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EFFECTS OF HIGH AMBIENT TEMPERATURE ON FISH SPERM PLASMA MEMBRANE INTEGRITY AND MITOCHONDRIAL ACTIVITY – A FLOW CYTOMETRIC STUDY

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Local extreme climatic conditions occurring as a result of global climate change may interfere with the reproduction of animals. In the present study fish spermatozoa were incubated at different temperatures (20, 25, 30 and 40 °C) for 10 and 30 minutes, respectively and plasma membrane integrity and mitochondrial membrane potential changes were evaluated with flow cytometry using SYBR-14/PI and Mitotracker Deep Red FM fluorescent dyes. No significant differences were found in plasma membrane integrity at either incubation temperatures or time points. Mitotracker Deep Red FM histogram profiles indicating mitochondrial activity showed significant ($p<0.001$) alterations in all cases of higher (25, 30 and 40 °C) temperature treatments as compared to the samples incubated at 20 °C. Our results indicate that fish spermatozoa exposed to high temperatures suffer sublethal damage that cannot be detected with conventional, vital staining techniques.

Keywords: Fish sperm – plasma membrane integrity – mitochondrial activity – high temperature – flow cytometry

INTRODUCTION

Local extreme climatic conditions occurring as a result of global climate change may disturb the physiological functions of wild and farm animals, involving their reproductive physiological processes [3]. It is known for example that extremely high or low temperatures result in disturbances in spermatogenesis causing several sperm defects [2].

Heat stress – besides changes at cellular level – can overthrow the sex-hormone balance of the organism and significantly affect the physiological process of animal production as well [22].

A number of animal species living at different temperate zones show strict seasonal reproduction where the main factor that triggers the onset of reproductive sea-

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son is the length of the daily light period instead of temperature. This latter does not fully follow the change of the day length which is a year-to-year precisely occurring phenomenon. This way there is a chance that the animals do not meet optimal temperature conditions in their reproductive period [4, 5].

The renaissance of ecological farming opens new fields for the old, traditional breeds, selected for extensive production methods. These breeds show more or less seasonality in reproduction, though this seasonality is not negligible in intensive type breeds either.

A majority of teleost fish species are seasonal breeders and may especially be sensitive to extreme environmental conditions, due to their external fertilization. Their reproductive success – and as a consequence, their fitness to reach brooder age – can be influenced by the extreme temperature affecting the gametes (eggs and sperm) either in the nature or during artificial propagation, gamete collection and storage [18, 24].

Our aim in this study was to assess the usefulness of flow cytometry to detect the changes of plasma membrane integrity and mitochondrial transmembrane potential of fish spermatozoa, incubated at different temperatures (20, 25, 30 and 40 °C) for 10 and 30 minutes, respectively, to reveal if this increase of ambient temperature can lead to cell death or mitochondrial membrane depolarization.

MATERIALS AND METHODS

In order to decrease individual variance [21] pooled sperm of three male prussian carps (*Carassius auratus gibelio*, Bloch) were used in the experiment. Spermiation was induced by injecting carp pituitary extract into the body cavity and stripping was performed in anaesthesia using clove oil. Sperm samples were transferred to the laboratory immediately after collection and pooling. Sperm concentration was adjusted via suspending in phosphate buffered saline (PBS, Sigma-Aldrich P4417-100TAB) to achieve the optimal cell concentration ($<5 \times 10^6$/ml) for flow cytometric measurements. In order to avoid artefacts due to accidental activation of spermatozoa, activated/inactive state was monitored with an Olympus CX40 phase contrast microscope, and only inactive sperm samples were used for the subsequent analyses. Pooled sperm samples were split to eight subsamples, and they were incubated at 20, 25, 30 or 40 °C for 10 or 30 min, respectively. The experiment was repeated three times.

Plasma membrane integrity

The percentages of spermatozoa with intact plasma membrane were assessed with Live/Dead Sperm Viability Kit (Life Technologies, L-7011) according to Garner et al. [8]. Intact sperm cells were labelled with SYBR 14 and showed green fluorescence, dead cells were labelled with propidium iodide (PI) and showed red fluorescence. Events showing green and red fluorescent signal simultaneously were considered as moribund, dying spermatozoa (Fig. 1).

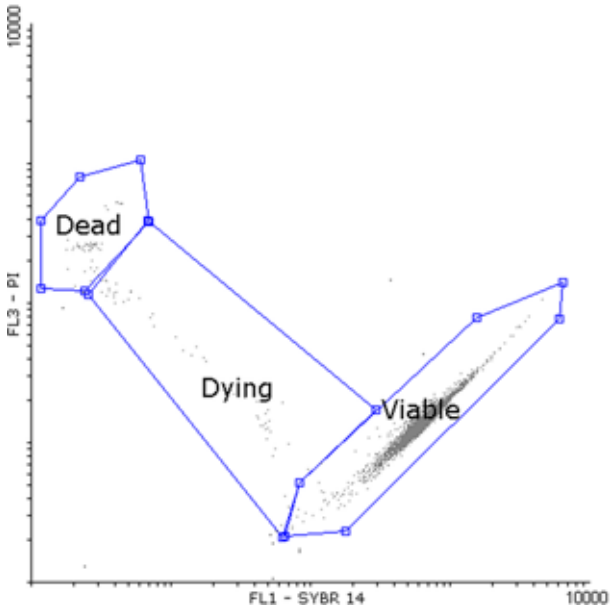

Fig. 1. Fluorescence intensities of viable, moribund and dead spermatozoa. Viable cells show high green, dead show high red signal, moribund, dying spermatozoa show high green and red fluorescence simultaneously. FL1: SYBR-14; FL3: PI

Mitochondrial activity

Mitochondrial transmembrane potential was evaluated with Mitotracker Deep Red FM (Life Technologies, M22426) fluorochrome according to Hallap et al. [12]. The fluorescent probe indicates high mitochondrial membrane potential with a high far red signal (Fig. 2).

Flow cytometry

Flow cytometric measurements were performed on a Beckman Coulter FC-500 flow cytometer equipped with a 488 nm Ar ion (20 mW) and a 635 nm red diode (25 mW) laser line. Fluorescent signals of SYBR 14, PI and Mitotracker Deep Red FM were evaluated on detectors FL 1 (525 BP), FL3 (620 SP) and FL 4 (675 BP), respectively. Forward and side scatter and fluorescent signals of 10,000 sperm events were recorded per sample using Beckman Coulter CXP acquisition software and stored as LMD files for subsequent analyses.

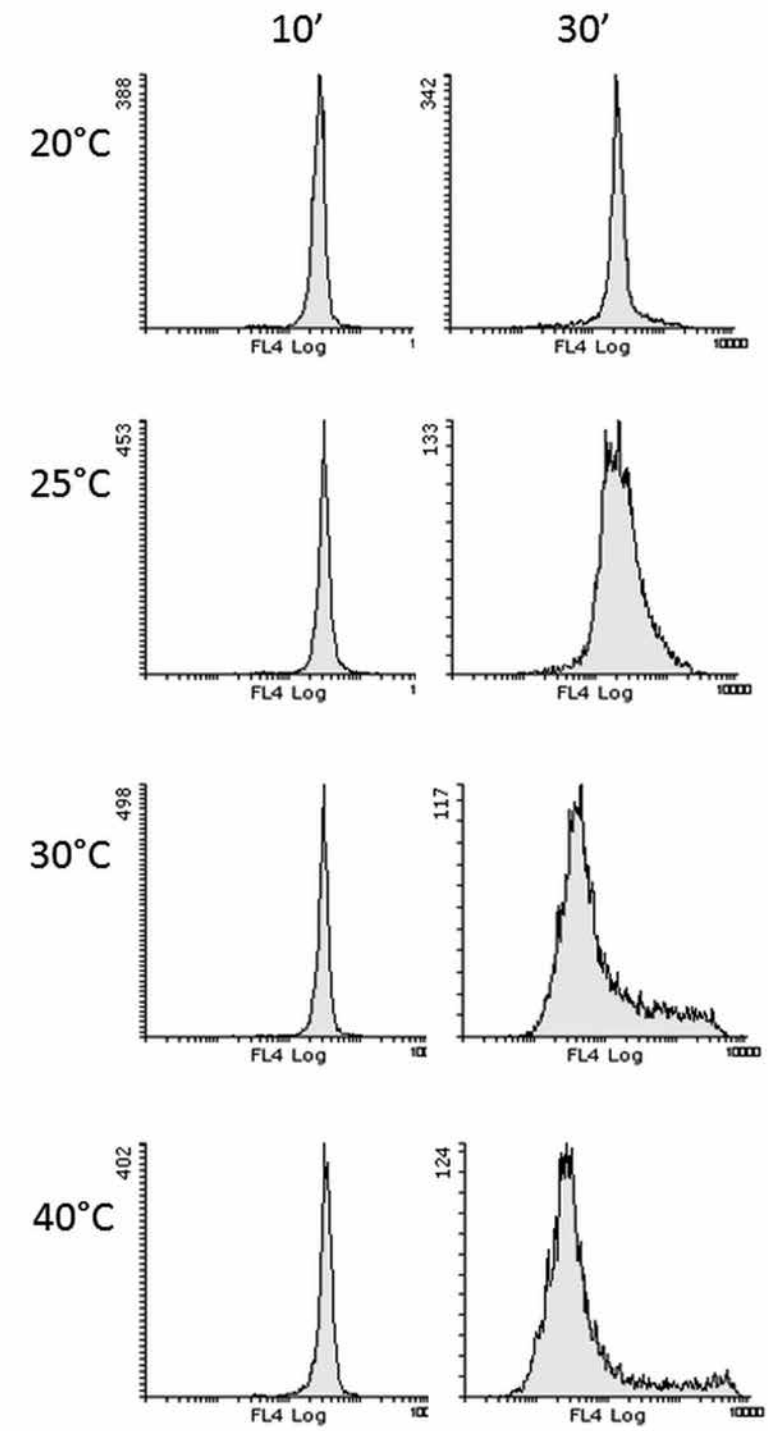

Fig. 2. Mitotracker Deep Red FM fluorescence intensity histograms of spermatozoa incubated at different temperatures for 10 or 30 minutes

Data analysis

The LMD files were analyzed with the free Flowing 2.5.1. software (www.flowing.com). The main outcome of the plasma membrane integrity analysis was the percentage of SYBR-14 positive events (interpreted as % viable). For the evaluation of mitochondrial membrane potential median fluorescence intensities (MFI – dimensionless value) of the Mitotracker Deep Red FM histograms were calculated. As spermatozoa contain more than one mitochondrion per cell and even within one cell there may be differences between individual mitochondria, in our opinion MFI may actually be a more sensitive indicator of the changes in mitochondrial membrane potential than the percentage of cells with high or low Mitotracker Deep Red FM fluorescence.

Viable % values were analysed with Repeated Measures ANOVA and post-hoc Newman-Keuls tests using Statistica for Windows 8.0 (StatSoft, Inc., 2007. STATISTICA data analysis software system, www.statsoft.com). Mitotracker Deep Red FM histogram profiles were compared with the Kolmogorov–Smirnov option of the Beckman Coulter CXP analysis software, applying sample incubated for 10 min at room temperature (20 °C) as control. Cumulative histograms were overlaid to establish the maximum absolute differences (D_{max}), and to reveal whether the differences between histograms are significant or not at $p < 0.05$ [6, 24].

RESULTS

Our results did not reveal significant differences between different incubation temperatures and exposure intervals in plasma membrane integrity. The ratio of cells with intact plasma membrane remained about 99% in all cases (Table 1).

Determining mitochondrial activity measurements, the MFI values of the Mitotracker Deep Red FM histograms increased in case of 40 °C treatment after 10 minutes exposition, while the other treatments did not influence MFI significantly. These values were 285, 287, 275 and 364 at 20, 25, 30 and 40 °C incubation temperatures, respectively.

Table 1
Percentages of viable spermatozoa incubated at different temperatures for 10 and 30 minutes

Temperature (°C)	10 min		30 min	
	mean	SD	mean	SD
20	99.67	0.23	99.77	0.12
25	99.46	0.42	99.70	0.20
30	99.70	0.10	99.83	0.06
40	99.67	0.21	99.73	0.21

Means and SD-s of three replicates.

The samples incubated at 30 or 40 °C showed significant fluorescence intensity decrease after 30 minutes exposition (MFI: 262, 296, 151 and 160 at 20, 25, 30 and 40 °C incubation temperatures, respectively).

The Kolmogorov–Smirnov test revealed significant ($p < 0.001$) differences in the Mitotracker Deep Red FM histogram profiles in all temperature treatments at both time intervals compared to the histogram of the sperm sample exposed to 20 °C for 10 minutes, regarded as control.

The D_{max}-values of the histogram comparisons are summarized in Table 2.

Table 2

Critical Kolmogorov–Smirnov D_{max} values of the Mitotracker Deep Red FM histograms of the control measurement (incubation at 20 °C for 10 min) and spermatozoa incubated at different temperatures and times

D_{max}	A-B	A-C	A-D	A-E	A-F	A-G	A-H
Mean	0.31	0.32	0.57	0.56	0.58	0.60	0.67
SD	0.26	0.09	0.31	0.25	0.30	0.29	0.23

Means and SD-s of three replicates. A: 10', 20 °C; B: 30', 20 °C; C: 10', 25 °C; D: 30', 25 °C; E: 10', 30 °C; F: 30', 30 °C; G: 10', 40 °C; H: 30', 40 °C.

DISCUSSION

Nearly 100% plasma membrane integrity of the freshly collected fish semen was showed earlier [19] and the high live-cell ratio was also observed in another species like carp [13]; striped bass [9, 10]; and zebrafish [11]. The plasma membrane lesion is a late end point of necrotic cell death. In this experiment, the intact cell ratio did not decrease in the semen samples, not even in the case of cells incubated at 40 °C for 30 minutes.

The mitochondrial membrane potential changes let us conclude that under the present experimental incubation time and temperature settings, the cells suffered sublethal damage which cannot be detected with conventional vital staining techniques. This phenomenon could be clearly observed in the case of spermatozoa with 40 °C incubation, where the mitochondria of the cells became hyperpolarized after 10 minutes, then after 30 minutes the decrease of MFI indicated the depolarization of mitochondrial membrane as compared to the sperm cells incubated at room temperature. Similar tendency was observed in sperm cells incubated at 30 °C, but less definitely. These results were considered biologically relevant as the results of Kolmogorov–Smirnov test demonstrated that the Mitotracker Deep Red FM histogram profiles significantly differed at all experimental times and temperatures from the histogram profile of the control sample (10 minutes at 20 °C). The damage of sperm mitochondria might be initiators of a series of intracellular, degenerative processes. The main role of mitochondria of inactive fish sperm cells is to maintain the intracellular ATP levels, besides other homeostatic roles. Following the sperm activa-

tion the spermatozoa use the stored ATP, instead of de novo synthesis of ATP generated by mitochondria. The increase of oxidative phosphorylation was not experienced together with the activation [16].

Similar phenomena can be observed at some mammalian species, the spermatozoa, of which contain only a few mitochondria (e.g. human – approx. 10–15 mitochondria per cell), the ATP synthesis needed for motility is mainly generated by glycolysis, the mitochondrial ATP synthesis is mainly required for sustaining plasma membrane-homeostasis [7].

The spermatozoa of prussian carp contains only 10 mitochondria per cell [17], their role might be considered similarly as limited in active motility as in human spermatozoa. The damaged mitochondria are the main sources of intracellular oxidative lesions – the reactive oxygen species (ROS) released from the damaged, fragmented mitochondria may cause lipid-peroxidation and oxidative DNA lesions and finally DNA-fragmentation [1]. Moreover, mitochondria have an important role in the maintenance of Ca^{2+}-homeostasis – the mitochondrial membrane depolarization causes decreased Ca^{2+} influx [20]. This latter and the increase in intracellular Ca^{2+} level are key components of fish sperm activation [15] and necessary for capacitation in mammalian spermatozoa [14].

According to our opinion, the present findings may provide useful information from the point of view of environment protection and conservation, may provide a basis for future experiments on fish sperm physiology, incorporating further assays such as the detection of intracellular Ca^{2+} level changes, oxidative DNA lesions and DNA fragmentations in fish spermatozoa exposed to extreme temperatures, and can be useful even for the practical specialists of aquaculture.

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