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Original research article

The adaptation of rainbow trout to warmer water: Oxidative damage in the germinal line

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ABSTRACT

Contemporary evolution was observed in a feral rainbow trout (*Oncorhynchus mykiss*) population of a thermal stream (Valcheta) in Northern Patagonia, in terms of juvenile thermal tolerance and preferred temperature. Other authors showed that high-temperature treatment applied to male rainbow trout juveniles increased the thermal tolerance in the next generation. This implies a high mutation rate and/or a modified epigenetic inheritance. Comparisons were made among a) a rainbow trout strain adapted in terms of upper thermal tolerance and higher preferred temperature (Valcheta stream), b) a wild temperate stream population (Guillelmo stream), and c) two temperate farmed strains. We examined: Oxidative damage (lipid peroxidation) and activities of antioxidant enzymes; Catalase (CAT), Glutathione S-Transferases (GST), and Superoxide Dismutase (SOD), in liver, testicle, and spermatozoa. Semen fatty acid composition, sperm morphology, sperm motility, and fertilization performance in samples before and after the application of cryopreservation procedures were also evaluated. The observed responses, mainly related to the sperm membrane, reinforces the idea that ROS can affect the germinal line of male rainbow trout juveniles subjected to high water temperature. Our results suggest that the acquired thermal tolerance traits may be part of a wide spectrum of novel characteristics produced as a consequence of an enhanced mutation rate and/or a different DNA methylation pattern, induced by higher temperatures and mediated by ROS.

1. Introduction

Recent studies have pointed out the contemporary evolution of a rainbow trout (*Oncorhynchus mykiss*) feral population in a thermal stream (Valcheta) on Somuncura plateau, Northern Patagonia (Menni & Gómez, 1995), in terms of juvenile upper thermal tolerance and preferred temperature (Crichigno et al., 2018). The authors showed that juveniles of a single pooled F1 stock (Valcheta thermal-resistant male x farmed female), reared under standard hatchery conditions and selected for growth and thermal preference, presented higher thermal preference and higher thermal tolerance than the stock-farmed in temperate water (Crichigno & Cussac, 2019). As could be expected due to the late zygotic genome activation (Figueroa et al., 2018; Lindeman & Pelegri, 2010), paternal genome shows little effect on F1 embryonic performances (Crichigno et al., 2021). However, Butzge et al. (2021) showed that high temperature treatment applied to male rainbow trout juveniles increased the thermal tolerance in the next generation. This direct effect on the germinal line of a poikilotherm could be the basis of rainbow trout adaptation in the thermal Valcheta stream and in the experimental production of thermal resistant rainbow trout strains selected by high-temperature breeding (Ineno et al., 2005). Several biochemical differences were observed between normal and thermally selected strains (Ikeguchi et al., 2006; Itoi et al., 2001). Moreover, thermally selected strains contained higher levels of mRNAs encoding heat shock proteins (HSPs) compared to the normal strains (Ineno et al., 2019; Ojima et al., 2012; Tang et al., 2012). These differences imply mutation

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and selection, within a few decades, i.e. contemporary evolution (Stockwell et al., 2003). In addition, these differences could be the consequence of the modulation of DNA methylation or other epigenetic mechanisms, by the thermal regime (Koch et al., 2022; Venney et al., 2022, 2023), mediated by ROS (Zhang et al., 2022).

There is scarce information regarding the factors that originate the high mutation rate in rainbow trout populations. Reactive oxygen species (ROS) are one of the most frequent sources of mutation. The ROS produced by normal metabolism can oxidize and damage cell membranes, proteins, and nucleic acids; and DNA damages that are not repaired during cell proliferation lead to mutations (Chowdhury & Saikia, 2020; Mugoni et al., 2014; Nilsson & Liu, 2020; Tripathy, 2016). Fish sperm is particularly prone to oxidative damage due to the high content of polyunsaturated fatty acids (PUFA) present in their membranes. Oxidative stress has been shown to reduce several spermatozoa functions, impairing sperm motility, cell viability and functionality, and DNA integrity (Félix, Oliveira, & Cabrita, 2021). Temperature outside the optimal range can enhance generation of ROS and oxidative stress in sperm. Catalase (CAT), Superoxide Dismutase (SOD), and Glutathione S-Transferase (GST) are key components of the antioxidant defense system and play a fundamental role in detoxification of ROS to prevent oxidative damage (Lushchak, 2011; Birnie-Gauvin et al., 2017; Ighodaro & Akinloye, 2018). However, limited knowledge is available about the response of sperm antioxidant enzymes to thermally induced oxidative stress (Dadras et al., 2019).

Sperm activation begins in response to external signals that act at the level of its plasma membrane (Dzyuba et al., 2014). Membrane properties are determined, among other factors, by the degree of saturation of fatty acids (FA) (Engel et al., 2019). Quantities of saturated and unsaturated FA in rainbow trout seminal plasma and spermatozoa vary due to differences in strain, diet, or water temperature (Lahnsteiner et al., 2009; Vassallo-Agius et al., 2001). In the same way, the ability of rainbow trout spermatozoa to withstand cryopreservation showed a significant correlation with the previous acclimation of males (Labbé & Maisse, 1996).

Since the contemporary evolution of rainbow trout is highly valuable for aquaculture and recreation fisheries; a) the rainbow trout thermal-resistant population of Valcheta stream, b) a wild temperate population (Guillelmo stream), and c) two temperate farmed strains (CENSALBA and CEAN) were considered in this work. Oxidative damage (lipid peroxidation) and activities of CAT, GST, and SOD were measured in liver, testicle, and spermatozoa. FA composition of semen, sperm morphology, sperm motility, and fertilization performance in fresh and following the application of cryopreservation procedures were also analyzed. The effects of temperature and oxidative damage on membrane lipids in the germinal line are important evidence towards understanding the process of contemporary evolution in rainbow trout.

2. Materials and methods

2.1. Strains

Four rainbow trout strains were used; a) a wild population adapted to high temperature in Valcheta stream, in the Argentine Patagonia (n = 112, 40°57′41″ S, 66°38′21″ W, 489 m a.s.l., Conductivity = 450 μS. cm^{-1}, Mean Annual Air Temperature (MAAT) 1981–2010 = 14–16 °C, https://www.smn.gob.ar/clima/atlasclimatico) (Crichigno & Cussac, 2019; Crichigno et al., 2018), b) a wild temperate water population in a stream inlet to Guillelmo lake (n = 88, 41°21′43″ S, 71°29′44″ W, 889 m a.s.l., Conductivity = 64 μS. cm^{-1}, MAAT 1981–2010 = 8–10 °C), c) an autumn spawning strain farmed in the Centro de Ecología Aplicada del Neuquen (CEAN) (n = 100, 39°54′59″ S, 71°06′21″ W, 810 m a.s.l., MAAT 1981–2010 = 10–12 °C, https://www.cean.gob.ar/) and d) an autumn spawning strain farmed in the Centro de Salmonicultura Bariloche (CENSALBA) of the Universidad Nacional del Comahue (n = 22, 41°07′37″ S, 71°25′14″ W, 800 m a.s.l., MAAT 1981–2010 = 8–10 °C).

All experiments complied with the Guia para cuidado y uso de animales de experimentación guidelines (INTA, https://inta.gob.ar/sites/default/files/script-tmp-inta-_gua_cuidado_y_uso_de_animales.pdf) and the Protocolo N°2020-024 of the CICUAL-INIBIOMA (https://inibioma.conicet.gov.ar/comite-institucional-para-el-cuidado-y-uso-de-animales-de-laboratorio-cicual/). Capture permits were provided by Administración de Parques Nacionales, Argentina (DRPN- 1652- SEVASTEI, proyect N°1652) (https://www.argentina.gob.ar/parquesnacionales) and Secretaría de Ambiente y Cambio Climático, Provincia de Río Negro, Argentina (Res. N°684/SAyCC/2021) (https://ambiente.rionegro.gov.ar/).

2.2. Enzymatic activities and oxidative damage

Individuals from Valcheta and Guillelmo streams were captured by electro-fishing. Individuals from the Valcheta stream (n = 98), Guillelmo stream (n = 88), and CENSALBA stock (n = 13) were euthanized with an excess (0.1 g. L^{-1}) of Benzocaine and the dissected. Also, a group (n = 100) of CEAN juvenile individuals (total weight 1.9–5.4 g) were maintained for more than 30 days in conditions of temperature and conductivity resembling Guillelmo stream (low Temperature, 6 °C and low Conductivity, 64 μS. cm^{-1}) and Valcheta stream (high Temperature, 21 °C and high Conductivity, 450 μS. cm^{-1}). Later, they were euthanized with excess Benzocaine, and then dissected. Levels of CAT, GST, and SOD, and lipid peroxidation were measured in cytosolic fraction (CF) of liver and testicle. Regarding spermatozoa, 400 μL of semen was centrifuged and the pellet mass was used as reference.

The CAT activity was determined with the method of Aebi (1983) by quantification of the hydrogen peroxide degradation, measuring the absorbance at 240 nm. Briefly, a final volume 50 μL of CF were added to a final volume of 2.95 mL of a solution of 100 μL of H_2O_2 in 50 mL of 50 mM buffer PiK, and the absorbance was read every 10 s during 1 min. The activity of CAT (UCAT) was expressed as, (Chance & Maehly, 1955).

UCAT (μmol/ (min·mg·protein)) = Slope. V_{final} / (ε. OP. V_{sample}. [mg of protein/mL]), with ε = 39.4/(mol/L·cm), OP = 1 cm, and Slope = min^{-1}

The activity of GST (UGST) was quantified measuring the rate of 2,4-Dinitrophenyl-S-Glutathione (DNP-SG) synthesis as the conjugation product between 1-cloro-2,4 Dinitrobenzene (CDNB) and reduced Glutathione (GSH) (Habig et al., 1974). The absorbance was read at 340 nm every 10 s during 2 min in 100 mM phosphate buffer, pH 6.5, with 100 mM GSH and 100 mM CDNB as substrates. Activity was expressed as,

UGST (μmol/min)/mg protein = [Slope. Cuvette volume] / sample volume.ε . OP. (mg protein/ mL)

Slope = absorbance/min
ε = coefficient extinction of DNP-SG = 9.6 mmol/L^{-1}·cm^{-1}
OP = 0.55 cm.

The SOD (USOD) activity was determined as the inhibition of Epinephrine self oxidation at alkaline pH (Misra & Fridovich, 1972). Briefly, 3 μL, 5 μL y 10 μL of cytosolic fraction were added into 50 mmol/L Glycine buffer (pH = 10.2 to 10.5, 25–30 °C), final volume 2.90 mL, with 0.9 mM of Epinephrine as substrate. The adrenochrome formation was measured at 480 nm each 10 s during 1 min in absence and presence of cytosolic fraction. The activity of SOD was calculated as,

USOD(mg protein)$^{-1}$ = ½ (slope in absence) / (mg of protein that reduces to 50% the speed of adrenochrome formation)

Enzymatic activity results were referring to the total protein content of the CF. It was quantified by the method of Bradford (1976), by incubation of 10 μL of CF into 200 μL of Bradford reagent, final volume. A calibration curve of bovine serum albumin (BSA) was performed. The absorbance was read at 465 nm and at 595 nm, and a 595 nm/465 nm ratio between absorbance from both, sample and BSA curve, was

calculated in order to linearize the calibration BSA curve and improve the accuracy of the original method (Ernst & Zor, 2010). Protein concentration was calculated as,

$$[protein] = (abs_{sample\ at\ 595\ nm} / abs_{sample\ at\ 465\ nm}) \cdot (\text{y-intercept BSA curve}_{595\ nm/465\ nm} / \text{slope BSA curve}_{595\ nm/465\ nm})$$

Lipid peroxidation was assessed by means of detection of substances (TBARS) reactive to Thiobarbituric acid (TBA) in 1 mg of tissue (Hermes-Lima et al., 1995). Tissue homogenate (400 μL) was incubated with 200 μL of 7% Phosphoric acid and 400 μL of 1% TBA in 50 mM NaOH, 0.1 mM Butylated Hydroxytoluene (BHT), at 90 °C during 30 min. Then, 1.5 mL of Butanol was added, and the solution centrifuged (3000 rpm) during 10 min. Supernatant was measured at 535 nm and the concentration of TBARS was calculated as,

$$TBARS(\text{nmol. g of tissue})^{-1} = 10^6 \cdot \text{Absorbance} / (\mathcal{E} \cdot P \cdot \text{tissue mass}), \text{ with}$$

$\mathcal{E}$ (extinction coefficient) = 1.55. 10^5/(mol. cm), and.
P (path length) = 1 cm.

2.3. Fatty acid composition of semen

Semen was obtained from 3- and 4-year-old rainbow trout males (n = 8); four ripe males from Valcheta stream and four ripe males from CENSALBA (Table 1). All males were acclimated at the same temperature in CENSALBA, and fed with the same commercial food from the juvenile period onward. The fish were anesthetized with 0.1% Benzocaine, and the semen was collected individually by stripping in 15 mL plastic tubes, with special care taken to prevent water, blood, urine, or feces contamination. Semen samples were selected for preliminary visual quality characteristics (low transparency and high cell density). The semen was immediately kept in a refrigerator (4 °C) in light absence condition and stored at −20 °C without dilution up to lipid analyses.

Semen samples (1.5 mL) were processed as follows: semen lipids were extracted using Folch methods with modifications (Folch et al., 1957). Briefly, 6 mL of 2:1 Chloroform-Methanol (v/v) mixture containing 50 ppm BHT were added to each tube containing semen, and homogenized for 30 min with a vortex (Thermo ®). Then 0.2 vol of 0.9% NaCl were added. After centrifugation at 1500*g*, the lower organic phase was transferred to a clean tube and dried under N_2 current. Lipids were derivatized to methyl esters after incubation in 2 mL of anhydrous Methanol and 0.5 mL H_2SO_4 (98% m/m) at 80 °C for 1 h. FA methyl esters (FAMEs) were extracted twice adding 1.5 mL of 5% NaCl and 3.5 mL of Hexane. Hexane was dried in a vial by N_2 stream. FAMEs were analyzed in an Agilent 7890B Gas Chromatograph coupled to a Mass Spectrometer (Agilent 5977A) in a Zebron ZB-WAX column (30 m × 0.25 mm × 0.25 μm). Operating conditions included: Injector 240 °C; column temperature 180 °C 30 min, 5 °C/min up to 240 °C, 240 °C 5 min; total run time: 47 min; MS full SCAN 50–550; injection volume 1 μL (1:50 split); heaters 240 °C. The retention times and mass spectra of each peak were compared with standards (Supelco 37 Component FAME Mix, Sigma ®) and with those available in database NBS75K (National Bureau of Standards) for identification and quantification of the major FA.

Table 1
Semen samples used for lipid analyses, fresh fertilization and cryopreservation.

Males	Lipid analyses	Fresh fertilization	Cryopreservation
CENSALBA2	X	X	X
CENSALBA4	X		
CENSALBA5		X	X
CENSALBA6	X		
CENSALBA7	X	X	X
CENSALBA8		X	X
Valcheta1	X	X	X
Valcheta3	X	X	X
Valcheta4	X		
Valcheta5		X	X
Valcheta6	X		
Valcheta7		X	X

2.4. Sperm morphology and sperm motility

Semen was obtained from fluent males (Valcheta, n = 10 and CENSALBA, n = 5) anesthetized with 0.05 g/L Benzocaine. Semen was refrigerated and carried to the laboratory. Spermatozoa were counted in a Neubauer chamber.

The semen (ca. 15 μL) was fixed in 5% buffered formaldehyde in the proportion of 1 : 1000 (semen: buffered formaldehyde). Samples were stained with Rose Bengal dye (3%) and observed under the microscope (1000 ×). A total of 100 sperm cells per male were selected randomly and classified as normal or abnormal, considering 26 malformations according to the studies of Billard (1983), Galo et al. (2011) and Miliorini et al. (2011).

Sperm motility was recorded in diluted fresh semen of Valcheta (n = 10, 1 μL of semen in 400 μL of Valcheta stream water, pH = 8.69, Conductivity = 518 μS/cm, room temperature) and CENSALBA males (n = 5, 1 μL of semen in 400 μL of tap water, pH = 7.3, Conductivity = 63 μS/cm, 9 °C). Video images were captured with a digital camera (Nikon D5300) and analyzed with Sperm Motility Tracker V2.0 (Buchelly Imbachí et al., 2022). Spermatozoa with curvilinear velocity, average path velocity, and velocity in straight line, above 20, 10, and 3 μm. s^{-1}, respectively, were considered motile. Besides those parameters, the amplitude of lateral head displacement, beat cross frequency (BCF), linearity, motility rate, progression (PROG), straightness, and wobble, were also measured. Those analyses were performed for 1 s (100 images) at different post-activation times (5, 10, 15, 20, 25, and 30 s), with two videos for each male.

2.5. Fertilization performance before and after cryopreservation procedures

Semen samples were obtained as detailed in 2.3 (Table 1). Fertilization controls were performed using fresh semen, diluted at a spermatozoa-to-oocyte ratio of 50.000:1 (see below), and a mix of ova from four CENSALBA females. No live eyed embryos were recovered using semen samples from CENSALBA5 and CENSALBA8 males for any protocols or fresh controls, so these males were discarded from the analyses.

A stock extender solution of Glucose-Methanol (GM), containing 0.36 M Glucose and 17.9% v:v Methanol (Ciereszko et al., 2014; Judycka et al., 2018; Nynca et al., 2014, 2015, 2017) was used. A dilution of 1:500 v/v of each semen sample in the extender was counted in a Neubauer counting chamber. Cylindrical plastic 0.5 mL straws were used for trout semen cryopreservation (IMV Technologies, L'Aigle, France). The average external diameter (d), length (l) and wall thickness (e) were d = 2.81 mm, l = 124 mm, and e = 0.21 mm, respectively. The 0.5 mL plastic straws were uploaded with a final concentration of 5. 10^8 spermatozoa x mL^{-1} in 10 mL of a 0.15 M Glucose and 7.5% v:v Methanol solution (Judycka et al., 2018; Nynca et al., 2017) (Table 2). Each filled straw was maintained at 8 °C for 10–15 min in a refrigerator.

Two cryopreservation protocols were used, varying the position of the straw above the LN_2 level. They were: Protocol A) height of 2 cm above the LN_2 level (external temperature of −124 °C), cooling rates of −91.4, −2.56, and −75.9 °C/min for V1, V2 and V3, respectively. Protocol B) height of 5 cm above the LN_2 level (external temperature of −60.8 °C), cooling rates of −61.47, −1.69, and −26.32 °C/min, for V1, V2 and V3, respectively. For the A and B protocols, the freezing times were 1.19 and 2.79 min, respectively (Fig. 1). The temperature of the nitrogen vapor at different heights was measured and recorded for all experiments using a type T thermocouple (Copper-Constantan). The time-temperature curve during the freezing process was recorded using another type T thermocouple placed at the central axis of the straw. The thermocouples were connected to an acquisition device (TESTO,

Table 2
Dilution of semen (8 samples) with extender (GM, containing 0.36 M glucose and 17.9% v:v methanol) to obtain final sperm concentration in the straw (5×10^8 spermatozoa. mL^{-1}).

Fresh sperm concentration (x 10^{10} spz. mL^{-1})	Final sperm concentration in the straw (x 10^8 spz. mL^{-1})	Dilution rate	Semen volume (mL)	H_2O volume (mL)	Volume (mL) of GM stock	Final volume (mL) of semen diluted with extender
1.54	5	30.8	1.299	4.501	4.2	10
1.76	5	35.2	1.136	4.664	4.2	10
1.68	5	33.6	1.190	4.610	4.2	10
1.00	5	20.0	2.000	3.800	4.2	10
2.92	5	58.4	0.685	5.115	4.2	10
3.92	5	78.4	0.510	5.290	4.2	10
2.54	5	50.8	0.787	5.013	4.2	10
1.94	5	38.8	1.031	4.769	4.2	10

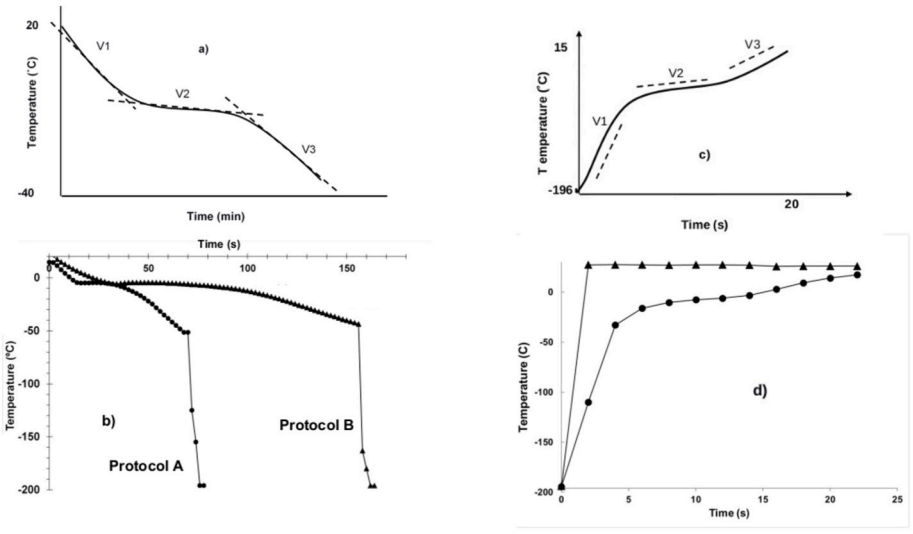

Fig. 1. Left; a) Typical freezing curves for straws cooled by suspension at a given height above the level of LN_2. V1, V2 and V3 are the cooling rates calculated from the slope for each time-temperature curve. b) Experimental time-temperature curves for 0.5 mL straws using Protocol A (straws held 2 cm above the LN_2 level ●) and Protocol B (straws held 5 cm above the LN_2 level ▲). For both Protocols, when the center of the straw reaches at least −40 °C they are plunged directly into LN_2 for long-term storage. Right; c) Typical thawing curve for 0.5 mL straws and warming rates (V1, V2, and V3). d) Experimental time-temperature curve during thawing of the straws in a thermostatic water bath set to a temperature of 27.3 °C. Temperature versus time at the center of the straw (●) and in the warm water bath (▲). The straws were removed when the center reached 15 °C.

Germany) and coupled to a computer. The cooling period was maintained until the center of the straws reached at least −40 °C, after which they were plunged into LN_2 until their use. A "safe" freezing time was assured when the internal temperature reached −69 °C where at least 95% of the water in the semen plus extender mixture in the straw was fully frozen (Santos et al., 2013a, 2013b). The cooling rates for each protocol were calculated based on the initial straight-line slope (Mazur & Seki, 2011) of each stage during the freezing process (Fig. 1).

On removal from the LN_2 tank the straws were immediately thawed by immersion in a temperature controlled warm water bath maintained at 16.6–28.0 °C. The straws were removed from the water when the center of the straw reached 15 °C in order to immediately proceed with fertilization trials (Fig. 1). Ova were collected from four CENSALBA females, and mixed together. The straws were thawed and then each semen sample (0.5 mL) was immediately added to a group of 120 ova (8 °C room temperature), and mixed in a 1 L plastic cup at a low spermatozoa-to-oocyte ratio (5. 10^4: 1). It must be noted that Cabrita et al. (2001) used 1.6. 10^7: 1 spermatozoa-to-oocyte ratio and Nynca et al. (2014), 10^5 : 1 . This ratio (5 . 10^4 : 1), was selected in order to avoid reaching 100% fertilization and maximize the resolution between protocols and strains. After 3 min each group of eggs was rinsed with abundant water, to discard excess of semen and extender, kept in water for 40 min in the absence of light to allow hardening of the chorion and then incubated in separate incubation trays with up-welling water flow. Accumulated thermal units (ATU) were considered as the sum of daily mean water temperatures (°C) obtained with a data logger (HOBO) over time. Fertilization success was established at 330 ATU (eyed embryos), counting all the live embryos in relation to the total number of eggs (Cabrita et al., 2001; Leitritz, 1959).

2.6. Statistics

Frequency data were analyzed using contingency tables, Chi-Square test and Yates correction (P = 0.05). Means were compared using *t*-test or ANOVA. Percent data (FA relative composition) were transformed using angular transformation (Y = arcsen((%. 100^{-1})$^{0.5}$)). When assumptions failed, the Mann Whitney *U* test and Kruskal–Wallis One-Way Analysis of Variance on Ranks (K–W) were used, and pairwise multiple comparison procedures (Dunn's method) were performed. Regression lines were used to show time or temperature related tendencies. When assumptions failed, Regression analyses were complemented with a Spearman rank order correlation or replaced by the analysis of residuals.

3. Results

3.1. Enzymatic activities and oxidative damage

3.1.1. Liver

GST (Mann-Whitney $U = 524$, $T = 455$, $n_{(Guillelmo)} = 22$, $n_{(Valcheta)} = 33$, $P = 0.006$) showed greater activities in Valcheta than in Guillelmo individuals (Fig. 2). However, CAT (Mann-Whitney $U = 1139$, $T = 2000$, $n_{(Guillelmo)} = 41$, $n_{(Valcheta)} = 58$, $P = 0.624$) and SOD (Mann-Whitney $U = 119$, $T = 255$, $n_{(Guillelmo)} = 16$, $n_{(Valcheta)} = 17$, $P = 0.552$) activities did not show significant differences between both streams. Lipid peroxidation (TBARS) showed a significantly greater incidence in Valcheta than in Guillelmo individuals (Mann-Whitney $U = 6683$, $T = 3730$, $n_{(Guillelmo)} = 78$, $n_{(Valcheta)} = 94$, $P = 0.001$, Fig. 2).

Within populations, CAT activity showed a significant dependence on capture temperature both in Valcheta (Regression, $Rsqr = 0.375$, $F = 34.129$, $P < 0.001$, Spearman rank order correlation = −0.665, $P <$

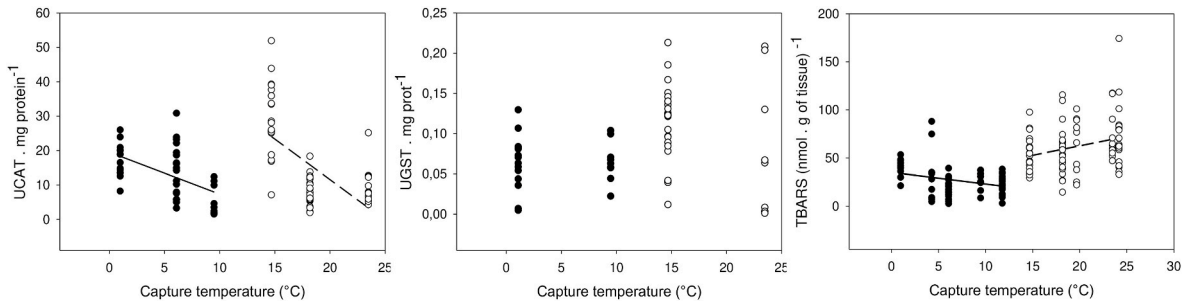

Fig. 2. CAT (UCAT) and GST activity (UGST), and lipid peroxidation (TBARS) levels in liver of rainbow trout of Valcheta (empty circles) and Guillelmo (black circles) streams regarding temperature. Regression lines are indicated to show the tendency.

0.000) and in Guillelmo (Regression, $Rsqr = 0.281$, $F = 15.214$, $P < 0.001$, Spearman rank order correlation $= -0.586$, $P < 0.000$). GST (Guillelmo: $t = 0.818$ with 20 degrees of freedom, $P = 0.423$, Valcheta: Mann-Whitney $U = 77$, $T = 105$, $n_{(23.5°C)} = 7$, $n_{(14.7°C)} = 26$, $P = 0.552$) and SOD activities (Valcheta: Mann-Whitney $U = 19$, $T = 89$, $n_{(14.7°C)} = 8$, $n_{(23.5°C)} = 9$, $P = 0.112$, Guillelmo: Mann-Whitney $U = 17$, $T = 83$, $n_{(9.5°C)} = 8$, $n_{(1.1°C)} = 8$, $P = 0.130$) did not show differences between capture temperatures. Lipid per-oxidation (TBARS) showed a significantly greater incidence at lower temperatures in Guillelmo (Regression, $Rsqr = 0.0982$, $F = 8.280$, $P < 0.005$, Spearman rank order correlation $= -0.295$, $P < 0.009$) and at greater temperatures in Valcheta (Regression, $Rsqr = 0.0810$, $F = 8.020$, $P < 0.006$, Spearman rank order correlation $= 0.275$, $P < 0.008$) (Fig. 2).

Juvenile individuals of CEAN stock showed differences between control (low water temperature and low water conductivity) and treated individuals (high water temperature and high water conductivity), being CAT ($t = -2.367$ with 9 degrees of freedom, $P = 0.042$), GST ($t = -3.032$ with 7 degrees of freedom, $P = 0.019$), and SOD ($t = -2.585$ with 9 degrees of freedom, $P = 0.029$) activities and TBARS abundance ($t = -3.825$ with 8 degrees of freedom, $P = 0.005$) significantly greater at high water temperature and high water conductivity than in control conditions (Fig. 3).

3.1.2. Testicle

GST activity was lower in Valcheta than in CENSALBA individuals (Mann-Whitney $U = 0.0$, $T = 51$, $n_{(CENSALBA)} = 3$, $n_{(Valcheta)} = 15$, $P = 0.009$, Fig. 4). However, CAT ($t = -0.231$ with 14 degrees of freedom, $P = 0.821$) and SOD (Mann-Whitney $U = 19$, $T = 25$, $n_{(CENSALBA)} = 3$ $n_{(Valcheta)} = 14$, $P = 0.850$) activities did not show significant differences between Valcheta and CENSALBA. Lipid per-oxidation (TBARS) showed a significantly greater incidence in Valcheta than in CENSALBA individuals ($t = -7.782$ with 13 degrees of freedom, $P = 0.001$, Fig. 4).

3.1.3. Spermatozoa

GST activity was lower in Valcheta than in CENSALBA individuals ($t = -4.476$ with 20 degrees of freedom, $P = 0.001$, Fig. 5) and SOD activity (Mann-Whitney $U = 11$, $T = 105$, $n_{(Valcheta)} = 8$, $n_{(CENSALBA)} = 11$, $P = 0.011$) was higher in Valcheta than in CENSALBA. CAT (Mann-Whitney $U = 72$, $T = 113$, $n_{(Valcheta)} = 10$, $n_{(CENSALBA)} = 13$, $P = 0.687$) activity did not show significant differences between Valcheta and CENSALBA.

Lipid per-oxidation (TBARS) showed a significantly greater incidence in Valcheta than in CENSALBA individuals (Mann-Whitney $U = 7.5$, $T = 111.5$, $n_{(Valcheta)} = 7$, $n_{(CENSALBA)} = 13$, $P = 0.003$, Fig. 5).

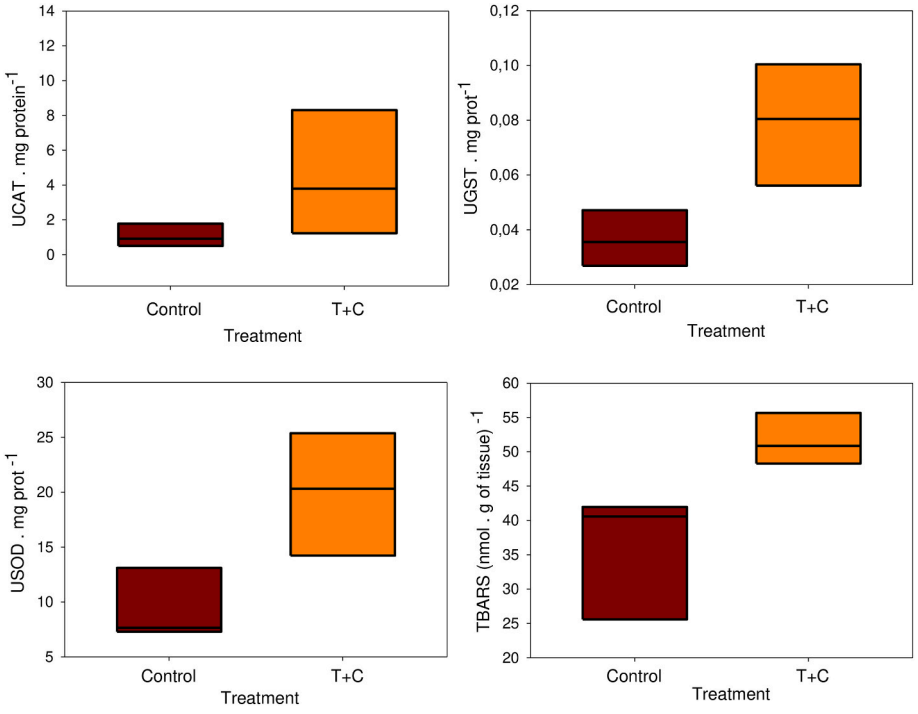

Fig. 3. GST activity (UGST) and lipid peroxidation (TBARS) levels in liver of rainbow trout of CENSALBA (Control) in relation to water Temperature and Conductivity (T + S). Means and standard deviation are indicated.

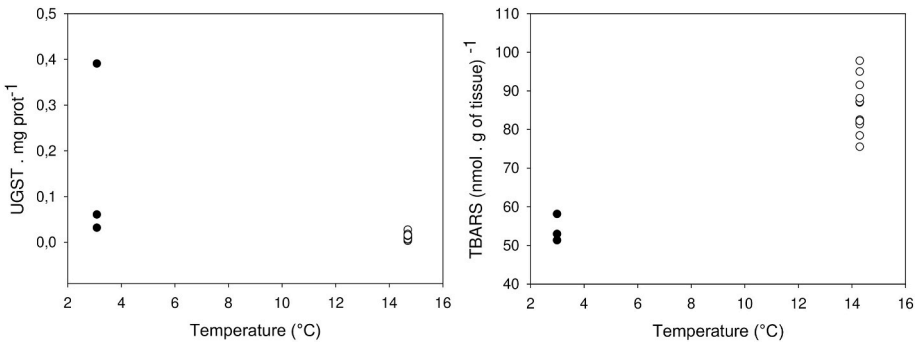

Fig. 4. GST activity (UGST) and lipid peroxidation (TBARS) levels in liver of rainbow trout of Valcheta stream (empty circles) and CENSALBA (black circles).

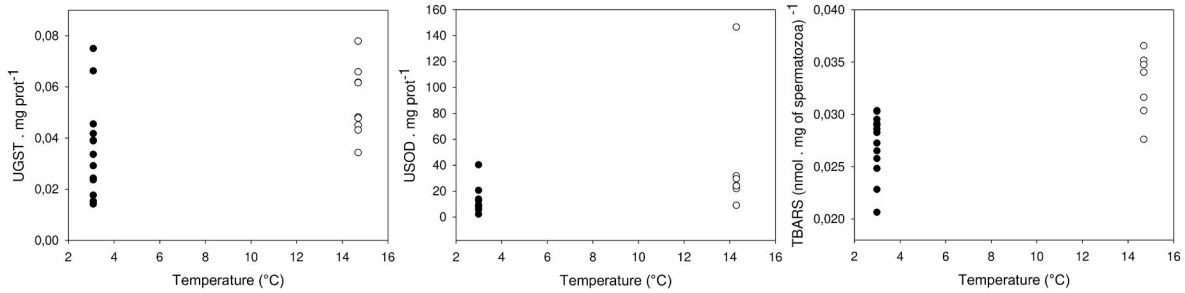

Fig. 5. GST (UGST) and SOD (USOD) activities, and lipid peroxidation (TBARS) levels in spermatozoa of rainbow trout of Valcheta stream (empty circles) and CENSALBA (black circles).

Table 3
Fatty acids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), MUFA + PUFA, n3 fatty acids (n3), n6 fatty acids (n6), and n3 highly unsaturated fatty acids (n3HUFA) relative composition (%), n3/n6 ratio, UFA/SFA ratio, and Unsaturation index (UI) of rainbow trout semen. Four semen samples from Valcheta population and four semen samples from CENSALBA stock were used (Table 1). Median, half range, Man Withney U (MW U) values, and significance of differences between Valcheta and CENSALBA samples are indicated.

Compound	Valcheta Median	Half range	CENSALBA Median	Half range	MW U	Sig.	Number in Fig. 5
C14:0	0.64	0.36	0.49	0.32	0	0.02	1
C15:0	0.15	0.09	0.11	0.08	0	0.02	2
C16:0	21.37	12.24	20.37	12.12			
C16:1n7	0.82	0.52	0.68	0.41	0	0.02	3
C16:1n9	0.04	0.03	0.04	0.03			
C17:0	0.28	0.27	0.21	0.12			
C17:1n7	0.10	0.08	0.08	0.05			
C18:0	4.93	3.02	4.87	2.77			
C18:1n9	9.82	5.46	10.23	5.94			
C18:1n7	3.29	1.71	3.73	2.16	0	0.02	4
C18:1n5	0.12	0.07	0.11	0.07			
C18:2n6	7.94	6.24	9.36	6.23			
C19:0	0.04	0.04	0.00	0.00	0	0.01	5
C19:1n9	0.04	0.03	0.03	0.02			
C18:3n3	0.17	0.13	0.24	0.18			
C20:1n9	0.25	0.22	0.18	0.16	1	0.04	6
C20:2n6	1.10	0.74	0.93	0.77			
C20:3n6	1.42	0.99	1.64	2.52			
C20:4n6	14.66	10.15	10.93	6.69	0	0.02	7
C20:4n3	0.08	0.06	0.24	0.51	0.5	0.03	8
C20:5n3	5.68	3.62	7.38	4.02	0	0.02	9
C22:4n6	0.50	0.41	0.28	0.21	1	0.04	10
C22:5n6	2.35	1.53	1.39	1.11	0	0.02	11
C22:5n3	0.74	0.58	0.67	0.46			
C22:6n3	23.47	14.54	25.83	18.44			
SFA	27.40	14.86	26.05	14.65			
MUFA	14.48	8.03	15.07	8.81			
PUFA	58.12	30.78	58.89	32.33			
MUFA + PUFA	72.60	37.31	73.95	38.55			
n3	30.15	17.25	34.36	22.23	0	0.02	
n6	27.98	17.94	24.53	15.44	1	0.04	
n3/n6	1.08	0.77	1.41	1.09	1	0.04	
n3HUFA	29.18	1.53	34.87	3.25	0	0.02	
UFA/SFA	2.68	0.10	2.84	0.16			
UI	2.85	0.04	2.95	0.13			

3.2. Fatty acid composition of semen

There were six major (>5%) semen FAs in both fish stocks, including Palmitic acid (16:0), Oleic acid (18:1n9), Linolenic acid (18:2n6), Arachidonic acid (AA, 20:4n6), Eicosapentaenoic acid (20:5n3), and Docosahexaenoic acid (DHA, 22:6n3); comprising more than 80% of the total FAs. Relatively abundant (>1%) FAs included Stearic acid (18:0), Vaccenic acid (18:1n7), Eicosatrienoic acid (20:3n3), and Docosapentaenoic acid (22:5n6). Fifteen other relatively scarce (<1%) FAs were also detected (Table 3).

Although all adult males were acclimated at the same temperature, fed with the same commercial food from the juvenile period onward, and semen samples were treated equally, the FA composition of the semen differed significantly between Valcheta and CENSALBA stocks (Table 3, Fig. 6). Differences involved two of the major FAs, the highly unsaturated fatty acids (HUFA) C20:4n6 and C20:5n3, and eight of the relatively abundant or scarce FAs. Particularly, the relative composition (%) was lower in Valcheta individuals for C18:1n7, C20:4n3, and C20:5n3 (Table 3, Fig. 6).

Additionally, the total n3 and n6 FA abundances, the n3/n6 ratio and the n3HUFA content, differed significantly between stocks (Table 3, Fig. 6). Omega 3 FAs percentages were significantly lower in Valcheta semen samples, particularly n3HUFAs, whereas omega 6 FAs were significantly higher in Valcheta samples; with a concomitant lower n3/n6 ratio in Valcheta than in CENSALBA semen.

3.3. Sperm morphology and sperm motility

The percentage of normal spermatozoa was significantly lower in Valcheta than in CENSALBA ($t = -5.579$ with 22 degrees of freedom, $P < 0.001$). The malformations with significantly different frequencies between Valcheta and CENSALBA are listed in Table 4.

The sperm motility allowed to observe two variables; BCF (Multiple Linear Regression, $F = 10.788$, $P < 0.001$) and PROG (Multiple Linear

Table 4
Sperm morphology and malformations. Means, statistics of *t*-test (*t*) or Mann-Whitney Rank Sum Test (*U*), degrees of freedom (DF), and significance (*P* =). Only malformations with significant differences between strains are indicated.

	Valcheta (%)	CENSALBA (%)	Statistic	DF	*P* =
Normal sperm	81.5	86.4	$t = -5.579$	22	<0.001
Bent tail in the final portion	0.0	4.4	$U = 130.5$	24	<0.001
Fractured tail in the proximal portion	5.2	1.0	$U = 27.0$	24	0.004
Fractured tail in the intermediate portion	2.9	1.5	$U = 27.0$	24	0.008
Flagellar coiling	0.0	0.0	$U = 49.0$	24	0.037
Macrocephaly	6.1	0.0	U = 0.0	24	0.001
Two tails	0.5	0.0	U = 49.0	24	0.037

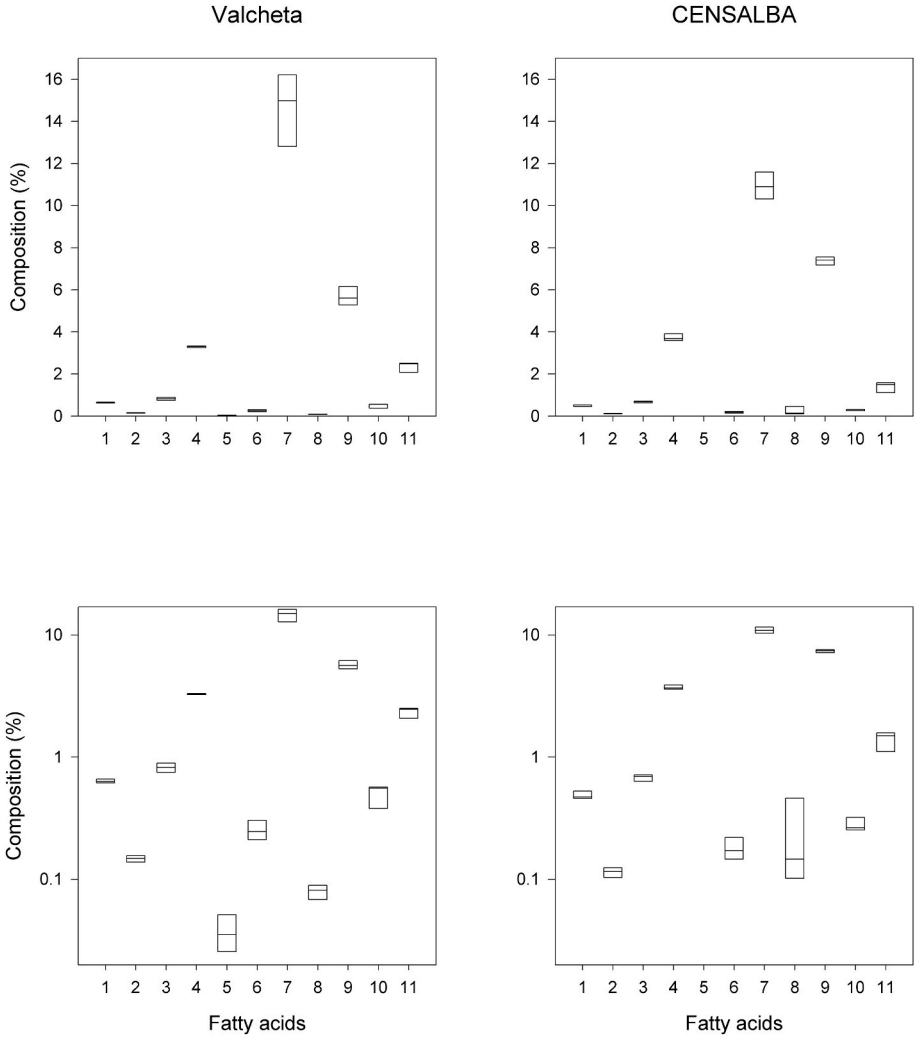

Fig. 6. Fatty acid composition (%, median and quartiles) of four semen samples from Valcheta population and four semen samples from CENSALBA stock. Only fatty acids with significantly different abundance (numbers in Table 3) are indicated: 1- C14:0, 2- C15:0, 3- C16:1n7, 4- C18:1n7, 5- C19:0, 6- C20:1n9, 7- C20:4n6, 8- C20:4n3, 9- C20:5n3, 10- C22:4n6, 11- C22:5n6. In order to improve visualization, percentages are indicated both in linear (upper panels) and in $\log_{10}$ scales (lower panels).

Regression, $F = 8.708$, $P < 0.001$) that were significantly explained by Time (5–30 s) and Strain (Valcheta and CENSALBA) (Fig. 7). However, as ANOVA assumptions failed, the residuals of the linear regression were obtained and compared between Valcheta and CENSALBA, without significant differences between strains for both BCF (Mann-Whitney $U = 587$, $T = 1063$, $n_{(CENSALBA)} = 25$, $n_{(Valcheta)} = 53$, $P = 0.422$) and PROG (Mann-Whitney $U = 676$, $T = 1053$, $n_{(CENSALBA)} = 26$, $n_{(Valcheta)} = 53$, $P = 0.896$).

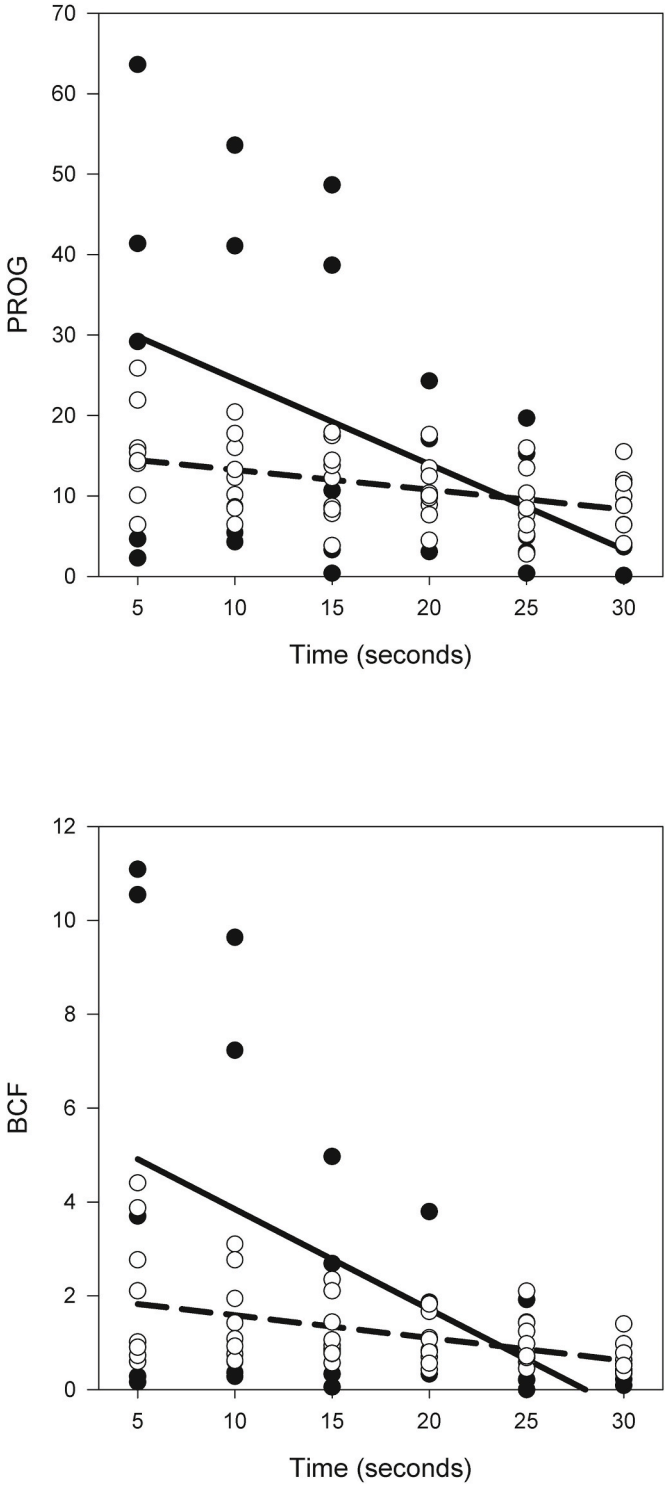

Fig. 7. Sperm motility along time. Beat cross frequency (BCF) and progression (PROG) of spermatozoa of Valcheta (white circles) and CENSALBA (black circles). Regression lines are indicated to show the tendencies.

3.4. Fertilization success before and after cryopreservation procedures

The probability of obtaining eyed embryos (fertilization success) depended on fish strains (Yates corrected Chi-Square test, $P < 0.05$), and was lower for Valcheta than for CENSALBA, including both fresh control fertilization assays and cryopreservation assays (Table 5, Fig. 8). CENSALBA showed a very low fertility percentage using protocol B. It can be observed that Valcheta showed low fertility values in both treatments (protocol A and B).

4. Discussion

In the present work, oxidative damage was measured through lipid peroxidation of FA chains (Hermes-Lima et al., 1995). It could be observed that the oxidative damage in liver, testicle and spermatozoa is consistently higher in rainbow trout individuals of Valcheta stream (thermal water) than in individuals of Guillelmo stream (temperate water) and higher than in farmed (temperate) individuals of CENSALBA. The same pattern could be observed in farmed (temperate) CEAN juveniles experimentally exposed to high temperature and conductivity in comparison to controls. It must be noted that, within the wild population of Guillelmo stream (temperate), the higher oxidative damage corresponded to lower temperatures. Nevertheless, the antioxidant system did not show a consistent pattern considering enzymes, strains and temperatures (Table 6).

It is known that quantities of saturated and unsaturated FAs in rainbow trout seminal plasma and spermatozoa differ due to differences in strain, diet, or the water temperature at which they are kept (Lahnsteiner et al., 2009; Vassallo-Agius et al., 2001). Also, changes in lipid composition may occur during short-term semen storage (Lahnstainer et al., 2009). However, the semen samples of Valcheta and CENSALBA were treated equally; the males were reared under the same temperature regime and fed in the same way for more than one year before the assay. In terms of FA composition, high proportions of unsaturated FAs were found in both Valcheta and CENSALBA, in agreement with previous studies on salmonid sperm (Pustowka et al., 2000; Sandoval-Vargas et al., 2021). Additionally, Valcheta semen showed a lower content of 20:5n3 than CENSALBA, which has been observed in trout fed with a low essential fatty acids (EFA) diet and may be related to a lower sperm activity (Watanabe et al., 1984). In fact, Valcheta semen showed a lower percentage of normal spermatozoa and a less variable motility, in terms of BCF and PROG. The n3HUFAs were also lower in semen of males fed with a low EFA diet, which affected the production of good quality fertilized eggs (Vasallo-Agius et al., 2001). Considering the same diet was given to both stocks, that cultivation temperature was the same, and that lipid peroxidation may probably affect equally both n3 and n6 PUFAs, the observed differential FA semen composition could be

Table 5
Number of eyed embryos/number of oocytes and percentage (into brackets), from two CENSALBA males and four Valcheta males, obtained in fresh control, 2 cm, and 5 cm treatments. Different letters indicate significant dependence of the percentage of eyed embryos obtained with treatments and strains (Yates corrected Chi-Square test, $P < 0.05$). Lowercase letters indicate comparison between treatments (within the same line). Capital letters indicate comparison between strains (within the same column).

Strains/Males	Fresh control	2 cm	5 cm
CENSALBA	**a, A**	**b, A**	**c, A**
CENSALBA2	92/105 (88)	12/106 (11)	0/105 (0)
CENSALBA7	34/106 (32)	23/105 (22)	9/105 (9)
Valcheta	**a, B**	**b, B**	**b, B**
Valcheta1	15/106 (14)	5/105 (4)	8/105 (8)
Valcheta3	60/104 (58)	1/105 (1)	1/105 (1)
Valcheta5	42/106 (40)	0/105 (0)	0/107 (0)
Valcheta7	31/107 (29)	0/105 (0)	0/104 (0)

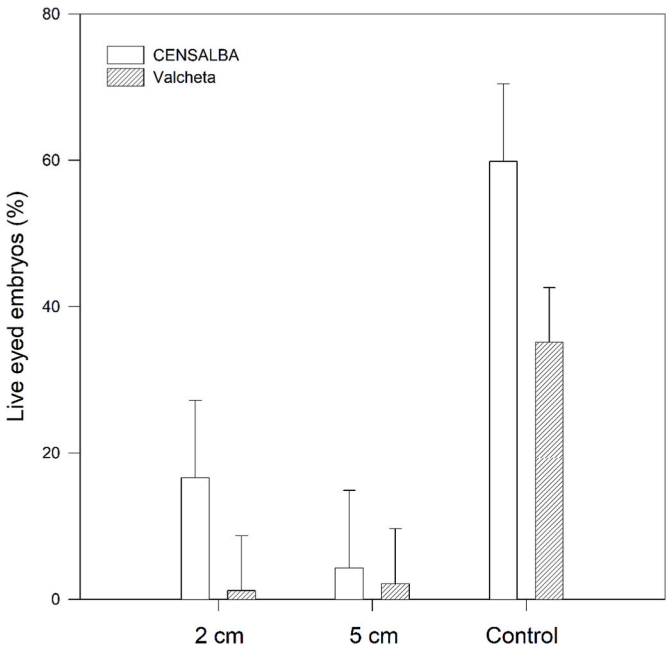

Fig. 8. Live eyed embryos (330 ATU) as percentage. Rainbow trout embryos were obtained from an oocyte pool of CENSALBA females; four semen samples were taken from Valcheta population, and two semen samples from CENSALBA stock. Semen samples were frozen at 2 and 5 cm over LN_2. Controls were obtained using fresh semen and an oocyte pool of CENSALBA females.

Table 6
Lipid peroxidation (LP) and enzimatic activities (CAT, GST and SOD) in liver, testicle and spermatozoa of wild (Valcheta and Guillelmo streams) and farmed (CENSALBA and CEAN) individuals in relation to temperature. The results (High or Low) corresponded with significant differences observed in the comparisons.

Liver				
	Valcheta		*Guillelmo*	
LP	High		Low	
CAT	–		–	
GST	High		Low	
SOD	–		–	
Temperature	*Low*	*High*	*Low*	*High*
LP	–	–	High	Low
CAT	High	Low	High	Low
GST	–	–	–	–
SOD	–	–	–	–
CEAN				
Temperature		*Low*		*High*
LP		Low		High
CAT		Low		High
GST		Low		High
SOD		Low		High
Testicle				
	Valcheta		*CENSALBA*	
LP	High		Low	
CAT	–		–	
GST	Low		High	
SOD	–		–	
Spermatozoa				
	Valcheta		*CENSALBA*	
LP	High		Low	
CAT	–		–	
GST	Low		High	
SOD	High		Low	

attributed to differences in lipid metabolism. Further analysis, e.g. lipid composition in different tissues and or the analysis of desaturases expression or activities, may serve to elucidate some of these hypotheses.

In terms of fertilization, a lower performance was observed for Valcheta semen, before and after the cryopreservation procedures. The lower fresh semen performance for Valcheta strain may be explained by the observed lower levels in EPA and or n3HUFAs (Vasallo-Agius et al., 2001, Watanabe et al., 1984); or also by the higher oxidative damage observed in Valcheta. Regarding cryopreservation, it is known that it damages sperm cells, affecting the plasma membrane, mitochondria, and chromatin structure (Cabrita et al., 2010; Labbé et al., 2001; Watson & Fuller, 2001; Watson & Morris, 1987). High levels of cholesterol and MUFAs were correlated with increased resistance to cryopreservation damage (Pustowka et al., 2000). However, significant differences on MUFAs in the total semen FAs between Valcheta and CENSALBA individuals were not found.

Plastic responses to temperature were reported for *O. mykiss* (Doctor et al., 2014, 2015). Moreover, as in all poikilotherms, the germ line of fishes is exposed to environmental temperature and high temperature can elicit transcriptomic changes (Rebl et al., 2020). Although the present work shows no evidence regarding the effects of ROS on mutation rate, the scientific literature (Chowdhury & Saikia, 2020; Nilsson & Liu, 2020) strongly supports this hypothesis. Also, a modulation of DNA methylation by the thermal regime (Venney et al., 2022, 2023), mediated by ROS (Zhang et al., 2022), may be occurring simultaneously, opening a wide universe of future studies (Gavery et al., 2018; Koch et al., 2022; Pittman et al., 2013).

When the process of adaptation to higher temperatures was observed in the Valcheta (feral) population (Crichigno & Cussac, 2019; Crichigno et al., 2018), the next step seemed to be the selection of a wild stock for growth and domesticity. However, Butzge et al. (2021) demonstrated that thermal tolerance could be reached in one generation in a farmed line. The authors considered that warm water temperature applied to juvenile rainbow trout may select F0 males and that each spermatogonia could respond in a different way. Our present results suggest that acquisition of thermal tolerance is probably only one of a wide spectrum of good or bad novelties produced as a consequence of a thermal-induced, ROS-mediated, increased mutation rate and/or epigenetic mechanisms (Venney et al., 2022, 2023; Zhang et al., 2022). Even though the exact mechanisms is yet not fully known, the procedure of Ineno et al. (2005) and the findings of Lagarde et al. (2023) confirm the possibility of selection procedures to obtain thermal tolerant strains in rainbow trout.

Submission declaration

The work described has not been published previously, it is not under consideration for publication elsewhere, its publication is approved by all authors and by the responsible authorities where the work was carried out, and, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Ethics approval

All animal experiments complied with the U.K. Animals (Scientific Procedures) Act, 1986, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) guidelines. Also, we works with protocols of animals used from CENSALBA hatchery, they were registered and enabled in RENACUA (https://www.argentina.gob.ar/inscripcion-como-productor-en-el-renacua) and SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria).

CRediT authorship contribution statement

Vianel Sevastei: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Sonia A. Crichigno:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **M. Victoria Santos:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Andrea Trochine:** Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. **Julio C. Painefilú:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Noemí Zaritzky:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Víctor E. Cussac:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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