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Research Paper

# First baseline for bioenergetic biomarkers in *Cnesterodon decemmaculatus* as test organism in ecotoxicological studies

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ABSTRACT

*Cnesterodon decemmaculatus* is a Neotropical teleost fish frequently used in ecotoxicological evaluations, whose biology has been thoroughly studied. Although there is considerable information on its response to different toxicants, no range of reference values has been so far established for the different biological parameters proposed as biomarkers of effect or exposure. Moreover, no study has yet examined the possible influence of the metabolic status of the exposed animals on their response to toxic stress. Therefore, the aim of this work was to provide a first baseline for a set of bioenergetic biomarkers in *C. decemmaculatus* adults exposed to a control medium under previously standardized conditions, and to assess their possible intrinsic seasonal variability. The responses of the biomarkers obtained from the controls were contrasted with those from the reference toxicant (Cadmio-Cd) and receiving waters (surface waters of the Reconquista River RR, Buenos Aires Province, Argentina). We conducted four 12-day assays (one in each season) of exposure to control media, (reconstituted moderate hard water, MHW) and two assays of exposure to Cd in MHW and surface river water (RR) in both summer and autumn. The variables recorded were: Food intake (In), fecal production (F), specific assimilation (A) and cumulative mortality, oxygen extraction efficiency (OEE), specific metabolic rate (SMR), ammonia excretion (N), ammonia quotient (AQ) and scope for growth (SFG). The seasonal variation shown by some physiological parameters, points to the need for establishing a baseline obtained from standardized media, preferably on a seasonal basis. Moreover, SFG and A appeared as the most sensitive biomarkers, emphasizing the importance to consider the metabolic status of the test organisms for the appropriate interpretation of results from ecotoxicological studies performed under controlled experimental conditions. The obtained results provide useful information on *C. decemmaculatus* as model species in ecotoxicological bioassays involving biomarkers of early effect.

## 1. Introduction

Many studies have emphasized the importance of biomarkers for evaluating the impact of contamination on biota (Amiard-Triquet and Berthet, 2015; Colin et al., 2016). However, biomarkers in organisms exposed to toxicants exhibit a substantial intrinsic variability related to different factors such as age, sex, reproductive and nutritional conditions, stress status, circadian rhythms and environmental (physicochemical) factors (Livingstone, 1993; Menéndez-Helman et al., 2015; Nahrgang et al., 2010; Sanchez et al., 2008). Such variability may be confounded with treatment effects, yielding misleading results. This problem is usually solved by comparing experimental treatments with parallel controls, which inevitably leads to an increase in the number of experimental animals used. Biomarker baselines are obtained from physiologically normal animals exposed to control media, thus constituting reference or basal values that describe the inherent variability of each parameter under specific experimental conditions (Baudou, 2019). On this basis, the results of the controls in successive assays are expected

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to be within the range of baseline values. Moreover, a comparison between the ranges of baseline values and post-exposure values is required to select appropriate biomarkers as endpoints. Indeed, the influence of the animal's metabolic status on stress responses should not be under estimated.

Some physiological variables used to estimate the energy needed for maintaining the homeostasis in the body are useful as bioenergetic or metabolic biomarkers. In animals, the index Scope for Growth (SFG) is a measure of net energy balance between the energy gained from assimilation (A), and the energy expended through specific metabolic rate (SMR) and ammonia excretion (E) (Scarlet et al., 2015; Zhou et al., 2001). In ecophysiological studies, these variables are usually used to determine the amount of energy available for counteracting toxic effects (Agbohessi et al., 2014; Bonifacio, 2017; Cazenave et al., 2006; Sancho and Ferrando, 2015). In fish, SFG has also been used for this purpose and has emerged as a valuable biomarker for ecotoxicity assessment (Baudou et al., 2017, 2019; Ferrari et al., 2011).

Cadmium (Cd) exhibits high toxicity to the aquatic biota, representing an important source of contamination for different trophic chains (Barhoumi et al., 2009; Mebane, 2006). This metal has been recommended as a reference toxicant (US EPA, 2002) and frequently used for ecotoxicological assessment (Ferrari et al., 2011; Rani et al., 2014). In fish, Cd is known to cause hypocalcemia (Sloman et al., 2003; WHO, 1992; Wright and Welbourn, 1994), gill morphological alterations (Ferrari et al., 2009; Thophon et al., 2003), alterations in swimming behavior (Baker and Montgomery, 2001; Eissa, 2009; Faucher et al., 2006; Sloman et al., 2003) and energy metabolic disorders (Baudou et al., 2017; Ferrari et al., 2011), among other deleterious effects.

The Reconquista River is an important peri-urban watercourse located in the province of Buenos Aires, Argentina. It is regarded as a model of river pollution and has been studied for more than 20 years from different approaches. Its surface waters are an archetype of receiving waters (Baudou et al., 2019; Defensoría del pueblo de la Nación Argentina, 2007; Ferrari et al., 2005; Giusto et al., 2014; Ossana et al., 2013, 2019; Salibián, 2006).

de la Torre et al. (1997) designed a lethality bioassay with receiving waters, Cd as positive control and the poeciliid *Cnesterodon decemmaculatus* (Jenyns, 1842) as test organism. Since then, this fish has been widely used in ecotoxicological studies at the regional level (Bonifacio et al., 2016; Carriquiriborde et al., 2007; de la Torre et al., 2002, 2007; Mastrángelo and Ferrari, 2013; Menéndez-Helman et al., 2012; Ossana et al., 2016, 2019; Vera-Candioti et al., 2013). Moreover, *C. decemmaculatus* was validated as model organism for toxicity assessment (IRAM, 2008), and a standardized method for its rearing at the laboratory was developed (Ferrari et al., 2017). On the other hand, Cd has been proposed as a reference toxicant for *C. decemmaculatus* in ecotoxicological studies (Mastrángelo and Ferrari, 2013).

The aim of the present study was to establish the first baseline for a set of metabolic biomarkers in adults of *C. decemmaculatus* exposed to a control medium under experimental conditions and to test the response of such biomarkers to a reference toxicant (Cd) and to samples of receiving water.

## 2. Materials and methods

### 2.1. Test organisms

We studied some biological parameters that provide useful information on the physiological status of *C. decemmaculatus*, which is used as an ecotoxicological model organism. All fish used in this study come from standardized stock cultures maintained in our laboratory (Ferrari et al., 2017) with food provided daily and under spring-like conditions.

We used adults (5:1 female:male proportion) of *Cnesterodon decemmaculatus* (mean weight ± SEM = 116.39 ± 49.32 mg and mean size ± SEM = 24.57 ± 3.83 mm; N = 45/treatment/assay). They were bred in aquaria containing chlorine-free tap water and supplied with continuous aeration, under controlled conditions (T = 23 ± 1 °C; 16:8 L:D photoperiod). Fish were fed ad libitum "TetraFin Goldfish Flakes" composed of (g/100 g): carbohydrates 30.0; proteins 42.7; fat 10.5; ashes 10.5; moisture 6.3, and a caloric value of 14.316 J $g^{-1}$. Fish used in the experiments have been cared according to the University Bioethics Committee (Universidad Nacional de Luján, DISP SE ACAD LUJ-0001438-18) criteria were followed and sacrificed in compliance according to the Guidelines for the euthanasia of animals (AVMA, 2013).

### 2.2. Exposure media and water sampling

The exposure medium (control) was reconstituted moderate hard water (MHW; pH 7.4–7.8; hardness: 80–100 mg $CaCO_3$ $L^{-1}$; alkalinity: 60–70 mg $CaCO_3$ $L^{-1}$; US EPA, 2002).

Cadmium (Cd; at a nominal concentration of 0.5 mg $L^{-1}$ MHW) solutions were prepared by dilution from a $CdCl_2{\cdot}2.5H_2O$ stock solution of 1000 mg Cd $L^{-1}$ in MHW. The effective Cd concentration was measured in samples taken regularly from the exposure solutions by atomic absorption spectrophotometer (Perkin Elmer; AAnalyst 200 model, quantification limit: 0.0048 ± 0.009 mg $L^{-1}$) equipped with hollow cathode lamps (lambda = 228.8 nm). The Cd concentration of 0.5 mg $L^{-1}$ was selected because of its subtoxic effect in our biological model under the experimental conditions used here (Baudou et al., 2017).

Samples of receiving water (RR) were collected from the headwaters of the Reconquista river (S 34° 41′ 03.5″ and W 58° 51′ 15.5″) in autumn and summer: The sample site is located just downstream from the water body created by the Roggero dam, in the upper basin of the Reconquista river. Although this reservoir was built to reduce overflow due to flooding, it could be considered a depuration system for material transported especially from La Choza, La Horqueta and Durazno streams; different contaminants (e.g., sewage water) enter the reservoir and La Choza stream contributes with the highest polluting load (Rigacci et al., 2013).

Conductivity, pH, dissolved oxygen (DO) and temperature of water samples were measured in situ using a portable device (HqD Field Case, Hach). Water samples were transported to the laboratory under proper refrigeration to measure hardness, alkalinity, ammonium ($NH_4^+$), chloride concentration ($Cl^-$), turbidity, conductivity, pH, 5-day Biochemical Oxygen Demand ($BOD_5$), Chemical Oxygen Demand (COD), and phosphates ($PO_4^{3-}$). Samples for the determination of As, Cu, Cr, Cd, and Pb were taken in plastic bottles and kept acidified with $HNO_3$ (pH ≤ 2), whereas those for screening of organochloride and organophosphorus pesticides were collected in amber-colored glass bottles. All measurements were performed in triplicate and determined according to standard methods (APHA, 2005), as described by Baudou et al. (2019).

Water quality at the sampling site of the river was determined using two indices: WQIa (Berón, 1984), which is an indicator of domestic pollution based on T, DO, $Cl^-$, $BOD_5$ and $NH4^+$, and WQIb (Lacoste and Collasius, 1995), which is an indicator of industrial pollution based on DO, COD and major metals (Cu) and a metalloid (As). Both indices are unitless and range from 0 to 10 (heavily polluted and unpolluted, respectively). The samples were centrifuged (5000 rpm, 5 min) to remove particulate suspended matter and stored at 5 °C. At the time of the bioassays, water samples were aerated and brought to experimental temperature (22–24 °C). Exposure began 24 h after water collection.

### 2.3. Experimental design

The experimental design is based on that used by Ferrari et al. (2011) for *Cyprinus carpio* juveniles, and later adjusted for the test species, *C. decemaculatus* (Baudou et al., 2017, 2019).

We conducted four bioassays with MHW, two with RR and two with Cd. Each assay was done in triplicate and included two successive periods: a 15-acclimation period and a 12-d exposure period under constant temperature (22 ± 1 °C) at 16:8 (L:D) photoperiod.

For the acclimation period, adults were randomly selected from stock cultures and distributed into glass aquaria of 15 × 15 × 20 cm (mean loading density of 500 mg $L^{-1}$) containing 2 L of culture medium (dechlorinated tap water gradually replaced by MHW until reaching 100% MHW after 7 days). Then, aquaria were placed in chambers under the same laboratory conditions as described above, and the fish were daily fed ad libitum.

At the beginning of the exposure period, fish were individually weighed and pooled together in groups of 10–15 individuals per aquarium.

Total biomass per aquarium was calculated as the sum of the initial weights. Daily feed ration was calculated to be 2% of the total biomass per aquarium and adjusted based on the daily mortality per replicate. The first feed ration was supplied 24 h after the beginning of the exposure. The experimental media were completely renewed every 96 h.

During the exposure period pH, DO, hardness and conductivity were measured periodically in the assay media of the MHW, Cd and RR groups.

For the baseline proposal, the bioenergetic parameters were obtained seasonally from fish exposed to the MHW medium. In summer and autumn, trials included parallel series of Cd- and RR-exposed fish.

### 2.4. Bioenergetic parameters estimates

The experimental procedure followed Baudou et al. (2017, 2019). Briefly, the following biological parameters were recorded daily in each aquarium:

*Fecal production (F):* prior to the daily food ration, feces were collected by siphoning, filtered and dried at 60 °C to constant weight (expressed as mg feces mg $biomass^{-1}$ $day^{-1}$).

*Food intake (In):* prior to food offer, dead animals were removed and weighed to recalculate the food ration based on updated biomass. Food was offered every morning for 1 h, and the excess of food was removed by siphoning, filtered and dried at 60 °C to constant weight. *In* was calculated as the difference between the weights of offered and remaining food (expressed as mg food mg $biomass^{-1}$ $day^{-1}$).

*Assimilation (A):* it was calculated as A = In − F; (Alcaraz and Espina, 1997; Ferrari et al., 2011). F, In and A were transformed into energy units and expressed as Joules per g wet weight per day (J $g_{ww}^{-1}$ $day^{-1}$).

*Mortality:* it was expressed as percentage of cumulative mortality over time per treatment.

About 10 fish from each group were randomly selected to measure oxygen consumption and ammonia excretion at the end of the exposure period. Each specimen was acclimated for one hour in an open flow-through respirometer. Then, it was replaced into a close-flow system for 1 h, where dissolved oxygen was measured at initial (DOi) and final (DOf) time. Additional samples were taken for determination of ammonia concentration at initial and final times when fish were in the closed-system respirometer. So, for each individual, the specific metabolic rate (SMR) was calculated as: $O_2$ consumed (mg) $g_{ww}^{-1}$ $h^{-1}$ and the oxygen extraction efficiency (OEE) was calculated as $OEE = (DO_i - DO_f) \times 100/DO_i$ per hour (Espina et al., 2000). In addition, the concentration of ammonia excreted (N) was expressed as μg $NH_4$ $g_{ww}^{-1}$ $h^{-1}$, and the ammonia quotient (AQ) as the ratio between moles of $NH_4^+$ excreted and moles of $O_2$ consumed (De Boeck et al., 1995; Owen et al., 1998). Finally, Scope for Growth was calculated as SFG = A − (SMR + N) and expressed as J $g_{ww}^{-1}$ $day^{-1}$ (Roast et al., 1999).

### 2.5. Statistical analysis

Prior to further analysis, the data were evaluated for the assumptions of normality and homoscedasticity using the Shapiro Wilks and Levene tests, respectively (Zar, 2010).

The results among controls and between exposed groups and controls were compared using One-way analysis of variance (ANOVA), followed by the Tukey's multiple comparison test (for parameters recorded daily) or by the parametric Kruskall–Wallis test with pair wise comparisons for the parameters recorded at final time (Zar, 2010).

We performed a frequency analysis and evaluated the goodness-of-fit of empirical distribution functions to theoretical ones for each physiological variable in the control media-exposed groups (MHW), considering the whole data set of the four assays. The tested hypothesis was that the variable under consideration exhibits a seasonally independent distribution and that its distribution function is of the normal or gamma type. The observed values for each class frequency were compared with the expected ones according to the specified model ($Chi^2$ $p \leq 0.05$).

The analyzes were performed with INFOSTAT software, version 2018 (Di Renzo et al., 2014).

To evaluate the effect of season and treatment, a type III mixed linear model with fixed effects was chosen with the restricted maximum-likelihood method using the SPSS software (α = 0.05) (26.0 IBM, 2019). In all cases, the effects of season, treatment and their interaction were evaluated. The variables intake, feces and assimilation, determined daily, were analyzed as repeated measures, while SMR, OEE, N, AQ and SFG, determined at end time, were treated as independent measures (see Supplementary data for a statistical summary of each comparison).

## 3. Results

### 3.1. Physicochemical parameters of assay media

The ranges of pH, DO, hardness, conductivity and Cd measured for each experimental group are shown in Table 1.

The analytical concentrations of Cd (mean ± SEM with number of measurements in brackets) measured every 48 h were 0.81 ± 0.06 mg $L^{-1}$ (18) and 0.66 ± 0.06 mg $L^{-1}$(18) for autumn and summer, respectively, and remained stable throughout the exposure period. Cd concentration in the MHW control was below the detection limit.

Table 2 shows the values of the physicochemical parameters and WQIs obtained from samples of receiving water from the Reconquista River used for the summer and autumn bioassays; water quality standards for the protection of aquatic biota were included for further comparison.

The values obtained for the physicochemical parameters in the bioassays of summer and autumn were below the guidelines for the protection of freshwater aquatic life. The OD values were higher than the guideline values in both seasons. In regard to the metalloid As and the metals Pb, Cr and Cu were higher in summer than in autumn, with the latter far exceeding the allowed upper limit of 5.25 μg $L^{-1}$. In regard to the water quality indices, WQIa and WQIb (using As and Cr) indicated no pollution (8.6–8.8) and slight pollution (7.0–6.3), respectively. Pesticide concentrations were lower than the detection limits of the analytical technique and were excluded from this table.

### 3.2. MHW baseline end points of bioenergetic parameters

We evaluated a possible seasonal variation in each biomarker parameter under the experimental conditions. Table 3 shows the mean, SEM and the variation coefficient (VC) of the parameters obtained from

**Table 1**
Physicochemical parameters in the exposure media of the bioassays with *Cnesterodon decemmaculatus*: control media (moderately hard water, MHW), Receiving Reconquista River water (RR) and Cadmium (Cd).

| | pH<br>pH units | OD<br>(mg $O_2$ $L^{-1}$) | Hardness<br>(mg $CaCO_3$ $L^{-1}$) | Conductivity<br>(μS $cm^{-1}$) |
|---|---|---|---|---|
| MHW: | 7.4–8.3 | 7.2–8.8 | 87.0–95.4 | 316.0–424.3 |
| RR: | 7.5–8.5 | 6.9–8.8 | 26.3–86.0 | 214.9–871.3 |
| Cd: | 7.4–8.1 | 7.4–8.6 | 87.1–97.0 | 327.9–344.5 |

Data expressed as ranges of values measured of values measured of 18 determinations.

**Table 2**
Physicochemical parameters and Water Quality Index (WQIs) for samples of receiving water from the Reconquista River.

| *In situ* | Summer | Autumn | Argentine Guidelines[a] |
|---|---|---|---|
| Temperature $H_20$ (°C) | 24.3 | 12.0 | ≤ 45 |
| Temperature air (°C) | 22.5 | 10.5 | |
| pH | 8.2 | 7.2 | 6.5–10 |
| Conductivity (μs $cm^{-1}$) | 854 | 778 | |
| DO (mg $O_2$ $L^{-1}$) air | 8.4 | 9.9 | |
| DO (mg $O_2$ $L^{-1}$) water | 6.9 | 8.8 | ≥ 5 |
| ***In the laboratory*** | | | |
| $BOD_5$ (mg $O_2$ $L^{-1}$) | 2.4 | 4.3 | ≤ 50 |
| DO(mg $O_2$ $L^{-1}$) | 8.4 | 7.6 | ≥ 5 |
| COD (mg $O_2$ $L^{-1}$) | 43 | 66.5 | ≤ 250 |
| $COD/BOD_5$ | 19.7 | 15.5 | |
| Chlorides (mg Cl $L^{-1}$) | 26.5 | 37.5 | ≤ 250 |
| Hardness (mg $CaCO_3$ $L^{-1}$) | 82 | 79 | |
| Alkalinity (mg $CaCO_3$ $L^{-1}$) | 313 | 233 | |
| Turbidity (UNT) | 231 | 91 | |
| Ammonium (mg N-$NH_4$ $L^{-1}$) | 0.07 | 0.07 | ≤ 1.37 |
| Phosphates (mg P-$PO_4$ $L^{-1}$) | 0.64 | NM | ≤ 1 |
| pH | 8.2 | 7.2 | 6.5–10 |
| **Metals/metalloid** (μg $L^{-1}$) | | | |
| Cr | **7** | 2 | ≤ 2.5 |
| Pb | **3** | ND | 1.59 |
| Cd | ND | ND | 0.028–0.09 |
| Cu | **503** | NM | 2.48–5.25 |
| As | **57** | 3 | ≤ 15 |
| **WQI** | | | |
| WQIa[b] | 8.6 | 8.8 | |
| WQIb[c] | 7.0 | 6.3 | |

NM: not measured; ND: not detected; values of metals and metalloid in bold are above the guideline levels for the protection of aquatic biota.

[a] Argentine Surface Water Guidelines for protection of aquatic life or maximum allowable content in effluents disposed in a water body.

[b] Berón (1984).

[c] Lacoste and Collasius (1995).

the MHW group for each assay in the four seasons. With regard to the parameters recorded daily, food intake (In) was significantly lower in winter than in summer ($F_{df3} = 29.83$, $p < 0.0001$), these values were significantly lower than those in spring and autumn. Fecal production (F) was significantly highest in the spring assay, ($F_{df3} = 11.74$, $p < 0.0001$) while Assimilation (A) was significantly highest (20–40%) in the autumn assay ($F_{df3} = 24.56$, $p < 0.0001$).

With regard to the parameters recorded at final exposure time, the lowest and highest values of oxygen extraction efficiency (OEE) were observed for the spring and winter assays, respectively ($H = 23.27$, $p < 0.0001$). The specific metabolic rate (SMR) was significantly higher in the autumn and spring assays than in the summer and winter ones ($H = 12.64$, $p < 0.0001$). Ammonia excretion (N) was significantly highest in the spring assay ($H = 11.08$, $p < 0.0113$). No significant differences in ammonia quotient (AQ) were found among season assays ($H = 4.94$, $p < 0.174$). Finally, Scope for Growth (SFG) was lowest in the spring assay and differed significantly from that obtained in the autumn and winter assays ($H = 10.12$, $p < 0.0175$).

There was significant individual variability for each studied parameter, with high variation coefficients (> 20% in most cases; Table 3). To overcome this problem, we calculated the relative frequency of each parameter for the whole data set of the four assays. Fig. 1 shows the frequency distribution of the biomarkers recorded daily, with a table indicating the best-fit distribution model for each of them. The frequency analysis for In reveals values belonging to the four seasons in the class intervals 1–5, whereas F has a very wide range of values, with 89% of the total records in the class intervals 1–3. Therefore, both In and F seem to be independent of the season. In contrast, the frequency distribution of A data shows that the highest values are grouped in the autumn assay, the lowest-intermediate values in the spring and summer assays, and the intermediate values in the winter assay. These results suggest that A may have a differential seasonal response.

Fig. 2 shows the frequency distribution of the biomarkers recorded at final time, with a table indicating the best-fit distribution model for each of them. For OEE, class intervals 1–3 contain 82% of data, with the lowest values being observed in spring and summer, whereas these are found in autumn and winter for class interval 2 data. These results may indicate that this parameter has a seasonal behavior. Regarding SMR, class intervals 2 and 3 include 68% of the data, with a similar proportion of records from the four assays. A similar result was observed for N and AQ, for which class intervals 1 and 2 contain 79% and 78% of the data from the four assays, respectively. Moreover, the latter biomarker shows a similar proportion of values from all the assays. These results suggest that SMR, N and AQ are independent of the season. Finally, the SFG values recorded in the spring and summer assays are distributed within the lower class intervals (1 and 2), whereas the classes 3 and 4 contain values mainly from the autumn and winter assays. This result suggests that SFG shows a seasonal behavior.

Table 4 shows a summary of the range of expected values for the studied bioenergetic biomarkers obtained under protocolized control conditions.

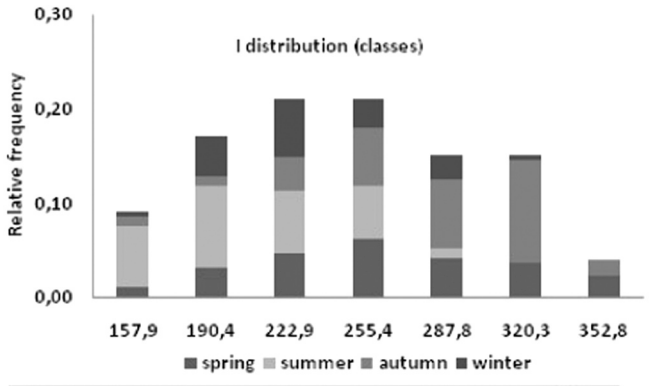
### 3.3. Bioassays of exposure to Cd and to receiving waters of the Reconquista River receiving water (RR) vs parallel controls with MHW

Table 5 shows the results of bioenergetic parameters recorded daily (In, F and A) and cumulative mortality at final exposure time for each treatment. The values of MHW are the same indicated in Table 3 for summer and autumn. For each assay, statistical comparisons were made between exposed groups and their parallel controls. In the summer and

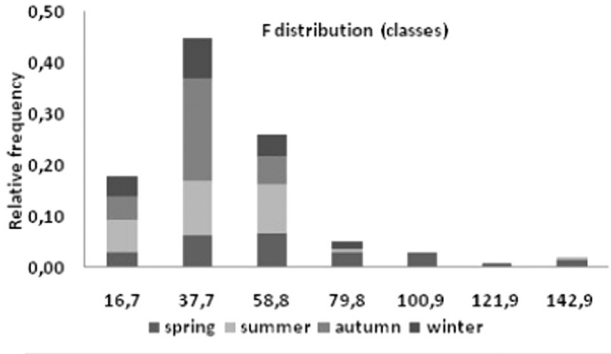
**Table 3**
Bioenergetic parameters (J $g_{ww}^{-1}$ $day^{-1}$) of adults of *Cnesterodon decemmaculatus* exposed to MHW control medium obtained from seasonal bioassays. In: food intake, F: fecal production, A: assimilation, SMR: specific metabolic rate, OEE: oxygen extraction efficiency (%), N: ammonia excretion, AQ: ammonia quotient, SFG: Scope For Growth.

| | Season | In | F | A | OEE | SMR | N | AQ | SFG |
|---|---|---|---|---|---|---|---|---|---|
| **Mean** | **Spring** | 260.76 (48)[a] | 63.24 (48)[b] | 197.51 (48)[b] | 2.58 (12)[a] | 115.55 (9)[a] | 29.11 (10)[a] | 0.28 (10)[a] | 51.1 (8)[a] |
| **SEM** | | 52.18 | 34.03 | 58.65 | 1.24 | 30.27 | 11.78 | 0.17 | 17.86 |
| **VC** | | 20.01 | 53.81 | 29.69 | 48.15 | 26.20 | 40.47 | 62.69 | 34.96 |
| **Mean** | **Summer** | 205.68 (56)[c] | 42.82 (56)[a] | 162.86 (56)[a] | 6.18 (11)[c] | 88.66 (9)[b] | 13.12 (8)[b] | 0.21 (7)[a] | 72.04 (4)[ab] |
| **SEM** | | 38.49 | 18.18 | 42.19 | 4.32 | 44.81 | 6.30 | 0.18 | 36.20 |
| **VC** | | 18.71 | 42.45 | 25.91 | 69.91 | 50.54 | 48.06 | 82.82 | 50.25 |
| **Mean** | **Autumn** | 280.64 (61)[a] | 40.1 (61)[a] | 240.54 (61)[c] | 7.50 (11)[bc] | 147.79 (9)[a] | 16.01 (10)[b] | 0.13 (10)[a] | 93.66 (7)[b] |
| **SEM** | | 46.85 | 17.69 | 50.60 | 1.55 | 45.85 | 7.98 | 0.09 | 26.43 |
| **VC** | | 16.70 | 44.13 | 21.04 | 20.61 | 31.02 | 49.88 | 71.79 | 28.22 |
| **Mean** | **Winter** | 233.37 (15)[b] | 39.38 (15)[a] | 194.00 (15)[b] | 9.93 (10)[c] | 83.86 (8)[b] | 14.22 (6)[b] | 0.19 (6)[a] | 100.36 (6)[b] |
| **SEM** | | 38.98 | 17.54 | 42.52 | 3.51 | 22.06 | 6.95 | 0.08 | 27.84 |
| **VC** | | 16.70 | 44.55 | 21.92 | 35.34 | 26.30 | 48.85 | 44.26 | 27.74 |

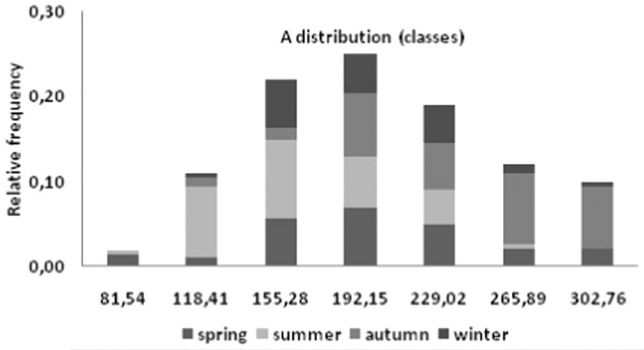
Data expressed as mean ± SEM; number of determinations in parenthesis. VC: variation coefficient. Different letters indicate significant differences between groups ($p \leq 0.05$).




| Parameter | Class | LL | UL | AC | AF | RF | $Chi^2$ | p |
|---|---|---|---|---|---|---|---|---|
| I | 1 | 141,67 | 174,15 | 157,91 | 18 | 0,09 | 0,01 | 0,06 |
| | 2 | 174,15 | 206,64 | 190,4 | 33 | 0,17 | 1,07 | |
| | 3 | 206,64 | 239,12 | 222,88 | 41 | 0,21 | 1,14 | |
| | 4 | 239,12 | 271,61 | 255,37 | 41 | 0,21 | 1,77 | |
| | 5 | 271,61 | 304,09 | 287,85 | 29 | 0,15 | 2,93 | |
| | 6 | 304,09 | 336,58 | 320,33 | 29 | 0,15 | 8,26 | |
| | 7 | 336,58 | 369,06 | 352,82 | 7 | 0,04 | 8,87 | |




| Parameter | Class | LL | UL | AC | AF | RF | $Chi^2$ | p |
|---|---|---|---|---|---|---|---|---|
| F | 1 | 6,13 | 27,18 | 16,66 | 36 | 0,18 | 0,77 | 0,1 |
| | 2 | 27,18 | 48,24 | 37,71 | 90 | 0,45 | 3,26 | |
| | 3 | 48,24 | 69,29 | 58,76 | 51 | 0,26 | 3,34 | |
| | 4 | 69,29 | 90,34 | 79,82 | 10 | 0,05 | 8,9 | |
| | 5 | 90,34 | 111,39 | 100,87 | 6 | 0,03 | 9,06 | |
| | 6 | 111,39 | 132,45 | 121,92 | 1 | 0,01 | 9,62 | |
| | 7 | 132,45 | 153,5 | 142,97 | 4 | 0,02 | 30,6 | |




| Parameter | Class | LL | UL | AC | AF | RF | $Chi^2$ | p |
|---|---|---|---|---|---|---|---|---|
| A | 1 | 63,1 | 99,97 | 81,54 | 4 | 0,02 | 1,97 | 0,18 |
| | 2 | 99,97 | 136,84 | 118,41 | 21 | 0,11 | 2,28 | |
| | 3 | 136,84 | 173,71 | 155,28 | 43 | 0,22 | 3,25 | |
| | 4 | 173,71 | 210,59 | 192,15 | 50 | 0,25 | 3,25 | |
| | 5 | 210,59 | 247,46 | 229,02 | 38 | 0,19 | 4,13 | |
| | 6 | 247,46 | 284,33 | 265,89 | 23 | 0,12 | 4,61 | |
| | 7 | 284,33 | 321,2 | 302,76 | 19 | 0,1 | 6,2 | |

**Fig. 1.** Frequency analysis for food intake (I), fecal production (F) and specific assimilation (A) for the MHW control groups of the four bioassays (N = 198). For each class interval of each parameter, the lower limit (LL), the upper limit (UL), the average per class (AC), the absolute frequency (AF) and relative frequency (RF) are given according to the best-fit distribution model. Statistical estimator $Chi^2$ ($p \leq 0.05$).

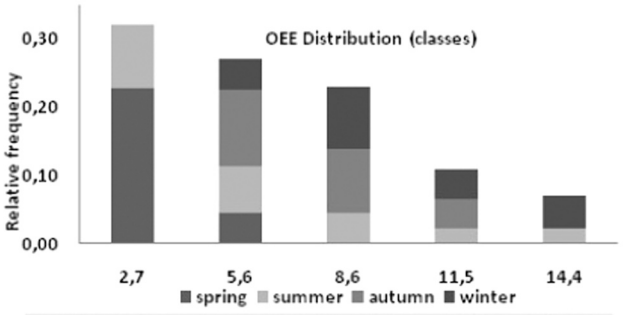
autumn bioassays, no significant differences in mortality were found in the RR-exposed group, while it was significantly higher in the Cd-exposed group compared to their controls.

In the RR-exposed group, In was significantly higher in the autumn assay ($F_{df2} = 111.35$, $p < 0.0001$), F was significantly higher in the autumn and summer assays ($F_{df2} = 191.11$, $p < 0.0001$), and A was significantly lower in the two assays in comparison with the control groups ($F_{df2} = 41.15$ and 28.89 for autumn and summer respectively, $p < 0.0001$). In the Cd-exposed group, both In and F were lower than the control in both assays ($F_{df2} = 111.35$ and 27.01 for autumn and summer respectively, $p < 0.0001$)-though the latter parameter was not significant in the summer assay-($F_{df2} = 54,51$, $p < 0.0001$), and A was significantly lower than the controls in the two assays ($F_{df2} = 41.15$ and 28.89 for autumn and summer respectively, $p < 0.0001$).

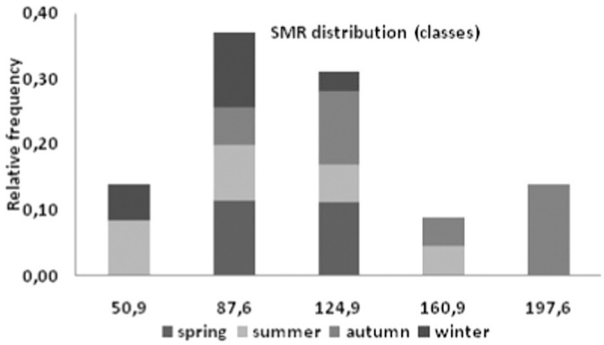
Table 6 shows the results of bioenergetic parameters determined at final time in the treated and control groups for summer and autumn assays.

In the Cd and RR-exposed groups, SMR did not differ significantly from their parallel control in the summer assay ($H = 4.22$, $p < 0.12$) and were significantly higher in the autumn assay ($H = 15.35$, $p < 0.0005$). In regard to % OEE, no differences were found between groups for summer assays ($H = 2.14$, $p < 0.3424$) and in autumn assay RR was lower than the others two groups ($H = 9.42$, $p < 0.009$).

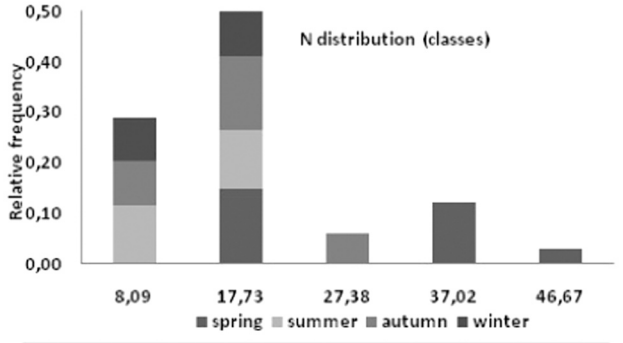
The RR-exposed groups showed significant increased in N in summer assay and Cd-exposed groups it was significantly higher in both assays, compared to their control ($H = 11.247$, $p < 0.0028$ and $H = 11.24$, $p < 0.0036$ for summer and autumn respectively). In the Cd-exposed groups, AQ showed no significant differences, while in the RR-exposed



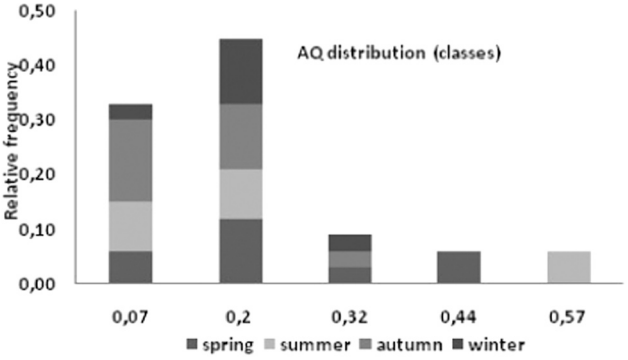

| Parameter | Class | LL | UL | AC | AF | RF | $Chi^2$ | p |
|---|---|---|---|---|---|---|---|---|
| OEE (44) | 1 | 1,25 | 4,17 | 2,71 | 14 | 0,32 | 0,17 | 0,65 |
| | 2 | 4,17 | 7,09 | 5,63 | 12 | 0,27 | 0,2 | |
| | 3 | 7,09 | 10,02 | 8,56 | 10 | 0,23 | 0,31 | |
| | 4 | 10,02 | 12,94 | 11,48 | 5 | 0,11 | 0,39 | |
| | 5 | 12,94 | 15,86 | 14,4 | 3 | 0,07 | 0,85 | |



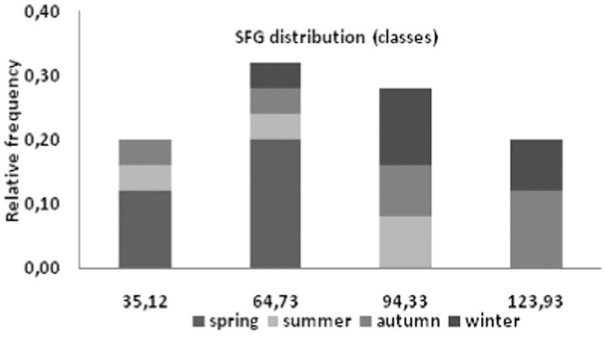

| Parameter | Class | LL | UL | AC | AF | RF | $Chi^2$ | p |
|---|---|---|---|---|---|---|---|---|
| SMR (35) | 1 | 32,58 | 69,26 | 50,92 | 5 | 0,14 | 0,27 | 0,21 |
| | 2 | 69,26 | 105,94 | 87,6 | 13 | 0,37 | 1,16 | |
| | 3 | 105,94 | 142,61 | 124,28 | 11 | 0,31 | 1,16 | |
| | 4 | 142,61 | 179,29 | 160,95 | 3 | 0,09 | 2,64 | |
| | 5 | 179,29 | 215,97 | 197,63 | 3 | 0,09 | 3,12 | |




| Parameter | Class | LL | UL | AC | AF | RF | $Chi^2$ | p |
|---|---|---|---|---|---|---|---|---|
| N (34) | 1 | 3,27 | 12,91 | 8,09 | 10 | 0,29 | 0,24 | 0,05 |
| | 2 | 12,91 | 22,56 | 17,73 | 17 | 0,5 | 2,38 | |
| | 3 | 22,56 | 32,2 | 27,38 | 2 | 0,06 | 5,4 | |
| | 4 | 32,2 | 41,84 | 37,02 | 4 | 0,12 | 6,12 | |
| | 5 | 41,84 | 51,49 | 46,67 | 1 | 0,03 | 6,23 | |




| Parameter | Class | LL | UL | AC | AF | RF | $Chi^2$ | p |
|---|---|---|---|---|---|---|---|---|
| QA (33) | 1 | 0,01 | 0,13 | 0,07 | 11 | 0,33 | 0,23 | 0,22 |
| | 2 | 0,13 | 0,26 | 0,2 | 15 | 0,45 | 1,58 | |
| | 3 | 0,26 | 0,38 | 0,32 | 3 | 0,09 | 2,71 | |
| | 4 | 0,38 | 0,51 | 0,44 | 2 | 0,06 | 2,75 | |
| | 5 | 0,51 | 0,63 | 0,57 | 2 | 0,06 | 3,03 | |




| Parameter | Class | LL | UL | AC | AF | RF | $Chi^2$ | p |
|---|---|---|---|---|---|---|---|---|
| SFG (25) | 1 | 20,32 | 49,93 | 35,12 | 5 | 0,2 | 0,01 | 0,61 |
| | 2 | 49,93 | 79,53 | 64,73 | 8 | 0,32 | 0,02 | |
| | 3 | 79,53 | 109,13 | 94,33 | 7 | 0,28 | 0,11 | |
| | 4 | 109,13 | 138,73 | 123,93 | 5 | 0,2 | 0,26 | |

**Fig. 2.** Frequency analysis for oxygen extraction efficiency (OEE), specific metabolic rate (SMR), ammonium excretion (N), ammonia quotient (AQ), and scope for growth (SFG) for the MHW controls of the four bioassays. For each class interval of each parameter, the lower limit (LL), the upper limit (UL), the average per class (AC), the absolute frequency (AF) and relative frequency (RF) are given according to the best-fit distribution model (number of measurements in brackets). Statistical estimator $Chi^2$ ($p \leq 0.05$).

**Table 4**
Range of expected values for the studied energetic biomarkers in laboratory-reared adults of *Cnesterodon decemmaculatus* exposed to MHW control medium during 12 days.

| Biomarker | Range of expected values | Seasonal behavior |
|---|---|---|
| In | 141–370 J $g_{ww}^{-1}$ $d^{-1}$ | – |
| F | 6.13–90.34 J $g_{ww}^{-1}$ $d^{-1}$ | – |
| A | 173.71–321.20 J $g_{ww}^{-1}$ $d^{-1}$ | higher values in autumn |
| OEE | 1.25–10.02% | lower values in spring |
| SMR | 70–140 J $g_{ww}^{-1}$ $d^{-1}$ | – |
| N | 3.27–22.56 J $g_{ww}^{-1}$ $d^{-1}$ | – |
| AQ | 0.01–0.26 | – |
| SFG | 20.32–138.73 J $g^{-1}$ $d^{-1}$ | higher values in winter and autumn |

In: food intake, F: fecal production, A: assimilation, SMR: specific metabolic rate, OEE: oxygen extraction efficiency, N: ammonia excretion, AQ: ammonium quotient, SFG: scope for growth.

groups it was significantly higher in the autumn assay in comparison to their control ($H = 5.24$, $p < 0.0721$ and $H = 6.88$, $p < 0.0315$ for summer and autumn respectively). Finally, the SFG had significant negative values in the Cd-exposed groups in both assays, while it did not differ significantly in the RR-exposed groups, compared to their controls ($H = 6.62$, $p < 0.0242$ and $H = 13.62$, $p < 0.0011$ for summer and autumn respectively).

According to the mixed model, the variables In, F and A differed both between treatments and between seasons (Table 7). In the case of F, it did not seem to differ significantly between seasons, but it showed a significant difference when the interaction season and treatment was considered, supporting the idea that season plays a considerable role in these variables.

Treatment had a significant effect on the variables measured at final time. On the contrary, season was only related to SMR and AQ, but the interaction between season and treatment was found to be related to all the variables.

## 4. Discussion

### 4.1. Baseline bioenergetic parameters from MHW-exposed *C. decemmaculatus* under experimental conditions

The rearing conditions of the animals used ensure that the cohorts used in the experiments are contaminant-free and that their genetic variability is lower than that in natural populations. Although under these rearing conditions the different endpoints are expected to be constant throughout the year, some bioenergetic parameters fluctuated seasonally. These variations, which may be genetically determined and intrinsic to the species, would affect the sensitivity of test animals to toxicants. Several studies have reported seasonal variation of enzyme activities in fishes. For example, a circannual pattern was observed for AChE activity in *C. decemmaculatus* (Bernal-Rey et al., 2020; Menéndez-Helman et al., 2015), for GST and CAT activity in *Boreogadus saida* (Nahrgang al, 2010) and for vitellogenin production in *Gasterosteus aculeatus* (Sanchez et al., 2008). Likewise, a study with *Pomatoschistus microps* collected along the Portuguese northwestern coast from five sites with different levels of contamination also found a significant seasonality effect on all the enzymatic activities (i.e. AChE, LDH, GST and EROD) analyzed (Monteiro et al., 2007). These findings underline the need to deepen our knowledge of the seasonal variability of biomarkers frequently used in biomonitoring, as well as their intrinsic variability in model species. This is the first study focused on baseline bioenergetic parameters for *C. decemmaculatus* under experimental conditions considering the four seasons.

Despite the difficulty of finding significant differences in some of the bioenergetic parameters due to their high considerable variability (Table 3), the frequency analysis allowed us to confirm or rule out the presence of a seasonal pattern. Food intake and Fecal production remained stable over seasons, while Assimilation increased in autumn and decreased in spring and summer (Fig. 1), suggesting a tendency to the accumulation of energetic resources during autumn, prior to food

**Table 5**
Bioenergetic parameters (J $g_{ww}^{-1}$ $day^{-1}$) measured daily and cumulative mortality at final time (%) for adults of *C. decemmaculatus* exposed to control medium (MHW), cadmium (Cd), and Reconquista River waters (RR) in summer and autumn assays.

| | | Parameters | | | |
|---|---|---|---|---|---|
| Bioassay | Treatment | Food Intake (In) | Fecal Production (F) | Specific assimilation (A) | Cumulative mortality (%) |
| Summer | MHW | 205.68 ± 5.14 (56)$^{a}$ | 42.82 ± 2.43 (56)$^{a}$ | 162.86 ± 5.64 (56)$^{a}$ | 9.47 ± 3.93 (10)$^{a}$ |
| | Cd | 140.25 ± 7.27 (28)$^{b}$ | 35.92 ± 3.60 (28)$^{a}$ | 104.33 ± 7.94 (28)$^{b}$ | 35.73 ± 4.14 (9)$^{b}$ |
| | RR | 184.29 ± 7.14 (29)$^{a}$ | 82.91 ± 3.54 (29)$^{b}$ | 101.38 ± 7.80(29)$^{bc}$ | 17.31 ± 4.39 (8)$^{a}$ |
| Autumn | MHW | 280.64 ± 6.00 (61)$^{a}$ | 40.10 ± 2.27 (61)$^{a}$ | 240.54 ± 6.48 (61)$^{a}$ | 7.56 ± 3.09 (13)$^{a}$ |
| | Cd | 167.87 ± 7.76 (28)$^{b}$ | 22.80 ± 3.42 (28)$^{b}$ | 145.08 ± 8.72 (28)$^{b}$ | 59.56 ± 4.98 (5)$^{b}$ |
| | RR | 319.78 ± 7.25 (32)$^{c}$ | 105.90 ± 3.20(32)$^{c}$ | 213.88 ± 8.16 (32)$^{c}$ | 4.03 ± 3.94 (8)$^{a}$ |

Data expressed as mean ± SEM; number of determinations in parenthesis. Different letters indicate significant differences between groups ($p \leq 0.05$).

**Table 6**
Bioenergetic parameters at final exposure time in adults of *C. decemmaculatus* exposed to control medium (MHW), cadmium (Cd) and Reconquista River water (RR) in summer and autumn assays.

| | | **Parameters** | | | | |
|---|---|---|---|---|---|---|
| **Bioassay** | **Treatment** | **SMR** | **EEO%** | **N** | **AQ** | **SFG** |
| **Summer** | MHW | 90.82 ± 20.64 (8)$^{a}$ | 6.38 ± 1.28(10)$^{a}$ | 13.12 ± 3.35 (8)$^{a}$ | 0.21 ± 0.19 (7)$^{a}$ | 72.04 ± 20.30 (4)$^{a}$ |
| | Cd | 117.12 ± 23.83 (6)$^{a}$ | 7.62 ± 1.65 (6)$^{a}$ | 24.47 ± 4.74 (4)$^{ab}$ | 0.64 ± 0.25 (4)$^{a}$ | −29,57 ± 20.30 (4)$^{b}$ |
| | RR | 40.42 ± 29.19 (4)$^{a}$ | 3.57 ± 2.03 (4)$^{a}$ | 62.90 ± 4.74 (4)$^{b}$ | 1.06 ± 0.25 (4)$^{a}$ | 16.85 ± 20.30 (4)$^{ab}$ |
| **Autumn** | MHW | 147.79 ± 35.34 (9)$^{a}$ | 7.50 ± 0.58(11)$^{a}$ | 16.01 ± 4.61 (10)$^{a}$ | 0.13 ± 0.04 (10)$^{a}$ | 93.66 ± 41.90 (7)$^{a}$ |
| | Cd | 392.9 ± 43.29 (6)$^{c}$ | 9.06 ± 0.78 (6)$^{a}$ | 53.66 ± 5.96 (6)$^{b}$ | 0.18 ± 0.05 (6)$^{a}$ | −301.48 ± 45.26 (6)$^{b}$ |
| | RR | 67.61 ± 43.29 (6)$^{b}$ | 4.71 ± 0.78 (6)$^{b}$ | 16.76 ± 5.96 (6)$^{a}$ | 0.29 ± 0.05 (6)$^{b}$ | 129.51 ± 45.26 (6)$^{a}$ |

Data expressed as mean ± SEM; number of determinations in parenthesis. Different letters indicate significant differences between treatment groups ($p \leq 0.05$).
SMR: specific metabolic rate.
EEO%: oxygen extraction efficiency.
N: ammonia excretion.
AQ: ammonia quotient.
SFG: Scope for Growth.

**Table 7**
Results of the mixed lineal model with fixed effects for *Cnesterodon decemmaculatus* exposed to moderate hard water (MHW), Cadmium (Cd) and Reconquista river (RR) water in summer and autumn.

| Parameters | | Treatment | | Season | | Treatment*Season | |
|---|---|---|---|---|---|---|---|
| | df | F | p | F | p | F | p |
| **In** | 196 | 144,09 | < 0.001 | 265,27 | < 0.001 | 23,12 | < 0.001 |
| **Feces** | 199 | 249,59 | < 0.001 | 0,44 | 0508 | 22,06 | < 0.001 |
| **A** | 190 | 82,24 | < 0.001 | 209,05 | < 0.001 | 5,35 | 0005 |
| **SMR** | 33 | 15,36 | < 0.001 | 17,11 | < 0.001 | 7,18 | 0003 |
| **EEO** | 37 | 5,05 | < 0.001 | 1,52 | 0225 | 0011 | 0,99 |
| **N** | 32 | 17,55 | < 0.001 | 1,15 | 0293 | 21,82 | < 0.001 |
| **AQ** | 31 | 7,37 | < 0.001 | 14,94 | < 0.001 | 3,41 | 0046 |
| **SFG** | 25 | 22,713 | < 0.001 | 1,83 | 0189 | 11,499 | < 0.001 |

df: degrees of freedom.
F: statistical.
Treatment*Season: interactions between treatment and season.
In: food intake, A: specific assimilation, SMR: specific metabolic rate, OEE: oxygen extraction efficiency, N: ammonia excretion, AQ: ammonium quotient, SFG: scope for growth.

scarcity in winter under natural conditions. Thus, assimilation capacity appears to have retained its "natural behavior" even under stable culture conditions, probably attributable to reproductive behavior and food availability in the wild. Feeding behavior is known to be influenced by both exogenous (e.g., time of day, seasonality and environmental conditions) and endogenous (e.g., involving the endocrine and sensory systems) factors (Hoskins and Volkoff, 2012; Volkoff et al., 2005; Volkoff and Wyatt, 2009).

In regard to the bioenergetic parameters determined at the end of the experiment (Fig. 2), only OEE and SFG showed seasonal variations. The decrease in OEE in the spring and summer assays suggests a lower effectiveness during the warmer months, while SFG increased in winter and autumn. Although SFG is not a specific biomarker, it integrates different bioenergetic variables thus providing a measure of the metabolic status of an animal and relevant information at population level. Recently, SFG proved to be useful in estimating the effect of receiving waters of the Reconquista River and Cd on *C. decemmaculatus* adults (Baudou et al., 2017, 2019) and the effect of Cd on *Cyprinus carpio* (Ferrari et al., 2011). In this study, SFG showed a seasonal pattern under toxicant-free conditions, with higher values in the colder months (during the reproductive-arrest period), as would be expected in the wild when food is not a limiting factor. Vega et al. (2004), who studied the Assimilation efficiency of *Chirostoma estor estor* fed on different diets, reported that assimilated energy is physiologically useful energy, which is channeled into metabolism maintenance, growth and reproduction. In the present study, the existence of differential seasonal responses could be established by SFG, even when the variables included in its equation could not.

Biomarker baselines, which are obtained from the animals in the control group, are useful reference values for comparison with the responses from animals under stress by exposure to toxicants. To achieve this aim, it is necessary to determine their range of values and possible seasonal variations, even under controlled laboratory conditions.

The marked seasonal variation shown by for A, SFG and OEE indicates the advantage of including parallel controls of MHW in ecotoxicological bioassays until establishing seasonal baselines for the proposed endpoints of effect.

### 4.2. Exposure bioassays

Except for the metals and metalloids, the values of the physicochemical parameters and water quality obtained from the environmental samples (Table 2) are in agreement with those reported in previous studies conducted in the Reconquista River, at sites close to our sampling site (Baudou et al., 2019; Ossana, 2011; Ossana et al., 2016, 2019; Rigacci et al., 2013). According to WQI results, both samples of receiving water can be classified as slightly polluted.

The cumulative mortality recorded for the different exposure media in the summer and autumn assays (Table 5), validates the use of moderately hard water (MHW) as an appropriate control medium for the protocol applied here.

In the Cd-treated groups, the mean cumulative mortality (about 50%) fell in the range expected for the Cd concentration and experimental design used here (Baudou et al., 2017). This supports the notion that *C. decemmaculatus* adults are more sensitive than *C. carpio* juveniles exposed to comparable experimental conditions, with a mean lethality of 1.6 mg Cd $L^{-1}$ after 12 d-exposure (de la Torre et al., 2000). In the present study, the cumulative mortality in the groups exposed to the environmental samples at final time is similar to that in their control groups. This result was expected considering that the test species is found from the sampling site in the headwaters of the river to 24 km downstream of it. In regard to the bioenergetic parameters recorded daily (Table 5), the decreased food intake in the Cd-treated groups may have indirectly led to a lower Assimilation efficiency, there by diminishing the available energy resources. Similar responses were observed for juveniles of *C. carpio* exposed to 0.15 mg Cd $L^{-1}$ (Ferrari et al., 2011), and for *C. decemmaculatus* exposed to concentrations ranging between 0.45 and 2.5 mg Cd $L^{-1}$ (Baudou et al., 2017). The negative impact of Cd on food intake and Assimilation efficiency has also been documented for *Silurus meridionalis* exposed to concentrations between 0.06 and 0.5 mg Cd $L^{-1}$ during 8 weeks (Li and Xie, 2019) and *Perca flavescens* from a lake polluted with Cd and other metals and metalloids (Couture and Rajotte, 2003). Likewise, the decreased Assimilation efficiency in the fish group exposed to the receiving water samples of the Reconquista River may have resulted by the presence of metals. Some metals (Cd, Hg, Zn, Cr y Cu) and pesticides dissolved in water are neurotoxic and inhibit sensory receptors in fish, thus affecting the olfactory, visual and lateral line systems and behavior (Baker and Montgomery, 2001; Strungaru et al., 2019; Tierney et al., 2010). In particular, a behavioral alteration of increased aggression has been described for *C. decemaculatus* exposed to cadmium, chlorpyrifos and glyphosate (Ferro et al., 2019; Bonifacio et al., 2020).

In regard to the bioenergetic parameters recorded at final exposure time (Table 6), the SMR was not affected in the groups exposed to receiving waters of the Reconquista River, whereas it significantly increased in the Cd-exposed groups. This response of increased SMR, which was also observed in *C. decemmaculatus* adults (Baudou et al., 2017) and *C. carpio* juveniles (Ferrari et al., 2011) exposed to Cd, may result from a higher energetic demand due to toxic stress.

The capacity of fishes to extract oxygen from water under stress conditions depends on the hemoglobin affinity for oxygen, the amount of water passing through the gills, and alterations in blood flow and gill morphology (Zhou et al., 2001). Ossana et al. (2019) demonstrated that *C. decemmaculatus* developed structural damage to the gill epithelium (e.

g., loose cell junctions, aneurysms in secondary lamellae and increased number of chloride and mucous cells) after a short-term exposure to waters of the Roggero Dam in the Reconquista river. Cd has a similar, though more severe, effect on fish gills (Ferrari et al., 2009; Thophon et al., 2003). Comparable effects on gill morphology were found in common carp exposed to copper nanoparticles (Vajargah et al., 2014). Respiration and ammonium excretion are expected to be affected in fish under toxic stress because gills are the site of both processes. Gill damage including aneurysms, lamellar fusion and hypertrophy of epithelial cells, among others, cause functional alterations that are used as histopathological biomarkers in studies of environmental evaluation mainly focused on metals and metalloids (Torres et al., 2010).

Measurements of ammonia excretion (N) are useful to assess nitrogen balance; N represents an important tool to evaluate the influence of the environment and feeding on protein metabolism (Fournier et al., 2003; Uliano et al., 2010). In fish, AQ, which is used to assess the contribution of proteins to total energy budget, estimates the proportion of proteins involved in respiration (De Boeck et al., 1995; Owen et al., 1998). Thus, AQ represents a reliable index of protein catabolism. In our study, the groups exposed to Cd showed no differences in AQ compared to the control, while AQ and N increased in the groups exposed to the receiving waters, suggesting high catalysis of proteins related to energy deficiency. Variations in N may not only be associated with protein metabolism but also with damage to the gill epithelium (Ossana et al., 2019). An increase in protein catabolism most likely helps adjust homeostasis to new environmental conditions.

Even in phylogenetically distant animals, the fact that the effects of different toxicants may converge in relatively similar responses (e.g., nervous system, behavioral, histopathological and biochemical alterations), has been frequently documented in the literature (Bonifacio et al., 2020; Da Cuña et al., 2020; Muñoz-Peñuela et al., 2021; Petrovici et al., 2020; Aliko et al., 2015; Vidal et al., 2018; Zambrano et al., 2018; among others). The adaptive response of animals to stress, particularly induced by toxicity, is a multifactorial and dynamic process, making the interpretation of endpoints more difficult. In this context, the integrative indexes have become useful tools, such as SFG, which has been adopted as an end point of toxicity in fish (Alcaraz and Espina, 1997; Baudou et al., 2017, 2019; Ferrari et al., 2011). In this study, SFG showed a seasonal fluctuation regardless of the stable experimental conditions, suggesting that SFG response is species-specific and probably influenced by the reproductive cycle. The Cd-exposed group showed a marked decrease in SFG in autumn, compared to the MHW parallel control (Table 6), thus indicating energy disbalance between energy gain from food intake and energy loss, influenced by seasonality. The SFG is a sensitive biomarker for detecting Cd exposure in different groups of animals, such as marine gastropods (*Nassarius festivus*) and teleosts (Baudou et al., 2017; Ferrari et al., 2011; Wo et al., 1999). The SFG of the groups exposed to receiving waters showed a non-significant decreasing trend and a large variability between seasons, which was possibly due to differences in their physicochemical profile. Finally, this study emphasizes the role of seasonality in the responses of all the biomarkers studied, including those for which there was a significant interaction effect between season and treatment.

This work attempts to provide useful information to be included in the elaboration of protocolized ecotoxicity tests for the evaluation of early effects, both for individual toxins or environmental samples, with *C. decemaculatus* as experimental model.

## 5. Conclusions

In brief, the appropriate use of biological responses as biomarkers for a given species should be based on knowledge of their behavior and variability under natural and experimental conditions, considering seasonal metabolic changes, reproductive cycles and nutritional status, among other variables. In addition, the selection of biomarkers for ecotoxicity assessment requires a methodology dealing with the homeostatic maintenance of the organism.

Under the experimental conditions of our study, the results obtained for *C. decemmaculatus* adults exposed to MHW as control medium allowed us to establish a first baseline for different energetic biomarkers. Some of them (A, OEE and SFG) evidenced seasonal variability, indicating the advantage of using parallel controls for comparison and of establishing seasonal baselines for improving the accuracy of effects analysis. The endpoint values of the control groups must be within the acceptable range established by their respective baselines.

The SFG is a practical index for use in ecotoxicological laboratory assays with *Cnesterodon decemmaculatus*.

Our study provides a first baseline for useful biological variables under controlled laboratory conditions, as well as important information on *C. decemmaculatus* as model species in ecotoxicological bioassays involving biomarkers of early effect. The obtained results provide useful information on *C. decemmaculatus* as model species in ecotoxicological bioassays involving biomarkers of early effect.

## CRediT authorship contribution statement

**Federico Gastón Baudou**: He's mainly carried out all the laboratory work and the determinations of the energetic biomarkers. **Bettina Lorena Eissa**: She's collaborated in the determinations of the energetic biomarkers and in the writing of the manuscript. **Natalia Alejandra Ossana**: She's collaborated in the writing of the manuscript and in the determinations of the physicochemical parameters in the laboratory and in the field and maintenance of the animal culture of *Cnesterodon decemmaculatus*. **Martina Maria Mastrángelo**: She's carried out the determinations of heavy metals and physicochemical parameters specially ammonia determinations. **Juan Pablo Ferro**: He's carried out the statistical analysis and collaborated in the maintenance of the experimental conditions. **Liria Belén Campos:** She's collaborate in the determinations of the biomarkers and in the maintenance of the experimental conditions. **Lucrecia Ferrari**: She's directed this project of research and wrote the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111639.

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