the isomers

Table 1 - Water quality (mean  $\pm$  standard error) of the four independent recirculation systems from a 45-day experiment with *Centropomus undecimalis* fed dietary citral supplementation.

	-----CITRAL (mL per kg of feed)-----			
	Control	0.5	1.0	2.0
Temperature (°C)	31.42 $\pm$ 0.06	31.49 $\pm$ 0.06	31.81 $\pm$ 0.04	31.62 $\pm$ 0.04
Dissolved oxygen (mg L <sup>-1</sup> )	4.73 $\pm$ 0.03	4.51 $\pm$ 0.07	4.76 $\pm$ 0.04	4.54 $\pm$ 0.02
Dissolved oxygen (%)	75.75 $\pm$ 0.05	73.22 $\pm$ 0.51	75.58 $\pm$ 0.64	72.04 $\pm$ 0.50
pH	8.17 $\pm$ 0.01	8.14 $\pm$ 0.01	8.19 $\pm$ 0.01	8.22 $\pm$ 0.01
Salinity (ppt)	31.03 $\pm$ 0.01	31.21 $\pm$ 0.05	31.15 $\pm$ 0.03	31.03 $\pm$ 0.01
Alkalinity (mg CaCO <sub>3</sub> L <sup>-1</sup> )	102.16 $\pm$ 12.41	102.66 $\pm$ 12.37	102.33 $\pm$ 5.87	102.24 $\pm$ 8.23
Nitrite (mg L <sup>-1</sup> )	0.27 $\pm$ 0.08	0.20 $\pm$ 0.05	0.29 $\pm$ 0.03	0.27 $\pm$ 0.05
Total ammonia (mg L <sup>-1</sup> )	0.29 $\pm$ 0.04	0.26 $\pm$ 0.03	0.25 $\pm$ 0.02	0.32 $\pm$ 0.07

was executed in an Agilent 6890A gas chromatography coupled with a 5973 mass selective detector using a HP-CHIRAL capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) and electron ionization mode at 70 eV. Helium was used as carrier gas in a flow rate of 1.0 mL min<sup>-1</sup>, injector temperature was set at 250 °C and detector at 280 °C. Oven temperature was kept at 40 °C for 4 min and raised to 240 °C at a rate of 4 °C min<sup>-1</sup>. Sample solutions of 1 µL (2:1000 in hexane, v/v) were injected in a splitless mode. Kovats retention indices were calculated using a homologous series of C8-C40 n-alkanes injected under the same conditions of the samples. The isomers were identified by mass spectra and Kovats retention index comparison with data from the National Institute of Standards and Technology Mass Spectral Library (NIST). Compounds relative percent was estimated by under peak area integration obtained from the chromatogram.

#### Diets and experimental design

Four diets based on the same initial formulation (MORI et al., 2019) were produced, dried in an oven at 40 °C; and subsequently, pelleted at 6 mm (Table 2). Different amounts of citral (0-control, 0.5, 1.0, 2.0 mL per kg of diet) were added to the mixture together with fish oil prior to the drying and pelletizing processes. Fish received the experimental diets to apparent satiety four times a day (9 a.m. and 1, 3 and 6 p.m.) for 45 days.

The feed was suspended 24 h prior to sampling and final collection of the experiment. The experimental design resulted in four groups (in triplicate).

#### Samples

After 45 days, three fish from each tank (n = 9 animals per treatment) were anesthetized with 50 mg L<sup>-1</sup> of benzocaine and euthanized by sectioning of the spinal cord. Stomach and intestine were removed and immediately frozen in liquid nitrogen. The tissues were stored at -20 °C for further analysis.

#### Zootechnical parameters

At 1, 22 and 45 days of experiment all Common sSnooks were anesthetized with 50 mg L<sup>-1</sup> benzocaine, measured and weighed to calculate weight gain (Wg), specific growth rate (SGR) and feed conversion (FC) as described by MICHELOTTI et al. (2018). Condition factor (FK) was calculated by the following equation:  $(FW/FL^3)^*100$ , where FW is the final weight and FL is the final length.

#### Digestive enzymes

Samples from stomach, anterior and posterior intestine were homogenized in an ice bath at ratio 1:10 (tissue: homogenization buffer) with an Ultraturrax. The homogenization buffer solution was composed by 20 mM Tris/10 mM phosphate, pH 7.0

Table 2 - Formulation (%) of the experimental diet and analyzed proximate average composition.

Ingredients	g kg <sup>-1</sup>
Starch	140
Soy lecithin	10
Vitamins and minerals (premix)*	5
Fresh squid	120
Fish meal	700
Fish oil	24
Vitamin C	1
Composition	(%)
Dry matter content	94.32
Protein	53.73
Ether extract	9.19
Mineral matter	20.73
Acid detergent fiber	2.04
Neutral detergent fiber	14.31

\*Vitamin and mineral mixture (security levels per kilogram of product) — folic acid: 250 mg, pantothenic acid: 5,000 mg, antioxidant: 0.60 g, biotin: 125 mg, cobalt: 25 mg, copper: 2000 mg, iron: 820 mg, iodine: 100 mg, manganese: 3750 mg, niacin: 5000 mg, selenium: 75 mg, vitamin A: 1,000,000 UI, vitamin B1: 1250 mg, vitamin B12: 3750 mcg, vitamin B2: 2500 mg, vitamin B6: 2485 mg, vitamin C: 28,000 mg, vitamin D3: 500,000 UI, vitamin E: 20,000 UI, vitamin K: 500 mg, zinc: 17,500 mg.

in 50% (v/v) glycerol. The extract was centrifuged and supernatant was utilized in assays as enzyme source.

Amylase activity was assayed in 0.2 M phosphate-citrate buffer, pH 7.0, 0.5% NaCl with a starch concentration of 2.5%. The reaction was stopped by adding Ba(OH)<sub>2</sub> 0.3 N and ZnSO<sub>4</sub> 5%. The experimental protocol was modified according to BERNFELD & COLOWICK (1955). The determination of starch hydrolysis was done following PARK & JOHNSON (1949). The absorbance was recorded at 660 nm. One unit of enzyme was defined as 1 mmol of glycosyl-glucose released from starch per min per mg of protein.

Lipase activity was assayed as described by GAWLICKA et al. (2000) and Chymotrypsin and trypsin assay was performed according HUMMEL (1959). Pepsin activity was assayed as described by HIDALGO et al. (1999). Enzyme activities were calculated as described by ALMEIDA et al. (2018).

#### Statistical analysis

Results are expressed as mean ± error. The Levene's test was performed to evaluate the homogeneity of variances of the data. Comparisons among treatments were made by one-way ANOVA followed by Tukey's test. All analyses were performed using Statistica Software 7.0 (Stat Soft, Tulsa, OK, USA), and differences were considered significant at P<0.05.

## RESULTS

#### Zootechnical parameters

Dietary addition of citral did not change significantly WG and FC compared to the control

group at 22 days, but WG was lower and FC higher in fish fed with 0.5 mL citral per kg of feed than in those fed 1.0 mL citral per kg of feed. At the same day the lowest SGR and FK were observed in fish fed with 0.5 mL citral per kg of feed. After 45 days the lowest WG and SGR and the highest FC were observed in fish fed 0.5 mL citral per kg of feed (Table 3).

#### Digestive enzymes

The amylase activity was significantly higher in groups 0.5 and 2.0 citral per kg of feed than in the control group. Lipase activity was higher in all groups fed with citral compared to control group. Chymotrypsin and trypsin showed no significant difference between groups. However, pepsin was significantly higher in fish fed with 0.5 citral per kg of feed than control group (Figure 1).

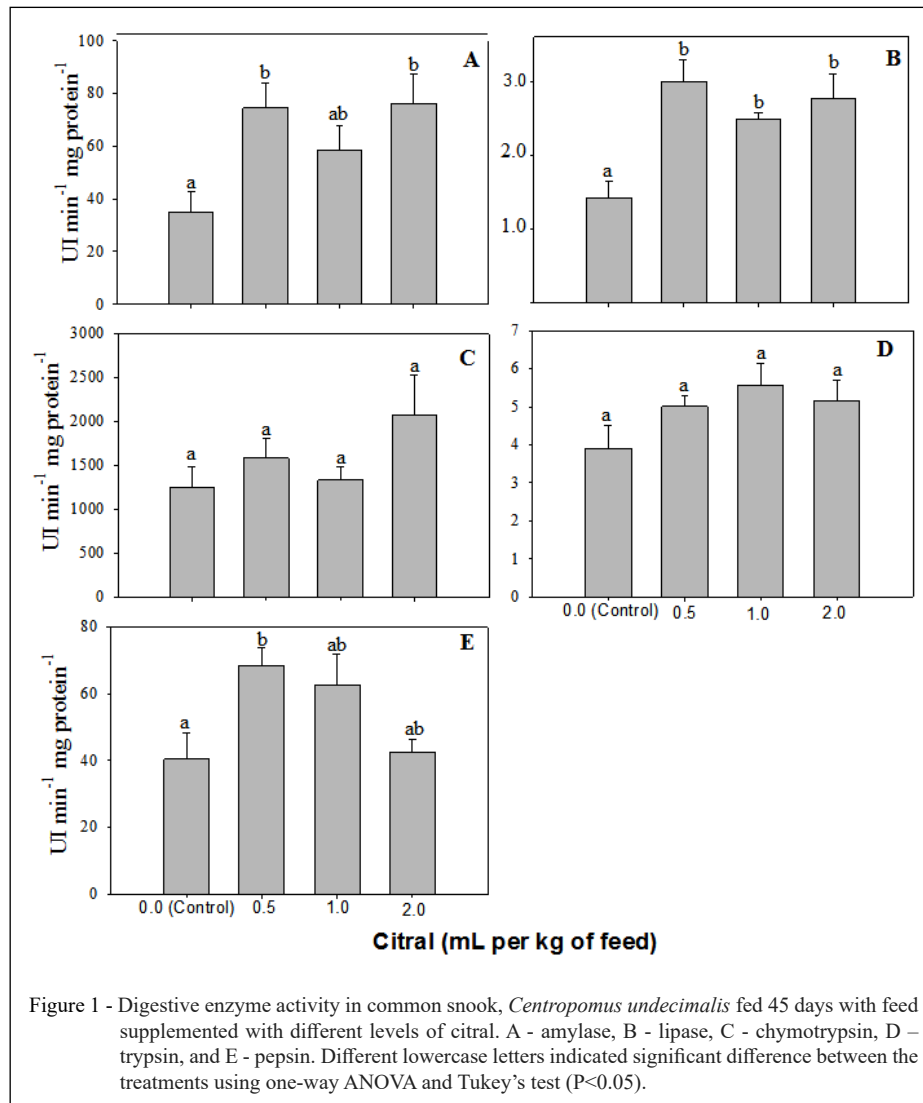
## DISCUSSION

The highest dietary insertion of citral did not affect significantly the Common Snook zootechnical parameters analyzed compared to control. In contrast, the lowest dietary insertion of citral (0.5 mL citral per kg of feed) was harmful, with the worst values of WG, SGR and FC. A similar amount of dietary citral (0.3 g citral per kg of feed) had no effect on the growth of Atlantic salmon (*Salmo salar*) after 30 days (JENSEN et al., 2015). Interestingly, dietary addition of the EO of ginger (*Zingiber officinale*) (5 or 10 g per kg of feed), which contains citral ( $\alpha$ -citral 23.9% and  $\beta$ -citral 17.2%), did not affect final weight of Nile tilapia (*Oreochromis niloticus*) after 55 days, but 15 g per kg of feed of this EO decreased final weight

Table 3 - Zootechnical parameters (mean ± standard error) of Common Snook, *Centropomus undecimalis* fed dietary citral supplementation.

		-----CITRAL (mL per kg of feed)-----			
		Control	0.5	1.0	2.0
WG (g)	22	3.86 ± 0.24 <sup>ab</sup>	2.72 ± 0.23 <sup>b</sup>	4.64 ± 0.24 <sup>a</sup>	4.08 ± 0.26 <sup>ab</sup>
	45	6.23 ± 0.52 <sup>a</sup>	2.14 ± 0.23 <sup>b</sup>	4.93 ± 0.06 <sup>a</sup>	5.91 ± 0.43 <sup>a</sup>
SGR (% day <sup>-1</sup> )	22	3.97 ± 0.16 <sup>a</sup>	3.11 ± 0.19 <sup>b</sup>	4.48 ± 0.14 <sup>a</sup>	4.12 ± 0.17 <sup>a</sup>
	45	2.87 ± 0.13 <sup>a</sup>	1.42 ± 0.07 <sup>b</sup>	2.22 ± 0.05 <sup>a</sup>	2.71 ± 0.22 <sup>a</sup>
FC (g g <sup>-1</sup> )	22	1.83 ± 0.08 <sup>ab</sup>	2.21 ± 0.09 <sup>b</sup>	1.46 ± 0.07 <sup>a</sup>	1.76 ± 0.05 <sup>ab</sup>
	45	1.41 ± 0.08 <sup>a</sup>	2.36 ± 0.13 <sup>b</sup>	1.77 ± 0.06 <sup>a</sup>	1.50 ± 0.12 <sup>a</sup>
FK (g cm <sup>-3</sup> )	22	0.86 ± 0.005 <sup>b</sup>	0.78 ± 0.006 <sup>a</sup>	0.84 ± 0.004 <sup>b</sup>	0.85 ± 0.02 <sup>b</sup>
	45	0.83 ± 0.003 <sup>a</sup>	0.78 ± 0.006 <sup>a</sup>	0.80 ± 0.018 <sup>a</sup>	0.80 ± 0.008 <sup>a</sup>

WG = weight gain (g), SGR = specific growth rate, FC = feed conversion ratio, FK = condition factor. Different lowercase letters indicated significant difference between the treatments using one-way ANOVA and Tukey's test (P<0.05).



compared to control fish (BRUM et al., 2017). Silver catfish fed 2 mL EO of *A. triphylla* per kg of feed ( $\alpha$ -citral 29.4% and  $\beta$ -citral 20.8%) improved growth of silver catfish (ZEPPEFELD et al., 2016, 2017), but did not change growth of zebrafish (*Danio rerio*) (ZAGO et al., 2018). Dietary addition of 1 or 3 mL of microencapsulated EO of *C. flexuosus* (contains  $\alpha$ -citral 45.7% and  $\beta$ -citral 32.1%), did not change growth parameters of silver catfish, but the duration of the experiment was only 30 days and the fish were adults. The group fed with 1 mL microencapsulated *C. flexuosus* EO per kg of feed increased carcass yield and protein deposition, but reduced the gonadosomatic index and fat deposition in comparison to the control group (RAMPELOTTO et al., 2018). Therefore, the

effect of citral and EOs containing this compound as major constituent on fish growth varies according to the species and other compounds present in the EOs. A recent study demonstrated that the addition of citral to the diet of *C. undecimalis* juveniles at equivalent levels and for the same period is not beneficial, since oxidative damage was verified to the liver and gills of the animals (MORI et al., 2019).

There are no studies dealing with dietary addition of citral and digestive enzymes. The dietary addition of the microencapsulated EO of *C. flexuosus* did not change the activity of intestinal trypsin and chymotrypsin in silver catfish (RAMPELOTTO et al., 2018). The same authors suggested that the increased carcass yield and protein content verified in this species

were not due to an improvement in protein digestibility. The only study that analyzed the effect of dietary EO supplementation and digestive enzymes activity in fish used the EO of cinnamon, which contains different main compounds (IMANI et al., 2017). The increase in the activity of the enzymes pepsin, amylase and lipase in Common Snook that received diets supplemented with citral may be due to an effort to improve the nutrient utilization efficiency. However, no clear relationship between the effect of citral on digestive enzymes activity and growth was observed.

## CONCLUSION

Results obtained indicated that in spite of increasing the activity of some digestive enzymes, dietary addition of citral does not improve common snook growth, even impairing it at 0.5 mL citral per kg of feed. Therefore, dietary supplementation with citral is not recommended for Common Snook.

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## BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The study was approved by the Ethics Committee on Animal Experimentation of Universidade Federal de Santa Catarina (UFSC) under registration n° PP00861/2013.

## DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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