# EFFECT OF TEMPERATURE ON MOTILITY AND ANTIOXIDANT ENZYMES OF COMMON CARP SPERMATOZOA

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**Abstract-** The effect of temperature on Cyprinus carpio spermatozoa in vitro was investigated with spermatozoa activated at 4, 14, and 24 °C. At 30 s post-activation, motility rate was significantly higher at 4 °C compared to 14 and 24 °C, whereas highest swimming velocity was observed at 14 °C. The thiobarbituric acid-reactive substance (TBARS) content was significantly higher at 14 °C and 24 °C than at 4 °C in motile spermatozoa. No significant differences in catalase and superoxide dismutase activity relative to temperature were observed. This study provides new information regarding effect of temperature on lipid peroxidation intensity and spermatozoon motility parameters in carp. The elevation of TBARS seen at higher temperatures could be due to inadequate capacity of antioxidant enzymes to protect the cell against the detrimental effects of oxidative stress induced by higher temperatures.

**Keywords-** Antioxidant enzymes, Carp, Lipid peroxidation, Spermatozoa

## I. INTRODUCTION

Spermatozoon motility is an important factor in fertilization capacity [1]. Most fish exhibit external fertilization, so spermatozoon motility will be strongly influenced by environmental temperatures, and it has been demonstrated that temperature affects spermatozoon motility rate and swimming velocity [2, 3, and 4]. A trade-off between increased spermatozoon velocity and decreased motility duration at higher temperatures has been reported for common carp Cyprinus carpio [5], grass carp Ctenopharyngodon idella [6], hake Merluccius merluccius [7], and burbot Lota lota [2].

The sperm activation process is accompanied by a series of cellular changes including a rise in respiration rate and rapid depletion of ATP [8, 9, and 10]. This might be associated with increase in reactive oxygen species (ROS) level, since ROS are usually generated endogenously through cell respiration or through interaction with exogenous factors [11].

Some changes in sperm viability might be related to ambient temperature [12], and temperature-induced stress has been associated with enhanced generation of ROS and oxidative stress in spermatogenic cells [13]. During times of environmental stress such as increased temperature, ROS levels can increase dramatically [13] which potentially leading to oxidative stress. Lipid peroxidation is an adverse effect of oxidative damage in fish spermatozoa associated with high poly-unsaturated fatty acid content resulting in susceptibility to ROS attack [14, 15, and 16]. In sperm, the measuring of TBARS content is currently used to evaluate lipid peroxidation level via quantification of malondialdehyde, which reacts with the thiobarbituric acid used as reagent [17].

Antioxidant enzymes present in spermatozoa counteract oxidative damage. In fish sperm, the primary enzymes for detoxification of ROS are catalase (CAT) and superoxide dismutase (SOD) [15, 17 and 18]; hence their activity will reveal oxidative status. Investigation of oxidative stress indicators during motility at different temperatures can provide valuable information about the effects of temperature on spermatozoon metabolism. The aim of this study was to evaluate the in vitro effect of temperature on carp sperm by analyzing lipid peroxidation (TBARS), the activity of CAT, SOD and motility characteristics.

## II. DETAILS EXPERIMENTAL

### 2.1. Semen collection

Cyprinus carpio reared at the fish farm of South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic were used for the study. Six mature males of 3-4 kg body weight were injected with 0.2 mL of a suspension containing physiological solution and lyophilized carp pituitary powder at 5 mg kg-1 body weight. After 24 h, sperm was stripped by abdominal massage into dry 20 mL vials and stored on ice up to 2 h before experimentation.

### 2.2. Physiological saline solutions used for inhibition and activation of motility

The spermatozoon motility-inhibiting saline solution consisted of 103 mmol L−1 NaCl, 40 mmol L−1 KCl, 1 mmol L−1 CaCl2, 0.8 mmol L−1 MgSO4, and 20 mmol L−1 HEPES (pH 7.8) [2]. Activation medium (AM) containing 10 mM Tris–HCl buffer, pH 8.0, 1 mM CaCl2, and 0.125% pluronic acid was used to trigger motility.

**2.3. Motility assessment**

Fresh sperm from each fish was added to a drop of AM (~50 µl) adjusted to 4, 14, or 24 °C on a microscope slide using the tip of a dissecting needle (~5µl) with which the sperm suspension was thoroughly mixed for 2 s. Immediately after dilution, spermatozoon motility parameters at each temperature were recorded at 0, 10, 20, 30, 60, 120, 180, and 240 s post-activation using a CCD video camera (Sony, SSCDC50AP, Japan) mounted on an inverted microscope equipped with a cooling stage (Olympus IX83, United Kingdom) and a X20 NIC contrast objective lens. Motility records were analyzed to estimate spermatozoon curvilinear velocity (VCL, µm s-1), percentage of motile spermatozoa after activation (motility rate), and motility duration in triplicate for each sperm sample. Temperature of the AM was adjusted by a thermoblock (HLC BO50/15, Germany) and monitored during the motility period by copper-constantan thermocouple (Omega, L-044T, Taiwan) via a data logger thermometer (Omega, HH127, Taiwan).

**2.4. Quantification of TBARS content**

In this experiment, two media were used to prepare samples for quantification of TBARS level. Samples (150 µL) of fresh sperm were incubated in 1350 µl of AM to suppress motility at 4, 14, and 24 °C. A solution containing 0.9% NaCl was used as non-activation medium (NAM) at 4, 14, and 24 °C. Subsequently, samples were centrifuged at 1000 x g for 5 min at 4 °C. The TBARS content of the supernatant was measured spectrophotometrically according to Asakawa and Matsushita [19]: Briefly, 0.025 mL butylated hydroxytoluene solution (22 mg in 10 mL ethanol), 0.025 ml ferric chloride solution (27 mg of FeCl3 6H2O in 10 mL water), 0.375 mL of 0.2 M glycine-hydrochloric acid buffer, pH 3.6, and 0.375 mL TBA reagent (0.5 % TBA and 0.3% sodium dodecyl sulfate) were added to 0.08–0.25 mL supernatant. The tubes were capped and heated for 15 min in a boiling water bath. After cooling, 0.25 mL glacial acetic acid and 0.5 mL chloroform were added. The mixture was vigorously shaken and centrifuged for 10 min at 1500 x g. The absorbance of samples was determined at 535 nm against a blank with deionized water substituted for the biological sample. A molar extinction coefficient of 1.56 105 M-1 cm-1 was used for calculation of TBARS content. The concentration of TBARS was expressed as nmol mL-1 supernatant. Measurements were taken in triplicate for each sample, and the average of the three measurements was used for the results

2.5. Extraction and evaluation of antioxidant enzymes

Before extraction of enzymes, 200 µL of fresh sperm was diluted in 1 mL of motility-inhibiting solution and centrifuged 1000 x g for 10 min at 4 °C. The sperm pellet was re-diluted in motility-inhibiting solution to remove the seminal plasma. Thereafter, the sperm suspension was centrifuged once more under similar conditions, and the sperm pellet was diluted in 1 mL of 0.1 mol L−1 Tris buffer (pH 8.0) containing 0.01% Triton-X-100 and 0.5% glycerol. Samples were homogenized and the suspensions were pooled, placed in an ice bath, and sonicated (Bandelin Electronic UW 2070, Germany) for 15 s. Insoluble particles were removed by centrifugation at 3000 x g for 10 min at 4 °C. Before analysis, extracted samples were stored at −80 °C until analysis.

The activity of CAT and SOD were determined at 4, 14, and 24 °C. The activity of SOD (EC 1.15.1.1) was measured by a spectrophotometer equipped with a thermo-regulated cuvette holder at 420 nm according to the procedure of Marklund and Marklund [20]. The inhibition of pyrogallol autoxidation by the sample was used to determine SOD activity. The autoxidation of 0.2 mM pyrogallol in air-equilibrated 50 mMTris–HCl buffer, containing 1 mM EDTA, pH 8.2, was inhibited by the addition of the assayed sample. One unit of the enzyme is generally defined as the amount of enzyme that inhibits the reaction (in this case, pyrogallol autoxidation) by 50%. The specific activity of SOD was expressed as unit mg−1 of protein.

The activity of CAT (EC 1.11.1.6) was measured by a spectrophotometer equipped with a thermo-regulated cuvette holder at 240 nm. The reaction medium contained 10 mM K+-phosphate buffer with 0.1 mM EDTA, pH 7.4, and 15 mM H2O2, according to the method of Marklund et al. [21]. The CAT activity was calculated from the H2O2 decomposition rate using the molar extinction coefficient 39.4 M-1 cm-1, and the specific activity was expressed as micromole min−1 mg−1 of protein. Measurements were carried out in triplicate for each sample.

**2.6. Statistical analysis**

The values of spermatozoon velocity were checked for distribution characteristics and homogeneity of dispersion using Shapiro–Wilk's and Levene's tests, respectively. As they were normally distributed with similar dispersion values, parametric one-way ANOVA was applied and Tukey's honest significant difference (HSD) test was used for comparing differences among groups. Because of the small number of observations (n = 6), nonparametric statistics using the Kruskal–Wallis test followed by the multiple comparison of mean ranks for all groups test were performed for comparison of motility rate, duration of motility, CAT and SOD activity, and TBARS content. Results were presented as mean ± standard deviation (SD), with significance at $P < 0.05$. All analyses and plots were conducted using the STATISTICA v. 12 computer program (Statsoft Inc., USA).

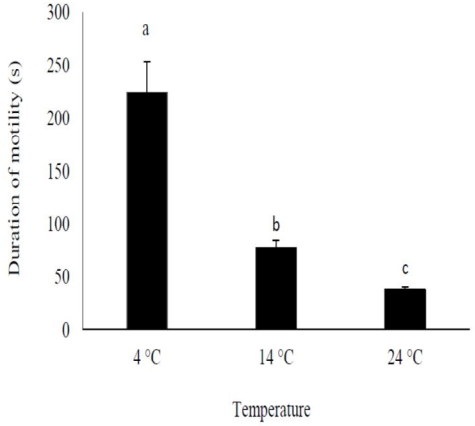



**Fig. 1. Effect of temperature on C. carpio spermatozoon motility duration. Data are mean ± SD. Different lowercase letters indicate significant difference (P <0.05).**

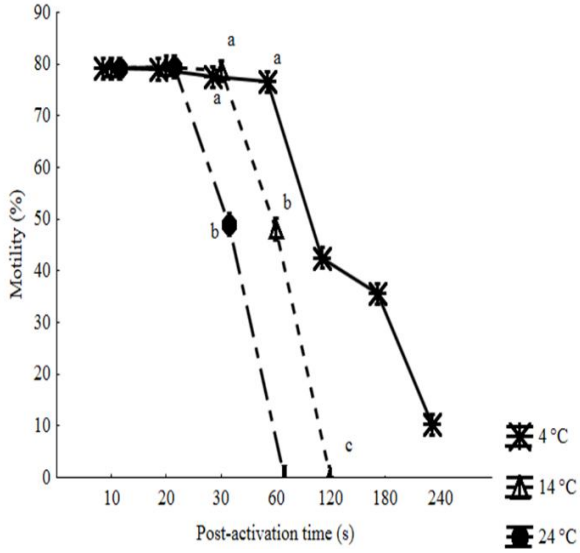



**Fig. 2. Effect of temperature on C. carpio sperm motility rate. Data are mean ± SD. Different lowercase letters indicate significant differences(P<0.05).**

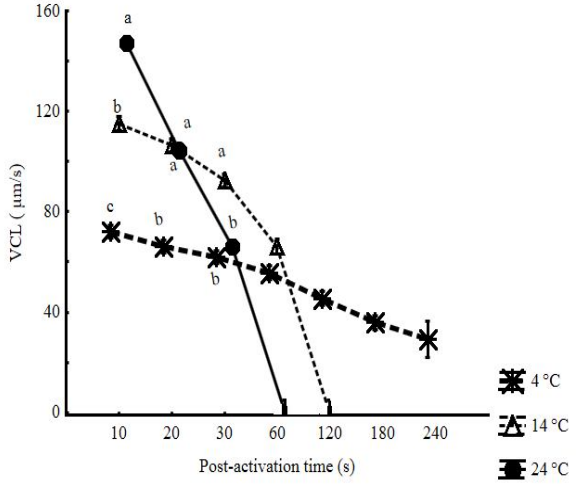



**Fig. 3. Effect of temperature on C. carpio spermatozoon velocity. Data are mean ± SD. Different lowercase letters indicate significant differences (P <0.05).**

## III. RESULTS AND DISCUSSION

Subjection of spermatozoa to ambient temperature transitions has been discussed in some literatures [2, 22 and 23]. In the current study, motility duration were significantly longer at 4 °C compared to 14 and 24 °C, supporting the crucial role of ATP availability at lower temperatures in prolonged motility [10 and 24]. In line with our results, it has been shown that duration of spermatozoon motility to be significantly increased by low temperature in different fish species [2].

The present results regarding swimming velocity are consistent with the findings of previous studies on Senegalese sole Solea senegalensis and European perch Perca fluviatilis, which showed elevation of swimming velocity at high temperatures [25 and 26]. Due to the limited energy resources of fish spermatozoa, the impact of the increased velocity associated with higher temperatures on metabolic processes leads to lower motility rate and duration [27].

According to our results, at 30 s post-activation, the highest motility rate at 4 °C and highest swimming velocity at 14 °C were observed. Lahnsteiner and Mansour [2] stated within 10 s of activation, motility rate and velocity in burbot spermatozoa were highest at 4 °C and 4-6 °C, respectively. However, the motility rate and velocity of brown trout Salmo trutta were considerably decreased at temperatures lower than 8 °C [2]. At 120 s, motility rate and velocity were also significantly higher at 4-6 °C than at ≥8 °C [2]. This implies that temperature may strongly influence spermatozoon motility parameters by its impact on energy resources [23].

Differences in enzyme activity can be attributed to temperature changes [28]; however, information regarding the effect of temperature on antioxidant enzyme activity in fish semen and precise mechanisms involved in temperature-related oxidative stress is scant. Most studies about the influence of temperature on oxidative stress have been tested on fish tissues. In the present study activities of SOD and CAT were observed at all temperatures, though no significant differences were detected at different temperatures, in contrast to findings for cattle and buffalo [29].

This could be attributed to insufficient activity of the antioxidant enzymes at higher temperatures [30]. The present findings are in agreement with the results obtained in sheep Sertoli cells in which SOD activity following heat stress of 32-42 °C was not significantly different from that in controls [31]. In addition, partial inactivation of SOD in ram spermatozoa under freezing condition (cold shock) has been reported by Marti et al. [32]. Activity of SOD in boar spermatozoa has been found to be

higher at temperatures of 20 and 45 °C than at 4, 50, 60, and 70 °C [33]. On the contrary, Horky et al. [34] showed that SOD activity in boar spermatozoa increased at higher temperatures, which could indicate activation of protective antioxidant mechanisms against free radicals. It is also noteworthy that CAT activity was found to exhibit considerable decline under summer heat stress compared to winter in rabbit semen [35]. The inconsistent results of these studies may be due to species differences in thermoregulation, temperature regime applied, tissues analyzed, and/or assays used to assess oxidative stress.

TBARS content can be elevated in the initial hours of oxidative stress induced by thermal shock [33], probably due to loss of cellular homeostasis accompanied by the formation of ROS [36]. Results of the present study have proved that lipid peroxidation in fish spermatozoa is highly sensitive to temperature. In AM solution, the level of TBARS was elevated significantly at 14 °C and 24 °C compared to that measured at 4 °C. However, this difference was not observed in immotile spermatozoa.

These discrepancies in TBARS level observed in spermatozoa exposed to AM solution at different temperatures is likely due to metabolic changes during motility leading to an increase in respiration rate and production of ROS [17]. Salehi Nezhad et al. [31] also reported an increased TBARS level in sheep Sertoli cells at higher temperatures. Influence of temperature on lipid peroxidation has also been shown in rabbit and mouse sperm [37 and 38], however no significant effect of temperature on TBARS levels was found in bovine epididymidis sperm stored at 4 and 34 °C [39].

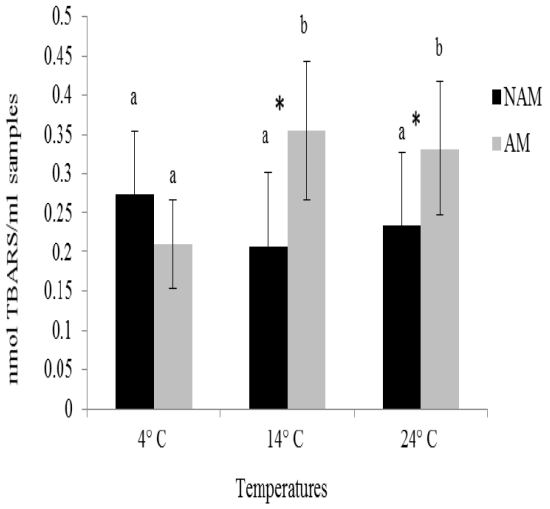



**Fig. 4. TBARS content of C. carpio spermatozoon at different temperatures. Asterisks indicate significant difference between AM and NAM solutions. Data are mean ± SD. Different lowercase letters indicate significant differences (P <0.05).**

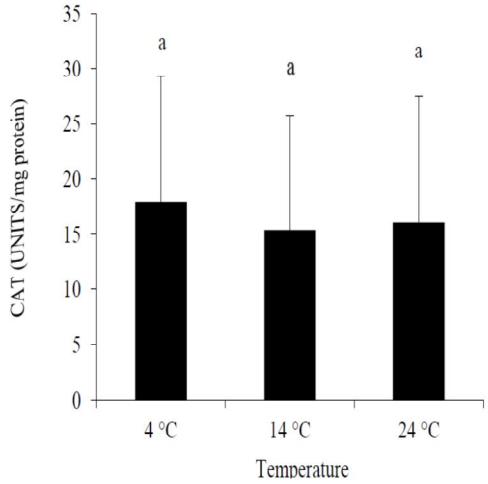



**Fig.5. Effect of temperature on catalase activity in C. carpio spermatozoa. Data are mean ± SD. Same lowercase letters indicate no significant difference (P >0.05).**

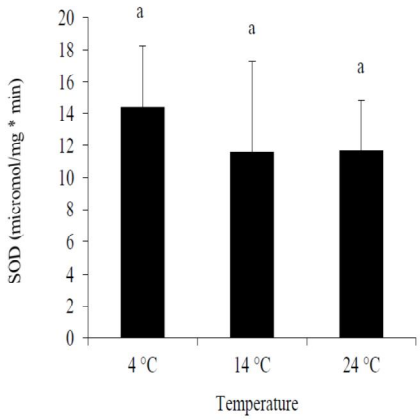



**Fig. 6. Effect of temperature on SOD in C. carpio spermatozoa. Data are mean ± SD. Same lowercase letters indicate no significant difference (P >0.05).**

## CONCLUSIONS

In conclusion, our results unequivocally demonstrate the great change in terms of sperm motility parameters and TBARS content at different temperatures. We found that elevation of TBARS content at higher temperatures (14 °C and 24 °C) is possibly due to inadequate capacity of antioxidant enzymes to protect the cell against the detrimental effects of oxidative stress induced by higher temperatures.

Consequently, excessive lipid peroxidation in sperm membranes result in a rapid loss of motility might directly influence fertilization success. However, further studies are required to elucidate the precise mechanisms involved and the relationship between motility and oxidative stress in carp sperm under different temperatures.

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