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Original Article

Effects of Cyanotoxins Produced by *Raphidiopsis raciborskii* T3 Strain and Temperature on The Reproductive Axis Biomarkers of Neotropical Catfish

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Abstract

Human activities are causing environmental degradation such as air and water pollution. These processes can increase cyanobacterial blooms, which are capable of producing cyanotoxins and have become a problem in public water supply reservoirs. Thus, this study aimed to evaluate the possible endocrine-disrupting effects of increased water temperature and cyanobacteria in fish. For this, the crude extract of *Raphidiopsis raciborskii* (formerly called *Cylindrospermopsis raciborskii*), a neurotoxin-producer, was produced. After the production of the extract and the animal's acclimation, male and female *Rhamdia quelen* fish were exposed to different treatments: control at 25°C, control at 30°C, extract equivalent to 100,000 cells of *R. raciborskii*.mL^{-1} at 25°C and extract 100,000 cells of *R. raciborskii*.mL^{-1} at 30°C. After 96 h, the fish were anesthetized and blood was collected and centrifuged to obtain plasma for hormone analysis. After being weighed and euthanasia, the liver was sampled for vitellogenin gene expression and the gonad for sexing. The data were analyzed to evaluate the effects of the isolated increase in temperature and the effect of exposure to the cyanobacterial extract under two different temperature conditions, separately between genders. Temperature alone was able to increase the expression of vitellogenin in the liver of females. Aiming at a current scenario, exposure to cyanobacteria extract at 25°C proved to be anti-androgenic in females, including increased vitellogenin gene expression and reduced testosterone levels. In the scenario of exposure to water at 30°C, fewer alterations were found, but a reduction in vitellogenin expression was observed for both sexes. These results indicate that the increase in water temperature can cause changes in reproductive levels. The same effect was observed at exposure to saxitoxins present in cyanobacterial extracts, with greater responses at a temperature of 25°C, was considered in this study as the current climate scenario. Thus, this study demonstrates that changes in water temperature and the presence of potentially toxic cyanobacteria can lead to population changes in *Rhamdia quelen* since these conditions are capable of altering important reproductive parameters in this species.

Keywords: Endocrine disruptor; global warming, *Rhamdia quelen*, saxitoxins, PSTs

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INTRODUCTION

Over 3.5 billion years old, cyanobacteria are part of a very ancient group of microorganisms that perform photosynthesis and are considered one of the main sources of oxygen on primitive Earth, having great importance in the formation of the biosphere (Banerjee *et al*., 2021). Despite being important in the production of oxygen on Earth, cyanobacteria can cause harm to the environment and human health when in excessive density (Banerjee *et al*., 2021). When these organisms reproduce excessively, we have a situation called "bloom" (Campos *et al*., 2024). These events tend to happen more frequently and intensely due to eutrophication and climate change. Eutrophication is a natural process characterized by the increase of nutrients such as phosphorus and nitrogen in the environment (Akinnawo, 2023). However, with global warming and the incorrect disposal of domestic and industrial waste and sewage, this event has been happening more frequently, which can increase the level of phytoplankton in lentic environments such as lakes and reservoirs (Allen *et al*., 2018; Banerjee *et al*., 2021; Akinnawo, 2023; Campos *et al*., 2024).

Among the organisms found in phytoplankton are cyanobacteria. It can be potentially toxic due to the production of a secondary metabolite called cyanotoxins, and their bloom events are known as Harmful Cyanobacterial Blooms (Cyano-HABs, Campos *et al*., 2024). An example of a cyanotoxin is saxitoxins (STX), often called paralytic shellfish toxins (PSTs), which are neurotoxins produced by some dinoflagellates and several genera of cyanobacteria, such as the genus *Raphidiopsis* (Abi-Khalil *et al*., 2017). Due to their mode of action, saxitoxins can alter the behavioral response which can result in changes in the reproductive fitness of a species (Banerjee *et al*., 2021). Many studies are carried out in marine environments, with PSTs-producing dinoflagellates, such as those of the genus *Alexandrium*. Some studies with gastropods and copepods have shown effects on the reproductive system, such as changes in the oviposition and hatching rate of *Chorus giganteus* and *Calanus helgolandicus* fed with *Alexandrium catenella* (Andrade-Villagrán *et al*., 2019; Abdulhussain *et al*., 2024). Gametes of the bivalve *Crassostrea* (*Magallana) gigas* exposed to *Alexandrium minutum* showed reduced viability, with sperm showing reduced motility (Castrec *et al*., 2021). In the case of fish, PSTs have already been detected in gonads of puffer fish *Arothron firmamentum* (Nakashima *et al*., 2004). However, the reproductive effects of saxitoxins are not yet fully elucidated about freshwater, especially with the addition of the variable of climate change.

One of the freshwater fish that can be used to study the toxic effects of cyanotoxins is the species *Rhamdia quelen* Quoy & Gaimard, 1824 (Heptapteridae). This is an omnivorous Neotropical catfish native to South America, found from southern Mexico to central Argentina (Gomes *et al.*, 2000). It is an eurythermic species, resistant to cold climates and rapidly growing in warmer temperatures (Montanha *et al*., 2011). There is scarce data available regarding the thermal physiology of this species, but it is known that its thermal ideal is between 22 and 28°C, and temperatures between 22 and 25°C favor their reproduction (Montanha *et al*., 2011). Furthermore, its fingerlings (1.29 ± 0.64 g) acclimated at 16 to 31°C have a lower lethal temperature of 7 to 14 °C and an upper lethal temperature of 33 to 34 °C (Chippari-Gomes *et al*., 1999). Studies also show that increasing the temperature up to 30 °C and exposure to PSTs can cause biochemical changes, damage to macromolecules, and changes in the hepatic proteome about energy production (Vicentini *et al*., 2024). Knowing this, some biomarkers can be used to study the potential for disruption of the reproductive axis. Estrogenic hormones, for example, can stimulate the production of the phospholipid glycoprotein vitellogenin in female fish. This protein will be the source of energy in the yolk, and in the eggs, so this motivation becomes extremely important in the reproductive process and is normally not produced in males (Brown *et al.*, 2023). In other words, hormone and vitellogenin levels can be considered biomarkers of endocrine disruption.

Although scarce, the literature includes some studies that evaluated these parameters in fish exposed to saxitoxins, such as those by Tian *et al.* (2014) and Haque *et al.* (2020). One of the cyanobacteria that produce this type of toxin is *Raphidiopsis raciborskii*, formerly called *Cylindrospermopsis raciborskii*. This species has already been described in the literature as causing blooms in public water supply reservoirs, causing several problems for local government entities (Calado *et al.*, 2017; Calado *et al*., 2020). In addition, *R. raciborskii* has a higher growth rate and cell viability at higher temperatures, such as in the range of 26 to 32°C, making the problem of blooms even greater in the scenario of global climate change (Zhen *et al*., 2023). Therefore, the present study aims to determine whether the crude extract of *Raphidiopsis raciborskii* is capable of altering hormone levels and vitellogenin liver expression in female and male freshwater fish, *Rhamdia quelen*, under two temperature conditions, simulating a current scenario and a temperature increase scenario. We intend to answer how PSTs affect the reproductive regulation axis of Neotropical fish species and evaluate the effect of global warming on these biomarkers.

MATERIALS AND METHODS

Cyanobacteria culture and crude extract

The *Raphidiopsis raciborskii* (formerly *Cylindrospermopsis raciborskii*) T3 strain was obtained from the Federal University of Rio de Janeiro and maintained in ASM-1 medium, pH 8, photoperiod 16h light/8h dark and controlled temperature (26 ± 1 °C). The culture, after growth, was centrifuged and resuspended in water to obtain the crude extract containing saxitoxins, according to Vicentini *et al.* (2024).

Experimental design

The fish used in this study came from the University of Western Paraná, and upon arrival at the Laboratory of Environmental Toxicology, they were acclimatized for one month under controlled conditions of photoperiod (12h/12h) and temperature (according to the experimental group), with constant aeration and daily feeding (Laguna® Brazilian Fish 32). There were 120 acclimatized animals, with 12.48 ± 1.03 cm and 15.75 ± 4.25 g of average total length and weight, respectively. These procedures and experiments, under number 1140, were approved by the Federal University of Paraná Animal Use Ethics Committee (CEUA).

The *Rhamdia quelen* was exposed to four different experimental conditions (30 animals per group and 10 per 50L glass aquarium): filtered water at 25°C (C25); filtered water at 30°C (C30); exposure to crude extract of R. racirborskii equivalent to 10^5 cells.mL^{-1} in filtered water at 25°C (CE25); and exposure to crude extract of R. racirborskii equivalent to 10^5 cells.mL^{-1} in filtered water at 30°C (CE30). C25 and C30 represent the control treatments under current temperature and water heating conditions, while CE25 and CE30 represent these treatments exposed to cell concentrations equivalent to cyanobacterial blooms in the environment under both thermal conditions. The experimental conditions were based on predicted water warming data, survival range for fish and cyanobacteria, and abiotic data from water bodies in which both species are found (Saker and Griffiths, 2000; Artaxo, 2014; Calado *et al*., 2019). The experiment was static, that is, the extract was added only once, which was considered time zero. Throughout the experiment, the animals were fed once a day with Laguna® Brazilian Fish 32, which did not significantly affect water parameters such as ammonia and nitrite. Details of this experiment and data are also described in Vicentini *et al.* (2024).

After 96h, the animals were anesthetized with benzocaine (0.1 µg.L^{-1}), and the blood was taken by the caudal vein with a heparinized syringe. For hormonal analyses, this blood was centrifuged at 2000x for 5 minutes and the plasma obtained was used for quantification of estradiol and testosterone levels. After euthanasia by medullary section, the fish, liver, and gonad were weighed, with the aid of an analytical balance. The livers were sampled for vitellogenin gene expression and the gonads for sexing.

Hepatosomatic and gonadosomatic indexes

The hepatosomatic (HSI) and gonadosomatic (GSI) indexes were calculated according to the following equation (Querol *et al*., 2002; Neves dos Santos *et al*., 2004):

$$HSI\ or\ GSI\ = \left(\frac{liver\ or\ gonad\ weight}{body\ weight}\right)\times 100$$

Gonadal histology

The gonadal fragments were fixed in ALFAC solution (80% alcohol, formaldehyde, and glacial acetic acid) for 20h, from the collection of material. Then, these samples were dehydrated in an alcoholic series (70%, 80%, 90%, and 100%), cleared in xylene and included in Paraplast®. Sections 7 to 8 µm thick were stained with hematoxylin-eosin (HE) and the slides were analyzed under light microscopy for sex determination, following Brown-Peterson *et al*. (2011) and Lowerre-Barbieri *et al*. (2011).

Hormones quantification

The plasma was used to quantify the levels of 17β – estradiol (25 µL) and 11 – keto testosterone (50 µL). The enzyme-linked Immunosorbent assay (ELISA) method was carried out using commercial kits and following their indications (17β – Estradiol from IBL International and 11 – Keto Testosterone from Cayman Chemical). The results were expressed in pg.mL^{-1}.

Vitellogenin (vtg) gene expression

For the expression of vitellogenin, a liver fragment of approximately 30 mg was used. For the extraction of total RNA from this material, the RNeasy minikit (QIAGEN) was used, following its instructions and using a volume of 30 µL of RNAse Free water for the elution of this RNA. This material was measured in nanodrop and 2 µg was treated with DNase I (Invitrogen) to ensure that there would be no DNA contamination in the samples. After a new measurement in nanodrop, the treated RNA was retro-transcribed into complementary DNA (cDNA) with the Reverse Transcription System (Promega), with 1 µg for the samples and a total of 0.5 µg for the negative control. The negative control was performed with a pool of samples from each group, without the addition of enzymes in the reverse transcription step, thus confirming that there is no DNA in the original sample, but only RNA.

For qPCR reactions for the *vtg* gene, cDNA (40ng), 10 µL of SYBR Green PCR Master Mix (Applied Biosystems), 1.2 µM of each primer (vtgFw: 5'-CATCATTGCTCGTGCTGTCA-3'; vtgRv: 5'-AGAGGCAACCACAACCTGTA-3, Fernandes *et al*. 2021) and RNAse Free water to complete the total reaction volume of 20 µL. For the relative analysis of the expression of a gene, it is necessary to use a reference gene. In this study, the elongation factor-1α reference gene was used. For the qPCR reactions for this gene, the same concentrations used for *vtg* were used, except for the primers, which were used at a concentration of 0.8 µM of each primer (ElFFw: 5'-GTTGGAGTCAACAAGATGG-3'; ElFRv: 5'-GGGTTGTAGCCGATCTTC-3', Silva de Assis *et al*., 2018). The efficiency curves of the primers used are found in the supplementary material (Figure 1).

For the qPCR analysis, plates in 96-well plates (MicroAmp®) were used, in which two replicates of each sample and each negative control of the groups were placed, in addition to the negative control of the plate (all reagents, except the sample). This analysis was performed in StepOnePlus Real-Time PCR System. Different cycles were used for each gene. For *vtg*: 2 minutes at 95°C and 40 cycles of 15 seconds at 95°C, 15 seconds at 59°C, 40 seconds at 72°C. For elongation factor-1α: 2 minutes at 95° C and 40 cycles of 15 seconds at 95° C, 15 seconds at 59° C, and 30 seconds at 72° C. Finally, the data were analyzed according to Livak & Schmittgen (2001).

Data analysis

Levenne and Shapiro-Wilk tests were used to test the assumptions of homogeneity of variances and normality, respectively. The data for each comparison (C25 x C30, C25 x CE25, and C30 x CE30) were subjected to the t-test or Mann-Whitney U-test, according to the assumptions. The responses (increase or decrease about the control) between temperatures were compared. Principal Coordinates Analysis (PCoA) was performed to integrate the results and show group patterns. The analyses were carried out in the R environment 4.2.2, with a decision rule of $p \leq 0.05$.

RESULTS AND DISCUSSION

Some indexes are frequently used to compare the reproductive condition of individuals or conditions. The hepatosomatic index, for example, can indicate the accumulation of lipids in the liver or the mobilization of these molecules to other organs, such as gonad, indicating the energy reserve status of the organism (Podolska *et al*., 2024). This mobilization, in the reproductive period, can be done for the gonads, reducing the hepatosomatic index so that gonadal development can occur (Becker *et al*., 2024). In this way, the gonadosomatic index can be used to describe the timing and/or duration of the spawning period of fish, such as the round goby, *Neogobius melanostomus* (Zeyl *et al*., 2014). Mainly in females, both indexes also indicate gonadal maturation since this process includes vitellogenesis, which occurs in the liver, and the transport of this synthesized vitellogenin to the gonads (Becker *et al*., 2024).

In the present study, the hepatosomatic index decreased with temperature increase in males (Figure 1A), while the gonadosomatic index increased in females and males in this same condition (Figure 1B). In addition, GSI increased for females exposed to *R. raciborskii* extract at 25 °C. These results indicate that both males and females are reallocating more energy to gonadal development with increasing temperature, indicating the proximity of a reproductive peak (Rodrigues *et al*., 2023). In the literature, this dynamic of temperature variation due to the seasonality is responsible for inducing spawning and the consequent reproductive period of the organisms (Duchaud *et al*., 2021; Huang *et al*., 2024). In this case, the crude extract of *R. raciborskii* in a 25°C scenario was able to cause the same effect in females, indicating energy reallocated to the development of the ovaries and a closer proximity to the spawning period. In other words, in an environment with a bloom of these cyanobacteria, females of *Rhamdia quelen* can alter their reproductive cycle, unlike males, leading to the desynchronization of this cycle, affecting the population dynamics of the species (Gomiero *et al*., 2007; Montanha *et al*., 2011).

However, reproduction encompasses more parameters than just gonadal development (Flores *et al*., 2024). The estradiol hormone did not change in exposure to the different conditions tested (Figure 2A and B). However, testosterone levels were significantly reduced in females exposed to *R. raciborskii* extract at 25°C scenario (Figure 2C). Differently, when exposed for 90 days to 0.1 and 1 $\mu g.L^{-1}$ of saxitoxins, the main cyanotoxin produced by this cyanobacterium, females and males of *Danio rerio* did not show changes in the plasma hormone levels of 17β-estradiol and 11-ketotestosterone under 26°C (Haque *et al*., 2022). Despite being a hormone related to male characteristics during the process of sexual differentiation in fish, testosterone has other important roles (Schaafsma & Groothuis, 2012). Testosterone levels are also associated with lower levels of oxidative stress that an organism will face, reducing cortisol in cichlid fish of the species *Astatotilapia burtoni* (Culbert *et al.*, 2023). The same occurred with female brown trout (*Salmo trutta*, L.), in which the reduction in testosterone levels was already related to the reduction in hydroperoxide levels during the spawning of these organisms (Hoogenboom *et al.*, 2012). Thus, the reduction in testosterone in females exposed to *R. racibosrkii* extract can lead to oxidative changes in the organism, altering the homeostasis, as demonstrated by Vicentini *et al* (2024) with the oxidative damage caused by cyanotoxins from *R. raciborskii* in *Rhamdia quelen*.

In addition to changes in indexes, temperature was also able to alter the expression of vitellogenin. In females, the relative expression of the vitellogenin gene

changes by significant increase of almost 6-fold in exposure to a thermal condition of 30°C. Unlike the present study, in which temperature directly influenced the increase in *vtg* gene expression since estradiol did not change, this relation between temperature and vitellogenin can be indirect. For fish of the species *Gadus chalcogrammus*, for example, the increase in temperature was not able to directly induce an increase in plasma vitellogenin. It was considered an indirect effect since the temperature increases estradiol and estradiol receptors are responsible for starting the vitellogenin synthesis cascade (Kim *et al*., 2019). Furthermore, this increase may mean greater energy expenditure by females for reproduction, since the production of vitellogenin demands a lot of energy for the organism (Colpo and López-Greco, 2018).

In the heating scenario, *vtg* gene expression upon exposure to cyanobacteria extract was significantly reduced by 7-fold (Figure 3A), for females, getting close to zero. In males, this relative expression changes a significant 3-fold reduction in the 30°C scenario in exposure to the cyanobacterial extract (Figure 3B). Thus, the combination of temperature and exposure to neurotoxin-rich cyanobacteria has an antiestrogenic effect on juveniles of the species *Rhamdia quelen.* About STX, the main cyanotoxin produced by *R. raciborskii*, chronic exposure of males and females of *Danio rerio* to 0.1 and 1 $\mu g.L^{-1}$ of STX was not able to alter the levels of the vitellogenin protein (VTG) in the plasma (Haque *et al*., 2022), different from what was found for the expression of the gene studied in the present study for the warming scenario. However, as in the present study, marine medaka (*Oryzias melastigma*) embryos treated with 840 and 1260 $\mu g \cdot L^{-1}$ 15 days after fertilization showed reduced expression of both the VTG protein and the *vtg1* gene (Tian *et al*., 2014).

For females exposed to *R. raciborskii* cyanotoxins in a global warming scenario, this no expression in vitellogenin may mean changes in their offspring. For organisms such as fish, vitellogenin is responsible for the presence of yolk in the oocyte, which leads to its maturation and serves as an energy source for the embryo until it becomes a larva and can feed itself. In other words, the reduction in vitellogenin may affect oocyte maturation and its fecundity (Qiao *et al*., 2016). As demonstrated for a female of *Danio rerio*, the reduction in the expression of a specific subtype of vitellogenin, *vtg1*, in addition to reducing their reproductive rate, can also cause malformations, reduced swimming capacity, and reduced food intake in their offspring (Sun *et al*., 2023).

As shown in the results of Vicentini *et al.* (2023 and 2024), females and males presented different responses, as observed by multivariate analysis, for temperature and cyanotoxins exposition (Figure 4). We observed that not only water heating but also exposure to *R. raciborskii* extract rich in neurotoxins is capable of altering the reproductive axis of juvenile *Rhamdia quelen*, with sex-related responses.

In the literature, increased temperature is associated with disturbances in gonadal maturation and development (Servili *et al*., 2020). Thus, temperature-related treatments for females and males are in different quadrants of the analysis (Figure 4), with the difference between controls becoming more pronounced in females. Females in this study had altered vitellogenin expression, which is an important biomarker of endocrine disruption in fish. There are few studies on the effects of warming specifically in males, but the available data indicate that the effects in females are more pronounced (Miranda *et al*., 2013). About cyanobacteria, it has been reported in the literature that compounds produced by them, such as their cyanotoxins, can interfere with the signaling of different intracellular receptors, and these signaling pathways may play an important role in hormonal regulation, reproduction, and development in vertebrates. Furthermore, among the compounds present in cyanobacteria, there is evidence of the presence of estrogenic compounds or compounds that interfere with the androgen receptors of organisms (Jonas *et al*., 2015). Combining information from cyanotoxins and abiotic factors such as temperature, female and male *Rhamdia quelen* respond differently.

CONCLUSION

The Neotropical species of fish, *Rhamdia quelen*, presented some alterations of biomarkers related to reproduction as a result of the increase in water temperature. Increased gonadosomatic index observed in females may indicate changes in the reproductive cycle, as well its increased expression of hepatic vitellogenin. The crude extract of *R. raciborskii*, a saxitoxin-producing cyanobacterium was anti-androgenic for *R. quelen* females in a water temperature at 25°C, reducing plasma testosterone levels and increasing expression vitellogenin gene. As temperature itself showed to be a stressor, it can justify the fewer effects observed in a scenario of exposure to cyanobacteria extract at 30°C. This study provided important data regarding the effects of two important global stressors, temperature and cyanobacteria, on a species of economic and ecological importance.

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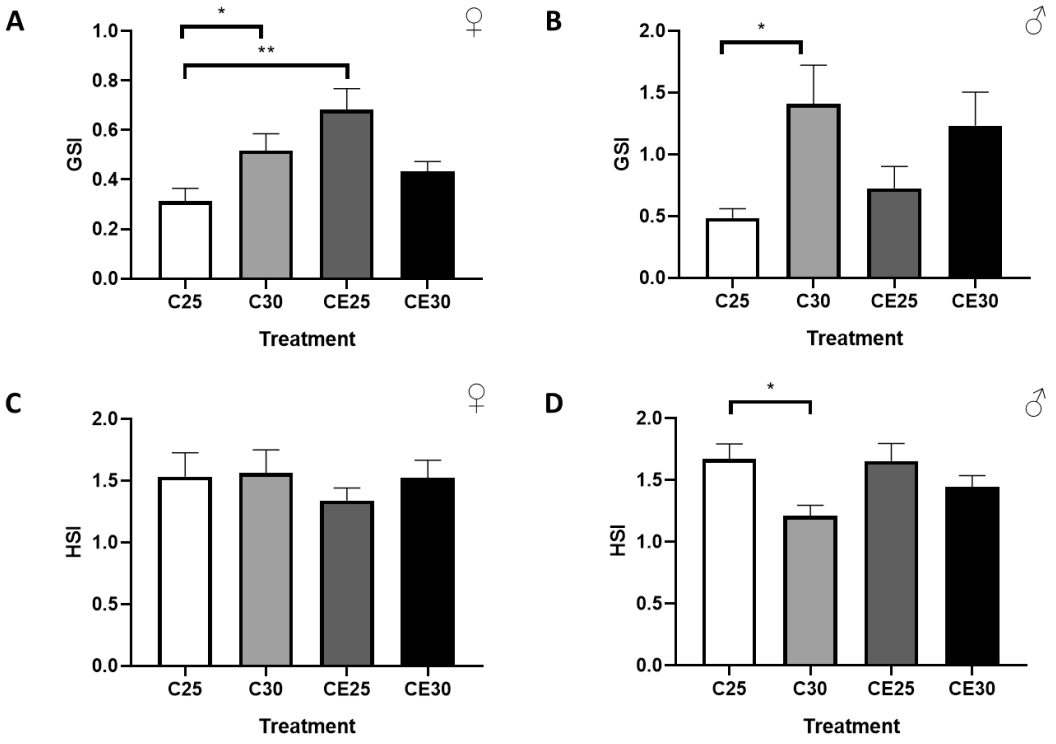

Figure 1. Gonadosomatic Index (A and B) and hepatosomatic index (C and D) levels, in females (A and C) and males (B and D) with exposure at 25°C and 30°C for 96 hours to crude *R. raciborskii* extract. * representing significant difference between the C25 and C30; and ** representing difference between the C25 and CE25.

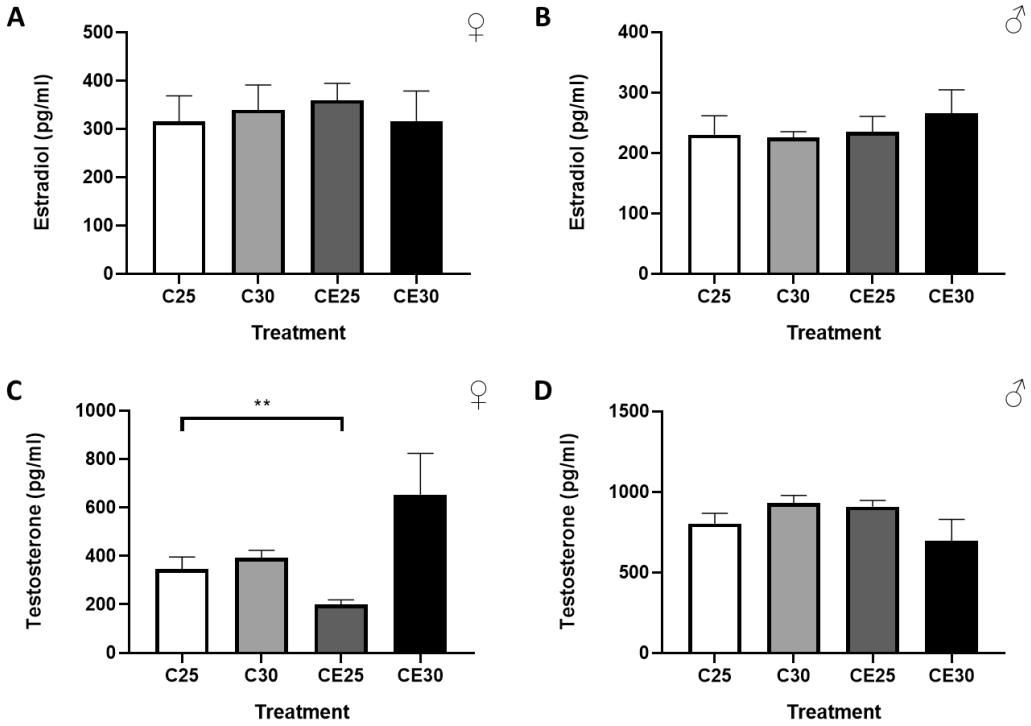

Figure 2. Estradiol (A and B) and testosterone (C and D) levels, in females (A and C) and males (B and D) with exposure at 25°C and 30°C for 96 hours to crude *R. raciborskii* extract. ** representing difference between the C25 and CE25.

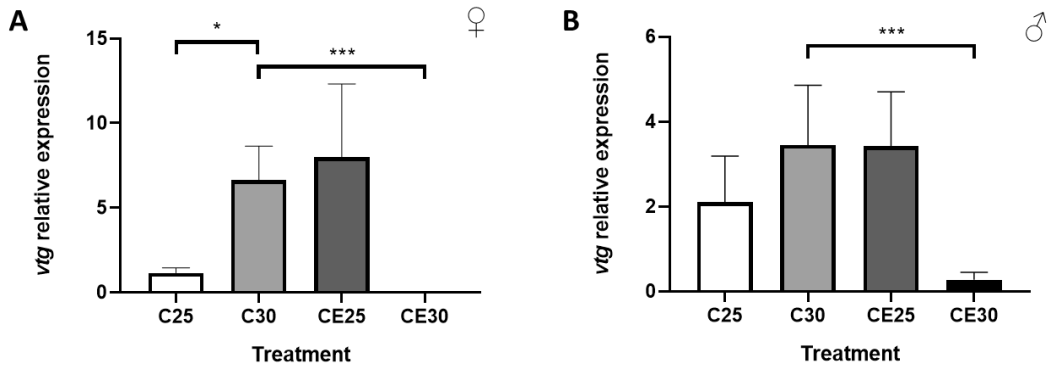

Figure 3. Vitellogenin gene expression in females (A) and males (B) with exposure at 25°C and 30°C for 96 hours to crude *R. raciborskii* extract. * representing significant difference between the C25 and C30; and *** representing the difference between the C30 and CE30.

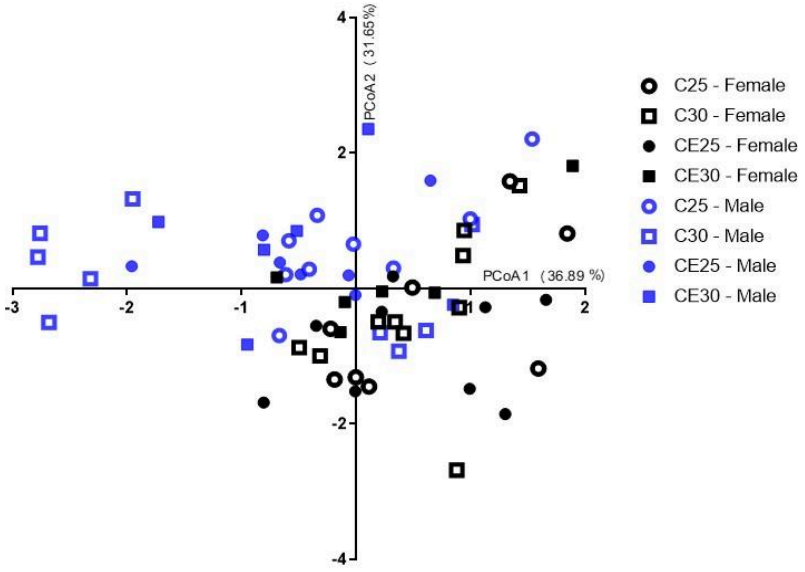

Figure 4. PCoA representation in females and males of *R. quelen* exposed at 25°C and 30°C for 96 hours to crude *R. raciborskii* extract, with the percentage of explanation on each axis.

CREDIT AUTHOR STATEMENT

MV: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Review & Editing, Visualization; **CLB:** Methodology – Cyanobacteria culture; **LASJC:** Methodology – Hormones quantification; **LAAC:** Methodology – Cyanobacteria culture, Supervision; **HCSA:** Resources, Writing – Review & Editing, Supervision; Project administration.

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