Fakulta rybářství
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Faculty of Fisheries
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of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Temperature dependency of sperm motility in different fish species

Teplotní závislost motility spermatu u různých druhů ryb

Hadiseh Dadras Asyabar

Czech Republic, Vodňany, 2018

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Name: Hadiseh Dadras Asyabar

Title of thesis:
Temperature dependency of sperm motility in different fish species
Teplotní závislost motility spermatu u různých druhů ryb

Ph.D. thesis, USB FFPW, RIFCH, Vodňany, Czech Republic, 2018, 90 pages, with the summary in English and Czech.
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz
ISBN 978-80-7514-072-2

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CHAPTER 1

GENERAL INTRODUCTION

Dadras, H., Dzyuba, B., Cosson, J., Golpour, A., Siddique, M.A.M., Linhart, O., 2017. Effect of water temperature on the physiology of fish spermatozoon function: A brief review. Aquaculture Research 48, 729–740.

It was allowed by publisher on 24th April, 2018 to include the paper in this Ph.D. thesis.

https://onlinelibrary.wiley.com/page/journal/13652109/homepage/permissions.html

My share on this work was about 70%.

Aquaculture Research

Aquaculture Research, 2017, **48**, 729–740 doi: 10.1111/are.13049

REVIEW ARTICLE

Effect of water temperature on the physiology of fish spermatozoon function: a brief review

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Abstract

Motility is a key factor in function of the spermatozoon and determines semen quality and fertilizing capacity. Effective motility occurs when sperm is diluted in a swimming solution, the adequacy of which is determined by factors varying according to fish species. Spermatozoon motility rate and velocity, as well as duration of the motility period, are influenced by the temperature of the water in which broodfish are held. Increase in temperature of swimming medium beyond the optimal increases cell metabolism, leading to an increase in velocity with rapid depletion of energy resources, promoting early cessation of movement. The aim of this review was to discuss current information on the influence of temperature on quantitative spermatozoon properties, which could affect sperm function. Our findings provide a greater understanding of fish sperm physiology and a biological foundation for the further development of spermatozoon motility investigations as well as reproduction technologies.

Keywords: flagellum, motility regulation, sperm physiology, sperm morphology

Introduction

Fish are ectotherms, with regulation of body temperature depending on external sources, and mostly have an external reproduction mode with spermatozoa and eggs released into water for fertilization. The alteration of water temperature commonly results in changes of some cellular parameters in ectodermic animals (Emri, Marian, Tron, Balkay & Krasznai 1998) although no reliable evidences are available about the changes of sperm functional parameters in fish at different temperatures. Consequently, any increase and/or decrease in the temperature in the habitats would have a significant influence on general metabolism and hence possibly reproductive efficacy (e.g. relative fecundity, number of spawning) (Wood & McDonald 1997). To date, few attempts have been made to evaluate direct or indirect impacts of climate change on aquaculture in terms of fish reproduction efficiency. The lower and upper ranges of temperature as well as the optimal temperature for fish species differ widely. Therefore, climate change might induce temperature variations which probably affect species-specific reproduction. External triggering of fish spermatozoon motility is highly dependent on the fish reproduction environment and species-specific reproductive behaviour (Dzyuba & Cosson 2014). Therefore, spermatozoon motility is expected to be strongly influenced by ambient temperature (Lahnsteiner & Mansour 2012; Thamizhselvi 2014). Studies have attempted to assess the relative influence of environmental cues that signal the occurrence of suitable spawning conditions for the regulation of

reproductive cycles of fish (De Vlaming 1972; Stacey 1979; Davies, Hanyu, Furukawa & Nomura 1986). One of the major environmental factors cueing reproductive activity of fish is water temperature (Balarin & Hatton 1979; Lowe-McConnell 1979; Eyeson 1983; Emit, Etcheri & Umoren 1989). It is generally accepted that rising water temperature within species-specific ranges stimulates reproductive development and spawning of fish. However, changes in temperature occur within the context of a natural ecosystem, and its relative influence cannot be easily determined (Resseguie & Kelsch 2008).

Spermatozoon motility is considered to accurately reflect sperm viability and fertilizing ability (Aas, Refstie & Gjerde 1991). The duration of motility and velocity of spermatozoa depend in part on the temperature of the swimming medium (Ginzburg 1972; Balarin & Hatton 1979; Morisawa, Suzuki, Shimizu, Morisawa & Yasuda 1983; Buck, Sinclair, Schapal, Cann & Levin 1999; Alavi & Cosson 2005a; Heuser, Raytchev, Krell, Porter & Nicastro 2009). In some species, high water temperatures lead to reduction in the duration of motility, but this reduction is compensated for by increased swimming speed compared to that of spermatozoa activated at lower temperatures and presenting longer duration of motility (Fig. 1). This reduction in duration of motility may be due to limited energy resources and to the influence of temperature on metabolic processes. In addition, temperature of the activation solution appears to be the most important physicochemical condition affecting spermatozoon motility (Bombardelli, Sanches, Baggio, Sykora, Souza, Tessaro & Pianal 2013); hence, a better understanding of the influence of water temperature is crucial to improvement of artificial reproduction methods (Alavi, Rodina, Policar, Kozak, Psenicka & Linhart 2007; Rodina, Gela, Kocour, Alavi, Hulak & Linhart 2007).

Babiak and Glogowski (1996) demonstrated that motility of spermatozoa is prolonged at lower temperatures. The duration of swimming time in some species such as salmonid spermatozoa has shown an inverse exponential relationship to water temperature (Lindroth 1947; Babiak & Glogowski 1996). A handful of studies have indicated that spermatozoon motility is prolonged at low temperatures (Lahnsteiner & Mansour 2012; Bombardelli *et al.* 2013), and this is generally accepted (Fig. 1). However, little specific information is available on effects of water temperature on spermatozoon motility parameters (Jezierska & Witeska 1999). The comparison of motility parameters during spermatozoon activation at different temperatures has revealed that the percentage of motile spermatozoa, as well as the initial flagellum beat frequency, increased, and, as a consequence, the duration of forward movement decreased when temperature of the swimming medium was increased (Billard & Cosson 1992).

Optimal temperature for enzymes involved in energy supply and flagellum movement leads to prolonging motility duration (Lahnsteiner & Mansour 2012). The effect of temperature on the energy status of spermatozoa is difficult to establish, as it can have multiple influences. Studies of the effect of

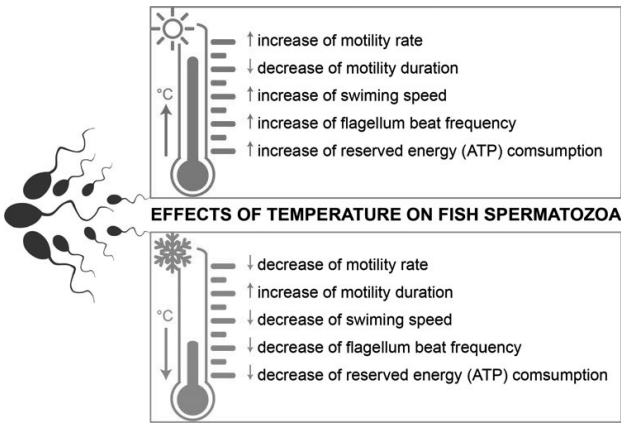

Figure 1 A conceptual model showing the effects of temperature on fish spermatozoa (data primarily from Billard & Cosson 1988, 1992; Perchec *et al.* 1995; Lahnsteiner *et al.* 1997, 1999; Cosson *et al.* 2010; Purchase *et al.* 2010; Lahnsteiner 2011; Lahnsteiner & Mansour 2012).

temperature on sperm biochemical composition have led to a better understanding of how movement characteristics of spermatozoa are related to temperature (Ishijima, Hara & Okiyama 1998). Temperature can affect the physical state of lipids, the properties of membranes, ion availability in cellular compartments, enzyme activity and consequently metabolic pathways (Hochacka & Somero 1971; Shaklee, Christiansen, Sidell, Prosser & Whitt 1977) and, more specifically, dynein motors of flagellum (Cosson 2008). The aim of this review was to discuss current knowledge in the effect of temperature on quantitative parameters of spermatozoon in different fish species.

Quantitative parameters

Spermatozoon motility rate and duration

Fish spermatozoa show several distinct features: (1) brief duration of motility (Billard & Cosson 1992), (2) immediate motility upon contact with an external medium, (3) specific differences between freshwater and marine species (Cosson, Billard, Cibert, Dreanno & Suquet 1999; Morisawa, Oda, Yoshida & Takai 1999; Cosson 2004) and (4) a variety of flagellum motility patterns exhibited among species and/or conditions (Cosson, Linhart, Mims, Shelton & Rodina 1995; Alavi, Rodina, Cosson, Psenicka & Linhart 2008). Flagellum movement is traditionally classified according to spermatozoan structure (Ishijima *et al.* 1998) on the basis that the mitochondria are elongate in fish with internal fertilization but are rudimentary and simple in fish with external fertilization (Alavi, Rodina, Viveiros, Cosson, Gela, Boryshpolets & Linhart 2009).

Motility is a characteristic function of spermatozoa, necessary to reach and penetrate the ovum. Spermatozoa of both freshwater and marine fish are immotile in the testis and in solutions of similar osmolality to that of the seminal plasma. In general, motility is acquired under the regulation of many extrinsic and intrinsic factors, and its characteristics reflect the specialized structure of the flagellum. Spermatozoa of fish inhabiting brackish and salt water generally swim for much longer periods than do those of freshwater species (Ginzburg 1963). However, the motile phase of spermatozoa of most fish species usually lasts for only 30 s to a few minutes (Billard, Cosson & Crim 1993). In most freshwater species, spermatozoa are generally motile for less than 2 min and, in many cases, forward movement is vigorous for less than 30 s (Morisawa & Suzuki 1980; Perchec, Cosson, André & Billard 1993; Billard, Cosson, Crim & Suquet 1995; Kime, Van Look, McAllister, Huyskens, Rurangwa & Ollevier 2001). In contrast, duration of spermatozoon motility in sturgeon is similar to that of many marine fish, with duration up to several minutes (Ginzburg 1972; Linhart, Mims & Shelton 1995; Alavi, Cosson, Karami, Amiri & Akhoundzadeh 2004; Cosson, Groison, Suquet, Fauvel, Dreanno & Billard 2008b).

The duration of spermatozoon motility has been reported to be significantly influenced by temperature, with an increase in duration at lower temperatures (Ginzburg 1972; Billard & Cosson 1992; Vladic & Jarvi 1997; Lahnsteiner, Berger & Weismann 1999; Alavi & Cosson 2005a; Lahnsteiner 2011; Lahnsteiner & Mansour 2012) (Table 1). Initial motility variables and motility duration are affected by temperature in an ecologically relevant range in perch (Lahnsteiner & Mansour 2012). These data confirmed that motility variables were increased at lower temperatures (range of 4–12°C) after 30 s in *Salmo trutta*, after 45 s in *Thymallus thymallus* (range of 4–16°C) and after 60 s in burbot *Lota lota* (range of 2–8°C) in water as the activating medium. In addition, a significant increase in motility and viability from 23°C up to 34°C in rosy barb *Barbus conchonius* (Thamizhselvi 2014). The percentage of motile spermatozoa in cultured Siberian sturgeon *Acipenser baerii* is influenced by temperature of water in holding tanks (Williot, Kopeika & Goncharov 2000). Temperature of milt along with that of the activating medium affected motility duration of sand sillago *Sillago ciliata* spermatozoa (Goodall, Blackshaw & Capra 1989). The duration of sperm activity was longest when milt was stored at 0°C and activated in medium of the same temperature compared with room temperature. High water temperature led to a reduction in motility duration, and temperature lower than 5°C (in range of 5–50°C) was associated with a longer period of spermatozoon motility in armoured catfish *Rhinelepis aspera* (Bombardelli *et al.* 2013).

Studies have also revealed that temperature has considerable effect on total duration and duration of forward movement in brown trout (Islam & Akhter 2011). Similarly, forward movement of lake trout *Salvelinus namaycush* spermatozoa was reported to cease completely within 29 s between 12.5 and 16°C and within 56 s at 2.25°C.

Table 1 Effects of temperature on the spermatozoa of different externally fertilizing fish species

Species	Spawning temperature	Effects of temperature on spermatozoa	References
Atlantic cod *Gadus morhua* Linnaeus, 1758	5–7°C	[] Velocity of spermatozoa increases at 6–11°C; compared to 3°C	Purchase *et al.* (2010)
Burbot *Lota lota* Linnaeus, 1758	1–4°C	[] The activity of malate dehydrogenase, pyruvate kinase, adenylate kinase and Mg^{2+}-ATPase shows alterations with changes in temperature.	Lahnsteiner and Mansour (2012)
		[] Too low/high temperatures affect on sperm motility	
		[] Spermatozoa become immotile at 2–5°C	Lahnsteiner *et al.* (1997)
		[] Spontaneous motility was observed at higher temperatures	
Common carp *Cyprinus carpio* Linnaeus, 1758	15–20°C	[] The duration of motility increases at low temperatures	Billard and Cosson (1992); Perchec *et al.* (1995)
		[] The ATP level decreases slowly at high temperatures	
European hake *Merluccius merluccius* Linnaeus, 1758	22°C	[] Flagellum beat frequency reduces with temperatures decrease	Cosson *et al.* (2010)
European perch *Perca fluviatilis* Linnaeus, 1758	7–8°C	[] The duration of motility increases at low temperatures	Lahnsteiner (2011)
Grass carp *Ctenopharyngodon idella* Valenciennes, 1844	20–30°C	[] The duration of motility increases at low temperatures	Billard and Cosson (1992)
Grayling *Thymallus thymallus* Linnaeus, 1758		[] Too low/high temperatures effect on sperm motility traits	Lahnsteiner and Mansour (2012)
Rainbow trout *Oncorhynchus mykiss* Walbaum, 1792	6–12°C	[] Duration of motility increases at low temperatures	Lahnsteiner *et al.* (1999)
		[] Flagellum beat frequency reduces with a decrease in temperature, but the duration of motility increases	Billard and Cosson (1988, 1992)
		[] A negative relationship were observed between ambient temperature and available ATP stores	
		[] The initial flagellum beating frequency rises with increasing of temperature	Cosson *et al.* (1985)
Rosy barb *Pethia conchonius* Hamilton, 1822	18–22°C	[] Longevity of spermatozoa increases up to 34°C	Thamizhselvi (2014)
Sand sillago *Sillago ciliata* Cuvier, 1829	*	[] The duration of motility reduces at high temperatures	Bombardelli *et al.* (2013)
Sea trout *Salmo trutta* Linnaeus, 1758	6–7°C	[] Significant effects on the motility period and duration of progressive movement	Schlenk and Kahmann (1938)
		[] Motility is affected by too low/high temperatures	Lahnsteiner and Mansour (2012)
		[] The activity of malate dehydrogenase, pyruvate kinase, adenylate kinase and Mg^{2+}-ATPase shows alterations with changes in temperature	
		[] Significant effect on overall duration of motility at 3–4°C	Vladic and Jarvi (1997)
Senegalese sole *Solea senegalensis* Kaup, 1858	*	[] The percentage of motility and initial velocity increases at high temperatures	Cavaleiro Diogo (2010)
Siberian sturgeon *Acipenser baerii* Brandt, 1869	12–16°C	[] The duration of motility decreases with increasing of temperatures	Williot *et al.* (2000)
Spot croaker *Leiostomus xanthurus* Lacepède, 1802	*	[] The activity of isozymes of malate dehydrogenase can be modified by the changes in ambient temperature	Schwantes and Schwantes (1982)

*Data not available.

Spontaneous motility in spermatozoa of burbot has been observed at temperatures above 5°C in seminal plasma (Lahnsteiner, Berger, Weismann & Patzner 1997). Vladic and Jarvi (1997) reported markedly longer motility duration of spermatozoa for brown trout and Atlantic salmon *Salmo salar* at 3–4°C, significantly longer than that observed at 16°C and 28°C. It has been confirmed that spermatozoa are motile for longer at 20°C than at 26°C or 30°C in common carp *Cyprinus carpio* and at 30°C in grass carp *Ctenopharyngodon idella* (Billard & Cosson 1992). A decreasing trend in spermatozoon motility duration was observed in common carp at lower temperatures of activation solution (Billard, Cosson, Perchec & Linhart 1995). However, no differences were found in the motility duration of brown trout spermatozoa at temperatures from 2 to 28°C, but in Atlantic salmon, motility duration decreased with increasing temperature (Vladic & Jarvi 1997). In brown trout, a decrease of 15% in motility rate was observed at 8°C compared with 4°C; in burbot, motility rate was reduced by 20% and the swimming velocity for 20 μm s^{-1} at 2°C compared with 4°C. In grayling, initial motility variables were not affected in the temperature range (6–8°C); at which, natural spawning occurs (Lahnsteiner & Mansour 2012). Among the four tested temperatures 10°C, 12.5°C, 15°C and 17.5°C in *A. baerii*, the highest and lowest spermatozoa motility was obtained at 10°C and 17.5°C (Williot *et al.* 2000). In streaked prochilod *Prochilodus lineatus*, motility duration was longest at 17°C and decreased at lower and higher temperatures (Romagosa, Souza, Sanches, Baggio & Bombardelli 2010). Considering the above-mentioned reports regarding influence of temperature on sperm motility duration, decreasing water temperature below ecologically relevant levels of species may prolong movement. Although the effect of temperature on sperm motility duration is species specific, overgeneralization should not be considered.

Spermatozoon velocity

There is little reported evidence that temperature differentially affects various characteristic parameters, such as progressive motility, straight-line velocity and curvilinear velocity (Cavaleiro Diogo 2010). In a study of Senegalese sole *Solea senegalensis*, data suggested that an activation solution at 20°C increased spermatozoon velocity within 60 s after activation, compared with 16°C (Cavaleiro Diogo 2010). In addition, temperatures that yield the highest swimming velocities were similar for water and SMPS (spermatozoon motility prolonging saline solution) at 4–6°C for brown trout and burbot and 8–16°C for grayling (Lahnsteiner & Mansour 2012). At 12.5°C and 16°C, the reported forward velocity of spermatozoa of grayling was high (160–164 μ s^{-1}) at 4-s post-activation in freshwater. After 8, 16 and 26 s, these values were 85–91, 24–33 and 2–5 μ s^{-1}, respectively (Lahnsteiner & Mansour 2012). Also, in European perch, motility rate and swimming velocity were higher at 4°C than at 8, 12 or 16°C after 60 min when temperature ranged from 12 to 20°C (Lahnsteiner 2011). Spermatozoon velocity did not increase in brown trout, burbot or grayling, but, in some cases, decreased with higher temperatures, while the effect on the linearity index was minor (Lahnsteiner & Mansour 2012). Atlantic cod *Gadus morhua* spermatozoa showed higher swimming velocity at 6–11°C than at 3°C and 21°C (Purchase, Butts, Alonso-Fernandez & Trippel 2010). This is because the energy resources of fish spermatozoa are limited, an increase in velocity associated with a temperature rise in the swimming solution leads to decreased duration of motility, and conversely, lowering the swimming temperature results in prolonged motility along with reduced velocity and flagellum beat frequency (Ginzburg 1972; Stoss 1983; Cosson, Billard, Gatti & Christen 1985). Temperature effects on the flagellum beat frequency of spermatozoa vary (Alavi & Cosson 2005a) which probably could be due to the adaptation of each species to environmental conditions.

Flagellum beat frequency

Motility characteristics are directly related to spermatozoon morphology and structure. Differences that can affect motility are observed among teleost species in flagellum length, sperm viability and number of spermatozoon mitochondria (Baccetti, Burrini, Gibertini, Mazzini & Zerunian 1984; Lahnsteiner & Patzner 2008). The spermatozoon flagellum beat frequency increases with a rise in temperature (Billard & Cosson 1988; Cosson, Groison, Fauvel & Suquet 2010). With a decrease in temperature, beat frequency is reduced, and the duration of motility increases (Billard & Cosson 1988). Reduced flagellum beat frequency and

longer duration of motility with decrease in temperature are likely associated with ATP stores (Cosson, Groison, Suquet, Fauvel, Dreanno & Billard 2008a; Cosson *et al.* 2008b). As the ATP reserves are depleted throughout the motile period, a gradual decrease in flagellum beat frequency and spermatozoon motility occurs (Christen, Gatti & Billard 1987; Cosson *et al.* 2008a,b). Higher flagellum beat frequencies require increased energy consumption, resulting in decreased motility duration due to limitations in the availability of ATP (Christen *et al.* 1987; Billard & Cosson 1988; Cosson *et al.* 2010).

The flagellum beat frequency increases with rise of temperature in spermatozoa of European hake *Merluccius merluccius* (Cosson *et al.* 2010). Billard and Cosson (1988, 1992) found that beat frequency of flagella in rainbow trout *Oncorhynchus mykiss* spermatozoa at different temperatures to be low at 5°C and to increase rapidly at about 14°C, while stabilize at values above 21°C. They also observed that initial frequency was 25 Hz at 5°C, 45 Hz at 15°C and more than 80 Hz at 25°C (Billard & Cosson 1988).

In spermatozoa of European hake, flagellum beat frequency was reduced with temperature decrease, while the duration of motility increased (Cosson *et al.* 2010). Similar results were obtained in spermatozoa of rainbow trout (Cosson *et al.* 1985; Billard & Cosson 1988, 1992). The temperature of an activation solution is known to affect spermatozoa, as it increases cell metabolism, causing an increase in velocity with more rapid depletion of scarce energy resources, promoting early motility cessation. This is due to the inability of fish spermatozoon mitochondria to synthesize ATP rapidly enough to compensate for its consumption by flagellum beating.

ATP content

Among fish species, longer motility duration and higher swimming speed are accomplished at high energy costs (Alavi & Cosson 2005b). Spermatozoon motility depends on the energy released via hydrolysis of adenosine triphosphate (ATP) by dynein ATPase to produce flagellum beating (Gibbons 1981). The energy-producing pathways, especially oxidative phosphorylation by mitochondria, are active during immotile storage and movement (Billard & Cosson 1990; Lahnsteiner *et al.* 1999) but are ineffective at maintaining ATP at constant levels (Christen *et al.* 1987; Lahnsteiner, Patzner & Weismann 1993; Lahnsteiner *et al.* 1999). This is a limiting factor in spermatozoon motility duration (Christen *et al.* 1987) and in sperm viability during storage (Lahnsteiner *et al.* 1999). Temperature rise leads to an increase in spermatozoon velocity but with shorter duration of motility, due to ATP exhaustion via hydrolysis by dynein ATPase (Billard & Cosson 1990; Perchec, Cosson, Andre & Billard 1995). It is generally accepted that longer duration of motility, which leads to higher fertilization success, is likely due to higher availability of ATP (Vladic, Afzelius & Bronnikov 2002). Some observations suggest a negative relationship of ambient temperature with available ATP stores *in vivo* (Billard & Cosson 1988, 1992). In common carp, spermatozoon motility duration was longer, and the decrease in ATP levels slower, at 2°C than at 22°C (Perchec *et al.* 1995).

Seminal plasma composition

Seminal plasma produced by the sperm duct provides an ionic environment that maintains the viability of spermatozoa after their release from the testes (Ciereszko 2008). Correlations between seminal plasma composition and spermatozoon motility have been shown in Atlantic salmon (Hwang & Idler 1969), common carp (Kruger, Smith, Van Vuren & Ferreira 1984), bleak *Alburnus alburnus* (Lahnsteiner, Berger, Weismann & Patzner 1996a), rainbow trout (Lahnsteiner, Berger, Weismann & Patzner 1998), Persian sturgeon *Acipenser persicus* (Alavi *et al.* 2004) and chinook salmon *Oncorhynchus tshawytscha* (Rosengrave, Taylor, Montgomerie, Metcalf, McBride & Gemmell 2009). Identification of the optimum temperature for spermatozoon enzyme activity may provide information on how spermatozoon metabolism is affected by temperature and whether all enzyme systems within the spermatozoon show similar responses. These data can provide insight into the metabolism of spermatozoa and contribute to optimization of enzyme assays that are used in basic research and for quality control of fresh and frozen-thawed semen (Lahnsteiner, Berger, Weismann & Patzner 1996b; Lahnsteiner *et al.* 1998; Cabrita, Sarasquete, Martínez-Páramo, Robles, Beirao, Pérez-Cerezales & Herráez 2010).

In general, enzyme activity increases along with temperature to a critical level and abruptly

declines with further increase in temperature (Bergmeyer 1985; Johnston & Dunn 1987). The activity of malate dehydrogenase, pyruvate kinase, adenylate kinase and Mg^{2+}-ATPase shows alterations with change in temperature (Lahnsteiner & Mansour 2012). Potential activity decrease due to suboptimal temperature can be compensated by an increase in enzyme-substrate affinity (Lahnsteiner & Mansour 2012). The activity of enzymes may also be affected by ions and cosubstrates, and the requirements for, and availability of which, can change with temperature (Hochacka & Somero 1971).

In fish sperm, temperature affects enzymes *via* enzyme-substrate affinity positive modulators increasing the enzyme-substrate affinity and the reverse (Hochacka & Somero 1971). Isozymes of a given enzyme can manage activity over a wide range of temperatures (Lin & Somero 1995), differing in their enzyme-substrate affinity, cosubstrate and ionic requirements (Gelman, Cogan & Mokady 1992). cAMP is believed to constitute the main signalling pathway to activate rainbow trout spermatozoa (Morisawa & Okuno 1982), but cAMP concentration has been shown to increase slowly, especially at low temperatures, and to peak after 100% spermatozoon motility (Cosson *et al.* 1995). Investigations of the thermos-stability of spermatozoon enzymes revealed that, in brown trout and burbot, highest activity of malate dehydrogenase, pyruvate kinase, adenylate kinase and Mg^{2+}-ATPase was at 4°C and corresponded with the optimum temperature for spermatozoon motility. Peroxidase activity showed an optimal temperature of 20°C in all species investigated (Lahnsteiner & Mansour 2012). In the spot croaker *Leiostomus xanthurus*, it was demonstrated that isozymes of malate dehydrogenase are present in differing quantities, and their activity can be modified by changes in ambient temperature (Schwantes & Schwantes 1982).

The effect of temperature on enzyme activity is generally complex (Lahnsteiner & Mansour 2012). Temperature is known to influence the phase disposition of lipids in cell membranes (Lee & Chapman 1987). Change in temperature shows an impact on membranes, particularly on the formation of lipid clusters that break down the lipid bilayer selective permeability (Watson 2000). Phospholipids contribute the largest lipid fraction of spermatozoon membranes, and of these, phosphatidylcholine and phosphatidylethanolamine are the major components (Mann 1964; Mann & Lutwak-Mann 1981).

Cooling is a major stressor, as a result of which membrane-bound phospholipids reorient themselves into a configuration that disrupts membrane function and permeability (Amann & Graham 1993; Lessard, Parent, Leclerc, Bailey & Sullivan 2000). As membranes cool, lipids undergo transition from their normal fluid to a liquid crystalline state, in which the fatty acyl chains become disordered (Parks & Lynch 1992; Watson 2000; Medeiros, Forell, Oliveria & Rodrigues 2002). Freshly collected boar spermatozoa are sensitive to a rapid reduction in temperature because of lipid phase transition and phase separation processes (Schulze, Henning, Rüdiger, Wallner & Waberski 2013). The peak phase transition temperature for phospholipids within the membrane of stallion, boar and bull spermatozoa are 20.7°C, 24.0°C and 25.4°C, respectively. The peak transition temperature for glycolipids in stallion spermatozoon membranes is 33.4°C compared with 36.2°C and 42.8°C for boar and bull, respectively (Parks & Lynch 1992). In mouse, a high proportion of immobile lipids in plasma membrane on the midpiece and spermatozoon head are temperature sensitive (Christova, James, Cooper & Jones 2002). Overall, there are little available data on the effect of temperature on enzyme activity and lipids in fish spermatozoon.

Spermatozoon morphological characteristics

Spermatozoa demonstrate extreme sensitivity to minute changes in temperature of surrounding media (Le Minh, Lim, Min, Park, Son, Lee & Chang 2011). Temperature influences on boar sperm physiology (Althouse, Wilson, Kuster & Parsley 1998; Vyt, Maes, Sys, Rijsselaere & Van Soom 2007) are well documented. The direct effects of high temperatures on spermatozoa are reported to be decrease in the percentage of live and morphologically normal spermatozoa (Casady, Myers & Legates 1953; Borg, Lunstra & Christenson 1993), alterations in acrosome and plasma membrane integrity (Meyerhoeffer, Wettemann, Coleman & Wells 1985; Borg *et al.* 1993; Sanocka & Kurpisz 2004; Safaa, Emarah & Saleh 2008) and spermatozoon chromatin stability (Paul, Murray, Spears & Saunders 2008; Pérez-Crespo, Pintado & Gutiérrez-Adán 2008). Mitochondria and spermatozoon membranes are known as sites

particularly vulnerable to damage from sudden temperature changes (Brouwers & Gadella 2003). Temperatures below the threshold for phase transition are associated with an increase in membrane permeability with subsequent leakage of cations, reduction of enzyme activity and alteration of membrane proteins (De Leeuw, Colenbrander & Verkleij 1990; Parks & Lynch 1992; Drobnis, Crowe, Berger, Anchordoguy, Overstreet & Crowe 1993).

Generally, cold shock damage is manifested as decline in cell metabolism, altered membrane permeability, loss of intracellular components, irreversible loss of spermatozoon motility and an increase in spermatozoon mortality. The damage to the cellular membranes is of highest significance, as it has a carry-over effect on other cell structures and functions (Lemma 2011). Lipid diffusion in the equatorial segment and post-acrosomal plasma membranes of mouse spermatozoa is responsive to changes in temperature, especially ranging from 20°C to 37°C, whereas the midpiece plasma membrane is considerably less so (Christova *et al.* 2002). The sensitivity of the mouse spermatozoon plasma membrane to changes in temperatures from 20°C and 37°C is of particular interest in relation to spermatozoon survival in the cauda epididymidis (Christova *et al.* 2002).

The quantitative measure of temperature effect on a given ion channel is described by the notation Q10, which denotes the fold-change in conductance over a span of 10°C. Specifically, large temperature effects (Q10 >4) have been documented for a number of ion channels such as the Shaker (K^+), ClC-0 (Cl^-), L-type (Ca^{2+}) and transient receptor potential (TRP) (Ca^{2+}) channels (McKemy 2005, 2007). Recently, TRPM8, a non-selective cation channel in spermatozoa, has been reported to be an important molecular thermo-sensor, as it can be activated by low temperature in mammals (De la Peña, Mälkiä, Cabedo, Belmonte & Viana 2005; McKemy 2005). In addition, the presence of thermo-sensitive TRP channels has been reported for the first time in the spermatozoa of early vertebrates as well as of aquatic animals (Majhi, Kumar, Yadav, Swain, Kumari, Saha, Pradhan, Goswami, Saha, Samanta, Maity, Nayak, Chattopadhyay, Rajakuberan, Kumar & Goswami 2013).

Conclusion

Temperature is one of the fundamental physical regulatory factor affecting the life of fish through control of all reproductive processes from gamete development, maturation, spawning, embryogenesis and hatching to larval development and survival. Several experimental data have been confirmed that some changes in the sperm temperature environment cause to initiate sperm motility. The direct effect of temperature on qualitative and quantitative spermatozoon parameters seems to be highly species specific. The available experimental evidences regarding the impact of or temperature of activating media used for sperm quality assessment are still insufficient to elicit general understanding of physiological mechanisms by which temperature affects sperm functionality. However, further studies are required to determine either direct or indirect physiological effect of temperature on fish spermatozoa.

Acknowledgments

This study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects 'CENAKVA' (No. CZ.1.05/2.1.00/01.0024), 'CENAKVA II' (No. LO1205 under the NPU I programme) and COST (No. LD14119), by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 134/2013/Z) and by the Czech Science Foundation (No. P502/15-12034S). The authors greatly appreciate valuable help from The Lucidus Consultancy with English editing and Prof. William Lee Shelton, University of Oklahoma, USA, for his valuable comments on the revised manuscript.

Conflict of interest

None of the authors have any conflict of interest to declare.

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GENERAL OVERVIEW

Knowledge of sperm physiology and regulation of sperm quality is essential to successful production in aquaculture systems (Cabrita et al., 2014). Sperm quality is affected by various factors (Billard et al., 1995), with spermatozoon motility regarded as one of its most reliable predictors. To evaluate sperm fertilizing potential, motility both *in vivo* and *in vitro* is commonly assessed. Spermatozoa are immotile in fish sperm ducts due to the high osmolality and the ion content of seminal plasma (Lahnsteiner et al., 1995) and become motile upon release into the surrounding aqueous environment during natural reproduction or into diluents during artificial reproduction (Darszon et al., 1999; Alavi and Cosson, 2006; Cosson et al., 2008 a,b). Spermatozoon activation occurs as a result of external factors including low K+ concentration in acipenserids and hypo-osmotic shock in freshwater teleost fishes (Linhart et al., 2003).

The external triggering of motility is dependent on the surrounding media. Immediately after activation, the volume of cytoplasm in freshwater fish spermatozoa increases due to hypotonicity. Common carp *Cyprinus carpio* spermatozoa increase in volume several-fold as a result of water influx in response to hypo-osmotic conditions (Perchec Poupard et al., 1997). Less dramatic changes occur in rainbow trout *Oncorhynchus mykiss* spermatozoa under similar osmotic conditions (Cabrita et al., 1999), suggesting a species-specific tendency of fish spermatozoa to swell in hypotonic conditions (Bondarenko, 2013; Dzyuba and Cosson, 2014).

Adenosine triphosphate (ATP) is the main source of energy for spermatozoon motility, and some studies in fish have reported a correlation between ATP level before activation and the motility percentage and fertilization rate (Bencic et al., 1999, 2000; Ingermann et al., 2003; Zilli et al., 2004). A high level of energy consumption has been observed in freshwater fish spermatozoa demonstrated by rapid activation of motility of short duration (Christen et al., 1983; Gibbons et al., 1985; Cosson et al., 1991, 1997, 1985, 2000; Perchec et al., 1995). For example, ATP stores in rainbow trout (Christen et al., 1987) and common carp (Perchec et al., 1995) were shown to be significantly decreased during the peak motility phase. Studies of the mechanisms of fish spermatozoon motility show that activation is accompanied by rapid depletion of ATP content, an increase of spermatozoon volume due to hypotonic swelling, and a rise in respiration rate (Robitaille et al., 1987; Perchec et al., 1995; Perchec-Poupard et al., 1997; Lahnsteiner et al., 1999; Dzyuba et al., 2001). These cell changes might be linked to an increase in reactive oxygen species (ROS) level endogenously induced by cell respiration (Saleh and Agarwal, 2002).

Sperm quality evaluation may involve understanding the mechanisms by which sperm behavior is influenced (Cabrita et al., 2014). It has been generally accepted that motility duration and velocity of fish spermatozoa depends on temperature of the swimming medium (Ginzburg, 1972; Balarin and Hatton, 1979; Morisawa et al., 1983; Buck et al., 1999; Alavi and Cosson, 2005; Heuser et al., 2009). The mechanisms involved in influence of temperature on sperm motility reflect a complex network of regulation. Species-specific differences in spermatozoon activation mechanisms with respect to their optimal spawning temperatures are of special interest.

The overall objective of this research was to determine the effect of temperature on spermatozoon function in different representative freshwater fish species via analyses of motility parameters, antioxidant enzyme activity, lipid composition, osmolality, and calcium ion requirements.

EFFECT OF TEMPERATURE ON SPERMATOZOON MOTILITY CHARACTERISTICS

Water temperature is a dominant environmental factor influencing function of spermatozoa in fish exhibiting external fertilization (Alavi and Cosson, 2005), yet temperature has been rarely investigated in studies of spermatozoon function. Since fish are ectothermic and usually exhibit an external mode of fertilization, spermatozoon motility function might be related to the ambient water temperature (Stoss, 1983; Lahnsteiner and Mansour, 2012). Sperm motility parameters, including percentage of motile spermatozoa, duration of motility, and swimming velocity, have been considered primary indicators of quality and fertilizing ability (Billard, 1978; Stoss, 1983; Cosson et al., 2008c). Because of the demonstrated relationship between temperature and spermatozoon metabolism, one might expect temperature to affect sperm swimming velocity (Mehlis and Bakker, 2014). Accumulating evidence has shown that changes in ambient temperature may have a significant effect on metabolism and reproductive potential of fish sperm (Ginzburg, 1968; Wood and McDonald, 1997; Stoss, 1983). The duration of motility and velocity of fish spermatozoa may depend on the temperature of activation medium (Ginzburg, 1972; Morisawa et al., 1983; Alavi and Cosson, 2005; Heuser et al., 2009). However, studies of the effect of temperature on spermatozoon motility traits have produced ambiguous results. Research on the effects of temperature on common carp spermatozoa demonstrated that increased temperature leads to higher velocity and shorter duration of motility (Perchec et al., 1995), while a negative relationship between temperature and velocity was reported in Senegalese sole *Solea senegalensis* (Beira˜o et al., 2011) and brown trout *Salmo trutta* (Lahnsteiner., 2012), and dependency of velocity on an optimum temperature was found in Atlantic cod *Gadus morhua* (Purchase et al., 2010) and burbot *Lota lota* (Lahnsteiner and Mansour, 2012). No influence of temperature on velocity of gilthead seabream *Sparus aurata* spermatozoa was observed by Lahnsteiner and Caberlotto (2012). Therefore, drawing general across-species conclusions with respect to sperm motility characteristics in relation to temperature is not possible, due to differences in optimum spawning temperature of the studied fish species.

Generally, sperm motility is initiated by a specific regulatory mechanism (Section 1.2) with a motility period of 30 s to 5 min. Motility duration of sturgeon sperm, including of sterlet *Acipenser ruthenus*, is longer than that of freshwater teleosts, possibly due to participation of metabolic enzymes in sterlet spermatozoon motility, which is not observed in other freshwater fish species (Dzyuba et al., 2016; Gazo et al., 2017). In the current research, we focused on freshwater fish species with distinct spermatozoon motility initiation mechanisms corresponding to their individual spawning temperatures. A comprehensive literature search revealed little information on the influence of temperature on fish sperm motility traits.

Effect of temperature on spermatozoon antioxidant response

The production of ROS is an inevitable consequence of aerobic activity in sperm cells. The ROS level may be enhanced due to an imbalance of ROS generation and scavenging activity, leading to spermatozoon damage (Sharma and Agarwal, 1996). High temperature may induce oxidative stress in spermatozoa, resulting in damage such as changes in enzyme activity and peroxidative insult affecting cell structures. Certain cell changes, including a rise in respiration rate and concomitant rapid depletion of ATP, are associated with the sperm activation process (Lahnsteiner et al., 1999; Boryshpolets et al., 2009) leading to an increase in ROS level (Saleh and Agarwal, 2002) accompanied by a reduction in spermatozoon motility and fertilizing ability (Aitken et al., 1989 a,b; de Lamirande and Gagnon, 1992). The enzyme protective system (oxidant defensive enzymes) counteracts cell damage caused by excessive production

of ROS (Mansour et al., 2006; Kefer et al., 2009; Lahnsteiner and Mansour, 2010; Cabrita et al., 2014). The major enzymes acting as oxidant scavengers to remove ROS include superoxide dismutase (SOD) and catalase (CAT) (Słowińska et al., 2013).

Quantification of thiobarbituric acid-reactive substance (TBARS) content is commonly used to assess lipid peroxidation level (Mansour et al., 2006). The TBARS content has been shown to be increased as a consequence of oxidative stress induced by thermal shock (Lushchak and Bagnyukova, 2006) resulting in formation of ROS and loss of cellular homeostasis (He et al., 2015). Various effects of temperature on TBARS content have been reported in mammalian spermatozoa (Foldesy and Bedford, 1982; Alvarez and Storey, 1985). A negative relationship of sperm quality with ROS level is attributed to lipid peroxidation potentially leading to reduction in sperm membrane integrity (Sikka et al., 1995; Lahnsteiner et al., 2010; Liu et al., 2014).

The unique structure of spermatozoa makes them vulnerable to oxidative stress, mainly due to the low volume of cytoplasm and large quantities of polyunsaturated fatty acids (PUFA) in the spermatozoon membrane, rendering them vulnerable to lipid peroxidation (Alvarez et al., 1987; Sikka, 2004; Słowińska et al., 2013). Studies of the effects of temperature on sperm biochemical composition are required for a better understanding of the relationship of movement characteristics of sperm with temperature (Ishijima et al., 1998). The exploration of optimum temperature for sperm enzyme activity may be an important step toward understanding how spermatozoon metabolism is influenced by temperature.

The *in vitro* assessment of patterns of metabolic activity in fish sperm during the motile period with respect to temperature and the comprehensive investigation of the effect of temperature on lipid peroxidation and antioxidant response may facilitate greater understanding of sperm function.

EFFECT OF TEMPERATURE ON SPERMATOZOON LIPID COMPOSITION

Sperm lipid composition is believed to play a crucial role in supplying energy for prolonged motility (Baeza et al., 2015), and hence can be associated with fertilization ability (Gholami et al., 2011). Phospholipids have been reported to be a major source of energy in sea urchin (Mita and Yasumasu, 1983; Mita, 1991). Studies of various sea urchin genera have demonstrated that endogenous phospholipid content decreases following initiation of spermatozoon flagellar movement (Rothschild and Cleland, 1952; Mohri, 1957; Mohri, 1964; Kozhina et al., 1978; Mita and Yasumasu, 1983).

Cholesterol is considered to regulate the lipid chain order (van Blitterswijk et al., 1987), controlling membrane properties and functions including fluidity and permeability to water and other molecules (Demel and De Kruyff, 1976; Hazel and Williams, 1990). Due to the particular structure of PUFAs, they are crucial participants in sperm cell membrane fluidity and motility (Israelachvili et al., 1980; Meizel and Turner, 1983; Conquer et al., 2000; Safarinejad et al., 2010; Baeza et al., 2015) as well as fertilization potency (Lenzi et al., 1996).

Environmental stressors including temperature lead to changes in physical properties of the membrane lipids in living cells (Los and Murata, 2004). It is generally agreed that the phase disposition of lipids in cell membranes is affected by temperature (Lee and Chapman, 1987). A trade-off between high PUFA and ROS production has been assumed in fish sperm, potentially leading to oxidative damage (Li et al., 2010; Cabrita et al., 2014). Changes in membrane properties and metabolic pathways of spermatozoa along with lipid peroxidation have been shown to be associated with oxidative damage mediated by the ambient temperature (Hochacka and Somero, 1971; Shaklee et al., 1977). Available data concerning changes in fish spermatozoa lipid composition relative to temperature are scarce. The question remains

whether the motility patterns of fish spermatozoa at different temperatures are associated with changes in lipid profile.

TEMPERATURE DEPENDENCY OF SPERM ACTIVATION

In all freshwater fish species studied thus far, physiological factors including osmolality, ions, and pH are considered to contribute to the activation of spermatozoon motility. It has been demonstrated that the rate and duration of sperm motility are influenced by intracellular parameters including concentrations of ions, particularly Ca2+ (Morisawa, 1985; Cosson et al., 1989) and pH (Krasznai et al., 1995; Lahnsteiner et al., 1996) along with extracellular temperature (Billard, 1986; Williot et al., 2000; Alavi and Cosson, 2005) and pH (Billard, 1986; Cosson and Linhart, 1996; Alavi and Cosson, 2005).

Eurasian burbot *Lota lota*, a unique freshwater fish species, has demonstrated sperm motility triggering in seminal plasma with elevated temperature (Lahnsteiner et al., 1997). Two studies have shown contradictory effects of temperature on the activation of sperm motility (Lahnsteiner et al., 1997; Zuccarelli et al., 2007). According to Lahnsteiner et al. (1997), sperm of Eurasian burbot became spontaneously motile in seminal plasma at temperatures >5 °C. No sperm activation was observed in North American burbot *Lota lota maculosa* sperm when the temperature was raised from 2 °C to 10 °C (Zuccarelli et al., 2007). The atypical effect of temperature on Eurasian burbot sperm function suggests a species-specific phenomenon that is not observed in other freshwater fish. According to the above cited studies, Ca2+ ions and osmolality, either alone or together, in combination with temperature, may be involved in regulation of sperm activation in burbot. The available data regarding the effect of temperature on activation of burbot spermatozoon motility imply that further studies are required to identify either a direct or indirect physiological effect of temperature. To address this, research was conducted to assess underlying key physiological factors contributing to activation of spermatozoon in Eurasian burbot.

RESEARCH GOALS

The primary goal of this research was to assess the effects of temperature on spermatozoon motility in different freshwater fish species and comprised three objectives:

1. To determine the effect *in vitro* of temperature on motility parameters and antioxidant enzyme activity in common carp *Cyprinus carpio*, rainbow trout *Oncorhynchus mykiss*, and sterlet *Acipenser ruthenus* spermatozoa.
2. To ascertain the effect of temperature on lipid composition of activated spermatozoa of common carp *Cyprinus carpio*.
3. To identify patterns of spermatozoa motility activation in Eurasian burbot *Lota lota* in relation to temperature.

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CHAPTER 2

THE *IN VITRO* EFFECT OF TEMPERATURE ON MOTILITY AND ANTIOXIDANT RESPONSE OF SPERMATOZOA IN DIFFERENT FRESHWATER FISH SPECIES

2.1. Dadras, H., Dzyuba, V., Cosson, J., Golpour, A., Dzyuba, B., 2016. The *in vitro* effect of temperature on motility andantioxidant response of common carp *Cyprinus carpio* spermatozoa. Journal of Thermal Biology 59, 64–68.

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2.2. Dadras, H., Dzyuba, V., Golpour, A., Xin, M., Dzyuba, B., 2018. *In vitro* antioxidant enzyme activity and sperm motility at different temperatures in sterlet *Acipenser ruthenus* and rainbow trout *Oncorhynchus mykiss*. (Manuscript)

My share on this work was about 80%.

Journal of Thermal Biology 59 (2016) 64–68

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

The *in vitro* effect of temperature on motility and antioxidant response of common carp *Cyprinus carpio* spermatozoa

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ARTICLE INFO

Article history:
Received 8 January 2016
Received in revised form
3 May 2016
Accepted 7 May 2016
Available online 11 May 2016

Keywords:
Antioxidant enzymes
Carp
Lipid peroxidation
Spermatozoa

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.

1. Introduction

Spermatozoon motility is an important factor in fertilization capacity (Aas et al., 1991). 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 (Lahnsteiner and Mansour, 2012; Beirão et al., 2014; Dadras et al., 2016). A trade-off between increased spermatozoon velocity and decreased motility duration at higher temperatures has been reported for common carp *Cyprinus carpio* (Perchec et al., 1995), grass carp *Ctenopharyngodon idella* (Jezierska and Witeska, 1999), hake *Merluccius merluccius* (Cosson et al., 2010), and burbot *Lota lota* (Lahnsteiner and Mansour, 2012).

The sperm activation process is accompanied by a series of cellular changes including a rise in respiration rate and rapid depletion of ATP (Lahnsteiner et al., 1999; Dzuba et al., 2001; Boryshpolets et al., 2009). 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 (Li et al., 2009).

Some changes in sperm viability might be related to ambient temperature (Nandre et al., 2013), and temperature-induced stress has been associated with enhanced generation of ROS and oxidative stress in spermatogenic cells (Nichi et al., 2006a). During times of environmental stress such as increased temperature, ROS levels can increase dramatically (Nichi et al., 2006a) 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 (Li et al., 2010; Cabrita et al., 2014; Gazo et al., 2015). 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 (Mansour et al., 2006).

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) (Mansour et al., 2006; Lahnsteiner and Mansour, 2010; Cabrita et al., 2014); 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.

2. Materials and methods

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

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http://dx.doi.org/10.1016/j.jtherbio.2016.05.003
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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} $CaCl_2$, 0.8 mmol L^{-1} MgSO4, and 20 mmol L^{-1} HEPES (pH 7.8) (Lahnsteiner and Mansour, 2012). Activation medium (AM) containing 10 mM Tris–HCl buffer, pH 8.0, 1 mM $CaCl_2$, 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 (~5 μl) 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). Temperature fluctuation during motility analysis is shown in Fig. 1, with three replicate tests for each temperature.

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 × *g* for 5 min at 4 °C. The TBARS content of the supernatant was measured spectrophotometrically according to Asakawa and Matsushita (1980): Briefly, 0.025 mL butylated hydroxytoluene solution (22 mg in 10 mL ethanol), 0.025 mL ferric chloride solution (27 mg of $FeCl_3$ $6H_2O$ 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 × *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 10^5 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.

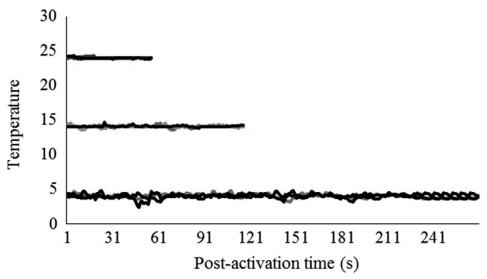

Fig. 1. Supplementary data about temperature fluctuation during spermatozoon motility analysis in Cyprinus carpio.

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 × *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 × *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 (1974). 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 mM Tris–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 H_2O_2, according to the method of Marklund et al. (1981). The CAT activity was calculated from the H_2O_2 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.

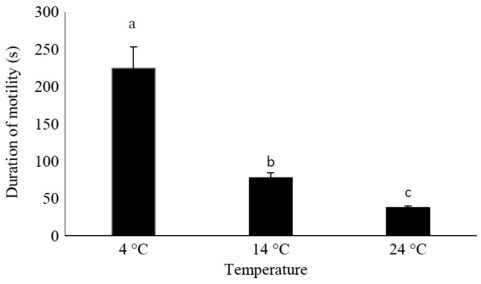

Fig. 2. Effect of temperature on *C. carpio* spermatozoon motility duration. Data are mean ± SD. Different lowercase letters indicate significant difference ($P < 0.05$).

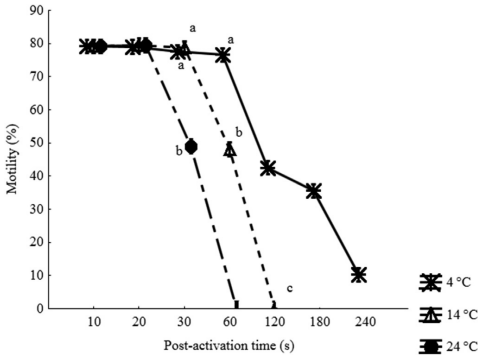

Fig. 3. Effect of temperature on *C. carpio* sperm motility rate. Data are mean ± SD. Different lowercase letters indicate significant differences ($P < 0.05$).

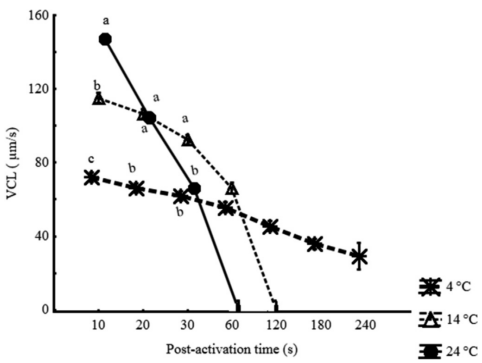

Fig. 4. Effect of temperature on *C. carpio* spermatozoon velocity. Data are mean ± SD. Different lowercase letters indicate significant differences ($P < 0.05$).

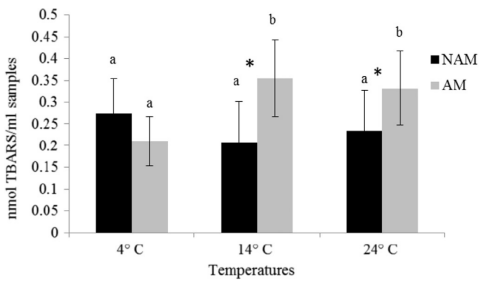

Fig. 5. TBARS content of *C. carpio* spermatozoon at different temperatures. Asterisks indicate significant difference between AM and NAM solutions. Data are mean ± SD. Different lowercase letters indicate significant differences ($P < 0.05$).

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).

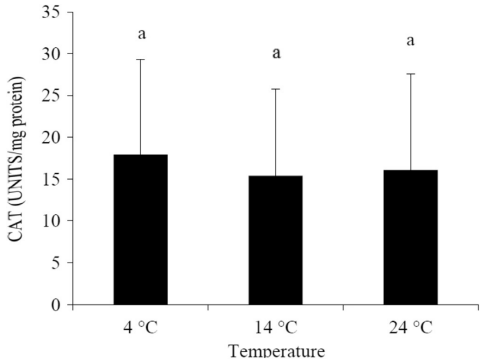

Fig. 6. Effect of temperature on catalase activity in *C. carpio* spermatozoa. Data are mean ± SD. Same lowercase letters indicate no significant difference (P > 0.05).

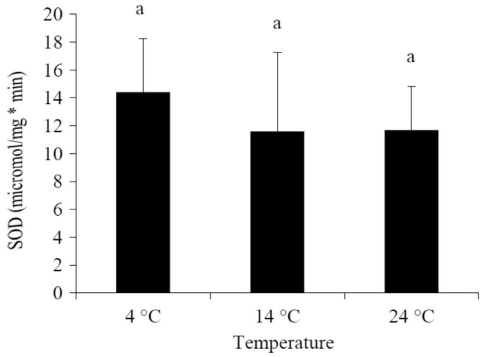

Fig. 7. Effect of temperature on SOD in *C. carpio* spermatozoa. Data are mean ± SD. Same lowercase letters indicate no significant difference ($P > 0.05$).

3. Results

3.1. Spermatozoon motility

The duration of spermatozoon motility was longest at 4 °C [Fig. 2]. The motility rate did not show differences relative to temperature within 20 s of motility initiation [Fig. 3]. At 30 s post-activation, motility rate was significantly higher at 4 and 14 °C than at 24 °C [Fig. 3]. However, the highest spermatozoon motility rate was observed after 60 s post-activation, at 4 °C [Fig. 3]. After 10 s, spermatozoa showed significantly higher velocity at 24 °C

[Fig. 4]. While, at 60 s post-activation the velocity was higher at 14 °C than at 4 °C [Fig. 4].

3.2. TBA-reactive substance content

The TBARS level was significantly elevated with AM at 14 and 24 °C compared to 4 °C and peaked at 0.35 nmol mL^{-1} supernatant at 14 °C. In contrast to spermatozoa in AM, TBARS concentration did not vary with temperature in non-motile spermatozoa. A significantly higher TBARS level was observed in motile spermatozoa compared to non-motile spermatozoa at 14 and 24 °C [Fig. 5].

3.3. Antioxidant enzyme activity

The activity of CAT and SOD are shown in Figs. 6 and 7, respectively. In contrast to TBARS, the level of SOD and CAT activity remained similar with all treatments.

4. Discussion

Subjection of spermatozoa to ambient temperature transitions has been discussed in some literatures (Ginzburg, 1972; Alavi and Cosson, 2005; Lahnsteiner and Mansour, 2012). 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 (Billard and Cosson, 1988; Cosson et al., 2010). 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 (Lahnsteiner and Mansour, 2012).

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 (Diogo, 2010; Lahnsteiner, 2011). 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 (Purchase et al., 2010). 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 (2012) 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 (Lahnsteiner and Mansour, 2012). At 120 s, motility rate and velocity were also significantly higher at 4–6 °C than at $\geq$8 °C (Lahnsteiner and Mansour, 2012). This implies that temperature may strongly influence spermatozoon motility parameters by its impact on energy resources (Alavi and Cosson, 2005). Differences in enzyme activity can be attributed to temperature changes (Heise et al., 2007); 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 (Nair et al., 2009). This could be attributed to insufficient activity of the antioxidant enzymes at higher temperatures (Lushchak and Bagnyukova, 2006). 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 (Salehi Nezhad et al., 2013). In addition, partial inactivation of SOD in ram spermatozoa under freezing condition (cold shock) has been reported by Marti et al. (2008). 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 (Orzołek et al., 2013). On the contrary, Horky et al. (2015) 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 (El-Tohamy et al., 2012). 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 (Lushchak and Bagnyukova, 2006), probably due to loss of cellular homeostasis accompanied by the formation of ROS (He et al., 2015). 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 (Li et al., 2010). Salehi Nezhad et al. (2013) 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 (Alvarez and Storey, 1985; Foldesy and Bedford, 1982), however no significant effect of temperature on TBARS levels was found in bovine epididymidis sperm stored at 4 and 34 °C (Nichi et al., 2006b).

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.

Acknowledgments

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects CENAKVA (No. CZ.1.05/2.1.00/01.0024), CENAKVA II (No. LO1205 under the NPU I program), by the Grant Agency of the University of South Bohemia in České Budejovice (No. 125/2016/Z), the COST Office (Food and Agriculture COST Action FA1205: AQUAGAMETE) and by the Czech Science Foundation (No. P502/15-12034S). The authors greatly appreciate valuable help from The Lucidus Consultancy with English editing.

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In vitro antioxidant enzyme activity and sperm motility at different temperatures in sterlet *Acipenser ruthenus* and rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

Influence of in vitro temperature on sperm antioxidant enzyme activity, thiobarbituric acid-reactive substance (TBARS) content, and motility parameters was evaluated in sterlet *Acipenser ruthenus* and rainbow trout *Oncorhynchus mykiss*. Sperm activation was conducted at 4, 14, and 24 °C in both species. Duration of motility was significantly greater at 4 °C than at 14 and 24 °C in both species. At 60 sec post-activation, the velocity of sterlet spermatozoa was highest at 24 °C. This trend continued to 420 s post-activation. In rainbow trout, at 10 s post-activation, the highest velocity was observed at 14 °C. Significantly higher catalase activity was seen at 4 °C in both species. No significant difference in spermatozoon superoxide dismutase activity among temperatures was observed. In sterlet, TBARS content was significantly higher at 24 °C compared to other temperatures, but in rainbow trout was highest at 4 °C. Results are presumed due to species-specific reaction at studied temperatures.

Keywords: *Antioxidant enzymes, Fish, Sperm function, Temperature*

INTRODUCTION

High quality sperm is a prerequisite for successful fertilization in fish breeding programs (Rurangwa et al., 2001, 2004). Sperm motility is the most evaluated criterion for fish sperm quality due to its correlation with fertility (Stoss, 1983; Aas et al., 1991). Temperature has been clearly demonstrated to be one of the principle environmental factors affecting sperm characteristics (Lahnsteiner and Mansour, 2012; Beirão et al., 2014; Dadras et al., 2016). Studies have shown that sperm quality parameters and fertilization potency in aquaculture depend on the temperature of the activating medium and rearing water (Billard et al., 1995; Williot et al., 2000; Dadras et al., 2016, 2017). The energy resources of fish spermatozoa are limited (Ingermann, 2008), and rise in temperature may lead to increased spermatozoon velocity and low duration of motility in common carp *Cyprinus carpio* (Perchec et al., 1995; Dadras et al., 2016). In contrast, studies have shown no effect of temperature on velocity of gilthead seabream *Sparus aurata* spermatozoa (Lahnsteiner and Caberlotto, 2012). A general trend of sperm motility relative to temperature cannot be determined, due to wide variation in experimental conditions and differences in fertilization modes of fish species.

Changes in energy metabolism of spermatozoa, such as elevation of respiration rate, resulting in rapid depletion of ATP have been reported during sperm activation (Lahnsteiner et al., 1999; Boryshpolets et al., 2009), when reactive oxygen species (ROS) are produced through cell respiration (Saleh and Agarwal, 2002). In addition, interaction of the cell with environmental stressors such as increased temperature potentially leads to exogenous ROS generation (Lushchak, 2011; Madeira et al., 2013; Kim et al., 2015). An imbalance of

production and elimination of ROS is detrimental to sperm function (Sharma and Agarwal, 1996), with quality negatively affected by increasing levels of ROS causing lipid peroxidation and damage to the sperm membrane (Sikka et al., 1995; Lahnsteiner et al., 2010; Liu et al., 2014). Spermatozoa are susceptible to oxidative stress, and potentially lipid peroxidation, due to the limited quantity of cytoplasm in mature sperm and large quantities of polyunsaturated fatty acids (PUFA) in the cell structure (Alvarez et al., 1987; Sikka, 2004). In aquatic animals, particularly fish, lipid peroxidation in spermatozoa is a serious issue due to the large quantities of PUFA (Li et al., 2010).

Temperature outside the optimal range can be a stressor that enhances generation of ROS and oxidative stress (Nichi et al., 2006a). The antioxidant system of spermatozoa includes oxidant defence enzymes catalase (CAT) and superoxide dismutase (SOD) that play prominent roles in detoxification of ROS to prevent oxidative damage (Lahnsteiner and Mansour, 2010; Cabrita et al., 2014).

Sterlet and rainbow trout are important commercial species with different spawning temperature ranges. Studies have demonstrated that sperm motility parameters, particularly motility duration, is influenced by temperature. It is generally expected that, alteration of metabolic activities correspond to sperm motility status at each temperature reveals different level of ROS production. Therefore, determination of activity of oxidative stress indicators during motility at different temperatures may reveal oxidative status of the spermatozoon relative to temperature.

The goal of this study was to determine the influence of temperature on motility characteristics, antioxidant enzyme activity, and lipid peroxidation of sterlet and rainbow trout spermatozoa *in vitro*.

MATERIALS AND METHODS

Fish

Semen was collected from six apparently healthy mature sterlet males (0.8–1.0 kg) held during the natural spawning season in 4 m^3 plastic recirculating system tanks in pond water with constant flow of 20 l min^{-1} (8-10 °C) at the hatchery of the Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. Prior to initiation of the experiment, water temperature was gradually increased to 14-15 °C over the course of 24 h, and fish were held an additional four days until beginning of experiment.

Six mature rainbow trout males (0.5-0.6 kg) obtained from ponds of the Anin fish farm, Klatovske Rybarstvi, Czech Republic were maintained in 4000 l aquaculture tanks at 4 °C for 14 days before stripping.

Sperm collection

Spermiation in sterlet was induced by intramuscular injection of carp pituitary powder dissolved in 0.9% NaCl solution at 4 mg kg^{-1} body weight. After 24 h, sterlet sperm were collected at the urogenital sinus by aspiration using a 4-mm plastic catheter connected to a plastic syringe. Trout semen samples were collected by gentle abdominal massage. The samples were carefully taken to avoid contamination of sperm with blood, urine, and faeces. Then sperm samples were stored on ice before and during experiments.

Media for inhibition and activation of sperm motility

Sperm motility inhibiting medium (SMIM) composed of 103 mmol l^{-1} NaCl, 40 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 0.8 mmol l^{-1} $MgSO_4$, and 20 mmol l^{-1} HEPES (pH 7.8) was prepared (Lahnsteiner and Mansour, 2012).

To trigger motility in both sterlet and rainbow trout sperm, an activation medium (AM) containing 10 mmol l^{-1} Tris–HCl buffer, pH 8.0, 2 mmol l^{-1} $CaCl_2$, and 0.125% pluronic acid was utilized.

For thiobarbituric acid-reactive substance (TBARS) analysis, a solution consisting of 10 mmol l^{-1} Tris–HCl buffer, pH 8.0, 20 mmol l^{-1} NaCl, and 1 mmol l^{-1} KCL was used as non-activation medium (NAM) for sterlet. The NAM for rainbow trout was modified to contain 0.9% NaCl and 28 mmol l^{-1} KCL.

Motility analysis

Sperm motility in both species were activated with adding fresh semen to AM solution (~50 µl) placed on a microscope slide (adjusted with desired *in vitro* temperatures) using the tip of a dissecting needle to mix within 2 s. Spermatozoon motility parameters at each temperature were recorded over 60, 180, 300, 420, 720, 1020 and 1320 s post-activation for sterlet and 10, 20, 30, 60, 70 and 80 s post-activation for rainbow trout using a CCD video camera (Sony, SSCDC50AP, Japan operating in 25 fps mode) mounted on an inverted microscope equipped with a cooling stage (Olympus IX83, United Kingdom) and a X20 NIC contrast objective lens. A thermoblock (HLC BO50/15, Germany) was used to adjust the temperature of the AM. Temperature of sample during the motility period was monitored via copper-constantan thermocouple (Omega, L-044T, Taiwan) using a data logger thermometer (Omega, HH127, Taiwan).

Spermatozoon motility duration, percentage of motile spermatozoa after activation and curvilinear velocity (VCL, µm s-1) were chosen for sperm motility analysis. The sperm motility records were performed in triplicate at desired temperatures. The recorded videos were analyzed by microimage analyzer (Olympus Micro Image 4.0.1. for Windows, Japan) to estimate spermatozoa motility parameters.

Antioxidant enzyme activity

Prior to enzyme extraction, ~200 µl of fresh rainbow trout sperm was diluted in 1 ml of SMIM and centrifuged (1000 x g) for 10 min at 4 °C. The sperm pellet was then diluted once more in SMIM to eliminate the seminal plasma. The sperm suspension was centrifuged again 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, 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. The extracted samples were kept at -80 °C until analysis.

For sterlet, 1ml of fresh sperm was diluted in 9 ml of SMIM and subsequently centrifuged (1000 x g) for 10 min at 4 °C. The remainder of the procedure was as described for rainbow trout semen samples.

The activity of CAT and SOD for both species were estimated at 4, 14, and 24 °C. Two temperature points (4 and 14 °C) have selected as close ranges to optimal spawning temperature of species interest. Additionally, an extremely high temperature point (24 °C) was applied to assess sperm function by increased water temperatures, a situation that might consider on species with higher spawning temperatures. The activity of SOD (EC 1.15.1.1) was measured by spectrophotometer equipped with a thermo-regulated cuvette holder at 420 nm following Marklund and Marklund (1974). To determine SOD activity at tested temperatures, inhibition of pyrogallol autoxidation by the sample was used. The autoxidation of 0.2 mmol l^{-1} pyrogallol in air-equilibrated 50 mmol l^{-1} Tris–HCl buffer containing 1 mmol l^{-1} EDTA, pH 8.2, was inhibited by the addition of the assayed sample. One unit of the enzyme is generally

defined as the quantity of enzyme that inhibits the reaction (pyrogallol autoxidation) by 50%. The specific activity of SOD was expressed as unit mg-1 of protein.

The CAT activity (EC 1.11.1.6) was measured using a spectrophotometer equipped with a thermo-regulated cuvette holder at 240 nm. A reaction medium containing 10 mmol l^{-1} K^+ phosphate buffer with 0.1 mmol l^{-1} EDTA, pH 7.4, and 15 mmol l-1 H_2O_2 was used according to Marklund et al. (1981). The CAT activity was calculated from the H_2O_2 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. The Bradford method (1976) was used to determine protein content. Measurements were carried out in triplicate for each sample.

Lipid peroxidation assay

Two sets of solutions were used to prepare samples for measurement of TBARS content after the required time for cessation of motility at each temperature. For rainbow trout, 150 µl of fresh semen was incubated in 1350 µl of AM at 4, 14, and 24 °C. The method was also used for sterlet spermatozoa with dilution of 1 ml of fresh semen in 9 ml of AM. The same dilution rate for each species was used to dilute fresh semen in corresponding NAM solutions. Samples were centrifuged at 1000 x g for 5 min at 4 °C. The TBARS content of the supernatant was measured spectrophotometrically with the method of Asakawa and Matsushita (1980): Briefly, 0.025 ml butylated hydroxytoluene solution (concentration in mol l^{-1}), 0.025 ml ferric chloride solution (27 mg of $FeCl_3$ $6H_2O$ in 10 ml water concentration in mol l^{-1}), 0.375 mL of 0.2 mol l^{-1} glycine-hydrochloric acid buffer, pH 3.6, and 0.375 ml TBA reagent (0.5 % TBA and 0.3% sodium dodecyl sulphate) were added to 0.4 ml supernatant. A boiling water bath was used to heat capped tubes ~15 min. 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 measured at 535 nm against a blank with deionized water substituted for the biological sample. To calculate TBARS level, a molar extinction coefficient of 1.56 105 M-1 cm^{-1} was used. The TBARS content 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 statistical analysis.

Statistical analysis

Before data analysis by ANOVA, the values of spermatozoon velocity were checked for normality of data distribution and homogeneity of variance using Shapiro-Wilk's and Levene's tests, respectively. The parametric one-way ANOVA followed by 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 Mann-Whitney U-test with Bonferroni correction were performed for comparison of motility rate, duration of motility, CAT and SOD activity, and TBARS content. All values were expressed as mean values ± standard deviation (SD), with alpha level of significance set at 0.05. All analyses and plots were conducted using the STATISTICA v. 12 computer program (Statsoft Inc., USA).

RESULTS

Spermatozoon motility

Sterlet *Acipenser ruthenus*

The duration of spermatozoon motility was significantly greater at 4 °C than at 14 and 24 °C. (Fig. 1 A). Within 60 sec of motility initiation, the percentage of motile spermatozoa at the investigated temperatures did not show differences (Fig. 2 A). At 180 sec, the percentage of motile spermatozoa was significantly higher at 4 °C than at 24 °C (Fig. 2 A). This trend was observed up to 1020 s post-activation (Fig. 2 A). At 60 s post-activation, the velocity was highest at 24 °C (Fig. 3 A) and at 180 sec post-activation (Fig. 3 A).

Rainbow trout *Oncorhynchus mykiss*

The duration of spermatozoon motility was significantly greater at 4 °C than at 14 and 24 °C (Fig. 1 B). At 10 s post-activation, no difference was observed in the percentage of motile spermatozoa among investigated temperatures (Fig. 2 B). At 20 sec post activation, the percentage of motile spermatozoa at 24 °C was significantly lower than at 4 and 14 °C (Fig. 2 B). At 10 s, spermatozoon velocity was significantly higher at 14 °C compared to other studied temperatures (Fig. 3 B), while, at 20 s, spermatozoon velocity at 14 °C had decreased to be significantly lower than that at 4 °C (Fig. 3 B).

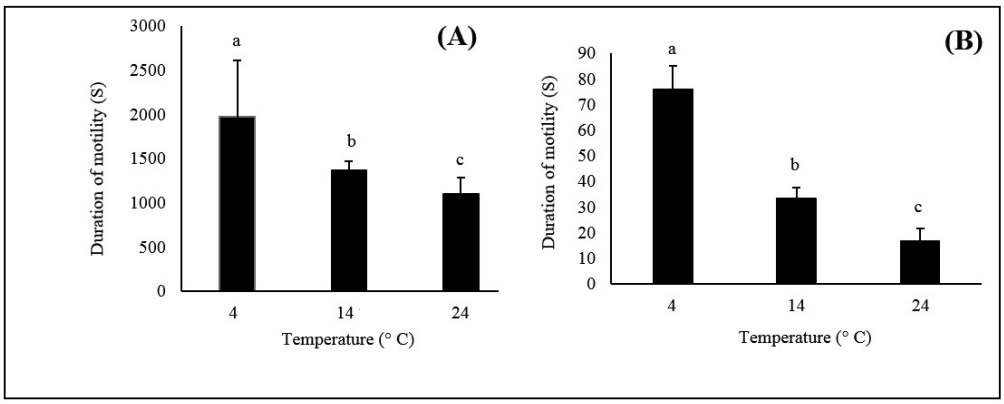


Fig. 1. *Effect of temperature on A. ruthenus (A) and O. mykiss (B) spermatozoon motility duration. Data are mean ± SD. Different lower case letters indicate significant difference (P <0.05).*

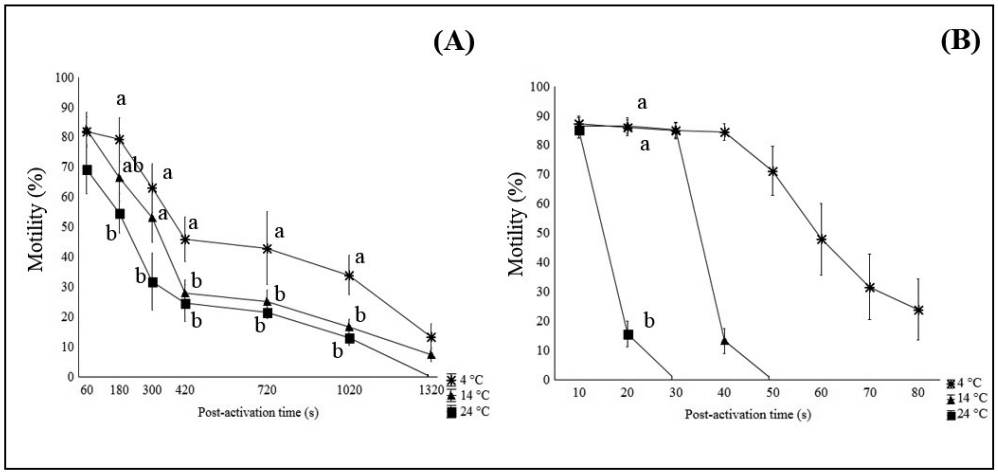


Fig. 2. *Effect of temperature on A. ruthenus (A) and O. mykiss (B) sperm motility percentage. Data are mean ± SD. Different lower case letters indicate significant difference within the same time point (P <0.05)*

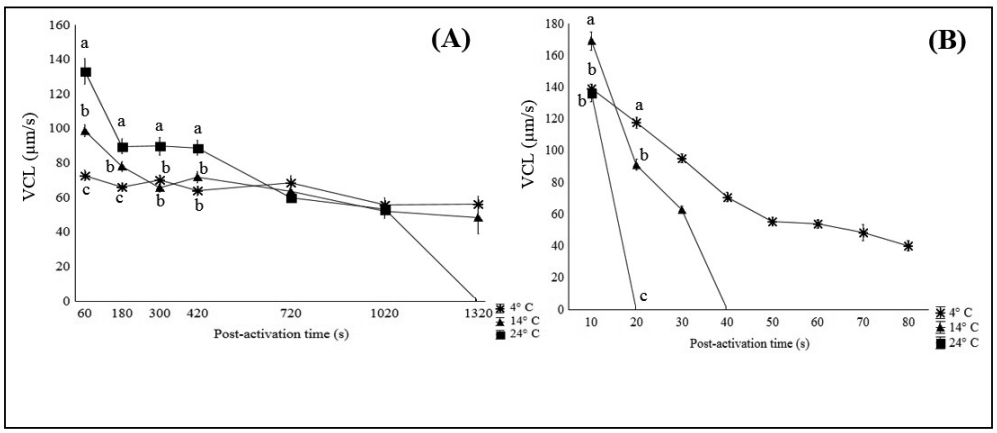


Fig. 3. *Effect of temperature on A. ruthenus (A) and O. mykiss (B) spermatozoon velocity. Data are mean ± SD. Different lower case letters indicate significant difference within the same time point (P <0.05).*

Antioxidant enzyme activity

Sterlet *Acipenser ruthenus*

Spermatozoon CAT activity was significantly elevated when sperm was activated at 4 °C compared to 14 and 24 °C (Fig. 4 A). The SOD activity showed no significant difference among temperatures (Fig. 4 B).

Rainbow trout *Oncorhynchus mykiss*

Spermatozoon CAT activity was significantly higher at 4 °C than at 14 and 24 °C (Fig. 4 C). Similar to sterlet, SOD activity was similar at tested temperatures (Fig. 4 D).

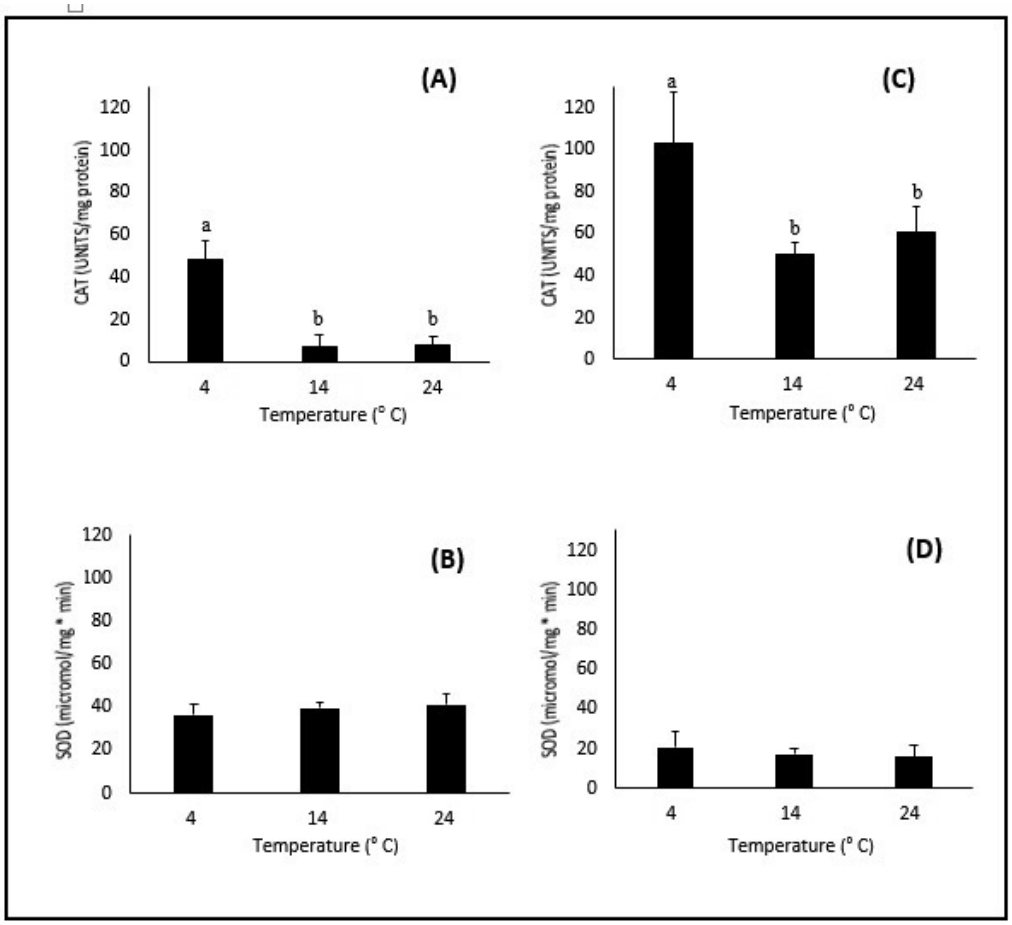


Fig. 4. *Effect of temperature on CAT and SOD activity in A. ruthenus and O. mykiss spermatozoon. (A): CAT activity in A. ruthenus spermatozoon, (B): SOD activity in A. ruthenus spermatozoon, (C): CAT activity in O. mykiss spermatozoon, (D): SOD activity in O. mykiss spermatozoon. Data are mean ± SD. Different lower case letters indicate significant difference (P <0.05).*

TBARS content

Sterlet *Acipenser ruthenus*

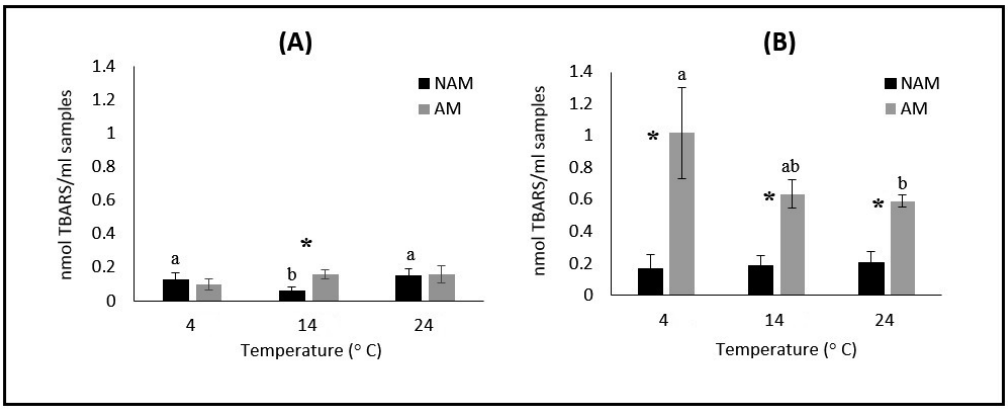
The TBARS content in motile spermatozoa showed no significant difference at different temperatures (Fig. 5 A). The TBARS level in non-motile spermatozoa was significantly lower at 14 °C compared to 4 and 24 °C (Fig. 5 A). There was a significant difference in TBARS content between non-motile and motile spermatozoa at 14 °C (Fig. 5 A).

Rainbow trout *Oncorhynchus mykiss*

The TBARS content did not show significant differences with respect to temperature in non-motile spermatozoa (Fig. 5 B). In contrast, the TBARS level was significantly elevated when sperm was activated at 4 °C (1 nmol ml^{-1} supernatant) compared to 24 °C (Fig. 5 B).

The TBARS level was significantly higher in motile compared to non-motile spermatozoa at all studied temperatures (Fig. 5 B).

Fig. 5. *TBARS content of A. ruthenus (A) and O. mykiss (B) spermatozoon at different in vitro temperatures. Data are mean ± SD. Different lower case letters indicate significant difference (P <0.05). Asterisks indicate significant difference between AM and NAM solutions.*



DISCUSSION

Numerous studies have addressed the effect of temperature on fish spermatozoon characteristics (Alavi and Cosson, 2005; Lahnsteiner and Mansour, 2012, Dadras et al., 2016), however the effect of temperature on spermatozoon enzyme activity and lipid peroxidation has been little explored. The present study showed spermatozoon motility duration in sterlet and rainbow trout to be significantly greater at 4 °C compared to 14 and 24 °C suggesting intensive ATP consumption by activated spermatozoa at higher temperatures (Perchec et al., 1995). We observed the highest VCL in sterlet sperm at 24 °C, while, in rainbow trout spermatozoa, highest VCL was at 14 °C. Our findings with respect to sterlet were in accordance with previously obtained results on common carp sperm motility (Dadras et al., 2016) at the same temperatures. Other studies have reported varying effects of temperature according to species. Negative effect of higher temperatures on VCL have been reported in Senegalese sole *Solea senegalensis* (Beira˜o et al., 2011) and brown trout *Salmo trutta* (Lahnsteiner, 2012); specific temperature dependency in Atlantic cod *Gadus morhua* (Purchase et al., 2010) and burbot *Lota lota* (Lahnsteiner and Mansour, 2012); and no influence of temperature on VCL in gilthead seabream (Lahnsteiner and Caberlotto, 2012). The reported differences might be attributed to species characteristics and to temperature ranges used in the studies (Mehlis and Bakker, 2014).

Several studies have reported that semen contains enzymes and metabolites considered primary ROS scavengers (Mansour et al., 2006; Lahnsteiner and Mansour, 2010; Cabrita et al., 2014). An antioxidative function has been demonstrated in semen of teleost fish (Ciereszko et al., 1999; Lahnsteiner et al., 2010), and the present study provides new insight into the effect of *in vitro* temperature on antioxidant enzyme activity. We observed considerable SOD and CAT activity at all temperatures, with no significant differences in SOD activity detected at different temperatures in either species. Similar results were observed in common carp spermatozoon SOD activity (Dadras et al., 2016). According to Lahnsteiner et al. (2010), CAT, which is responsible for protecting spermatozoa against oxidative damage, presents low

activity in seminal plasma and spermatozoa. In the present study, CAT activity was higher in both species at lower temperatures, which might be attributed to a decrease in activity with rising temperature caused by a progressive spontaneous inactivation of the CAT or may be the result of destruction of CAT by excess hydrogen peroxide (Morgulis et al., 1926). On the contrary, CAT activity in common carp spermatozoa showed no dependence on in vitro temperature (Dadras et al., 2016). The underlying cause of antioxidant status fluctuation in fish spermatozoa in stressful situations, particularly temperature-related, is not clearly understood. The function of antioxidant enzymes in spermatozoa at various temperatures is complex process (Lahnsteiner and Mansour, 2012) that might be modified via differences from the environmentally relevant spawning temperature. Since most cytoplasm is eliminated from the spermatozoon during the final stages of spermatogenesis (Alvarez et al., 1987), it is particularly susceptible to oxidative damage induced by thermal stress (Lushchak and Bagnyukova, 2006). Lahnsteiner et al. (2010) and Shiva et al. (2011) have suggested that the sperm antioxidant enzyme system could play an important role in preventing plasma membrane lipid peroxidation.

In the current study, no difference was revealed in TBARS content of sterlet motile spermatozoa at different temperatures, while, in rainbow trout motile spermatozoa, the highest level of TBARS was detected at 4 °C, suggesting that, despite the highest activity of CAT being at low temperature in both studied species, the enzyme activity was likely insufficient to protect the cell from oxidative damage. This is in line with Nichi et al. (2006b) who reported no significant influence of temperature on TBARS levels in bovine epididymis sperm stored at 4 and 34 °C. In contrast, TBARS content of common carp spermatozoa showed the opposite pattern at the temperatures used in the present study (Dadras et al., 2016). Numerous studies of different animals have observed irregular TBARS levels associated with fluctuating temperature (Foldesy and Bedford, 1982; Alvarez and Storey, 1985). It is assumed that sperm of rainbow trout has a higher fatty acid content, especially DHA (22:6, n-3), than that of sterlet. An explanation for sperm species-specific levels of TBARS may be the presence of discrepancies in antioxidant capacity and in fatty acid content, particularly of PUFAs.

CONCLUSION

Based on our results, enzyme activity and TBARS content of semen are affected by species-specific thermoregulation within a natural spawning temperature range for each species. Nonetheless, it is still difficult to draw general conclusions about the effect of temperature on sperm metabolism. Further studies are required to elucidate the effect of water temperature on sperm function and antioxidant capacity in fish.

ACKNOWLEDGMENTS

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects CENAKVA (No. CZ.1.05/2.1.00/01.0024), CENAKVA II (No. LO1205 under the NPU I program), CZ.02.1.01./0.0/0.0/16_025/0007370 Reproductive and Genetic Procedures for Preserving Fish Biodiversity and Aquaculture and by the Grant Agency of the University of South Bohemia in Ceske Budejovice (125/2016/Z).

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CHAPTER 3

ANALYSIS OF COMMON CARP *CYPRINUS CARPIO* SPERM MOTILITY AND LIPID COMPOSITION USING DIFFERENT *IN VITRO* TEMPERATURES

Dadras, H., Sampels, S., Golpour, A., Dzyuba, V., Cosson, J., Dzyuba, B., 2017. Analysis of common carp *Cyprinus carpio* sperm motility and lipid composition using different in vitro temperatures. Animal Reproduction Science 180, 37–43.

It was allowed by publisher on 22nd April, 2018 to include the published paper in this Ph.D. thesis.

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Animal Reproduction Science 180 (2017) 37–43

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Analysis of common carp *Cyprinus carpio* sperm motility and lipid composition using different *in vitro* temperatures

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ARTICLE INFO

Article history:
Received 19 September 2016
Accepted 24 February 2017
Available online 6 March 2017

Keywords:
Lipids
Motility
Sperm
Temperature

ABSTRACT

In fish, sperm quality is frequently associated with sperm motility variables. The response of sperm motility to different temperatures varies among species and plasma membrane lipid composition may contribute to variations in findings in previous research. In the present study, sperm motility and lipid composition were analysed between motile or immotile carp *Cyprinus carpio* sperm at different *in vitro* temperatures (4, 14 and 24 °C). The duration of the period over which sperm motility is sustained was longer at 4 °C compared with 14 and 24 °C; while sperm velocity was greatest at 24 °C. Motile sperm had lesser proportions of 18:3 (n-3) and 22:6 (n-3) fatty acids at 24 °C relative to immotile sperm. There was no difference in fatty acid composition of motile and immotile sperm at 4 and 14 °C. The total phospholipid content was less in motile than in immotile sperm at 24 °C. At 24 °C, phosphatidylcholine and phosphatidylserine proportions were less in motile than immotile sperm. It is concluded that lipid composition of motile carp sperm is affected by temperature, with greater temperatures associated with reduced lipid content, elevation of sperm curvilinear velocity and a decreased duration of the period over which motility is sustained.

1. Introduction

Fish sperm are unique in that, in most species, sperm cells remain quiescent in gonad seminal plasma (Morisawa and Morisawa, 1990; Golpour et al., 2015). Sperm become motile upon release into the surrounding medium and motility continues for a short period (Cosson, 2004), and this motility is temperature-dependent (Billard and Cosson, 1988; Diogo, 2010; Lahnsteiner and Mansour, 2012; Dadras et al., 2016; Dadras et al., 2017). Sperm motility is associated with the series of cellular changes, including increased sperm cell respiration rate and a marked decrease in ATP content (Dreanno et al., 1999; Dzuba et al., 2001; Boryshpolets et al., 2009; Dzyuba and Cosson, 2014). During the period over which sperm motility is sustained, changes in sperm cell viability might be related to surrounding temperature (Nandre et al., 2013), and stress induced by temperature has been associated with concomitant enhanced generation of reactive oxygen species (ROS) and oxidative stress (Nichi et al., 2006).

Membrane lipid composition has been proposed to have a role in specific functions, because it promotes the development of micro-domains in the sperm cell plasma membranes that are associated with differing membrane

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http://dx.doi.org/10.1016/j.anireprosci.2017.02.011
0378-4320/

fluidity and permeability and sperm cell fertilizing capacity (Wassall and Stillwell, 2009). In carp sperm, membrane lipid composition is of interest due to its specific properties associated with the stretch-activated mechanically sensitive channels (Krasznai et al., 2003) involved in activation of sperm motility (Krasznai et al., 1995), which might be associated with the surrounding membrane lipids (Ermakov et al., 2010).

Lipids are presumed to have a role in preservation of sperm and there are actions of lipids that are influenced by environmental factors (particularly temperature) that affect fish sperm after time of semen ejaculation (Bozkurt et al., 2008; Golpour et al., 2013). Plasma membranes contain a large proportion of polyunsaturated fatty acids (PUFA) which, probably due to their highly dynamic structure, actively contribute to the fertilization process (Lenzi et al., 1996). Oxidative damage of fish sperm occurs with increased sperm content of PUFAs which potentially lead to susceptibility to ROS damage to these cells (Li et al., 2010; Cabrita et al., 2014). Accordingly, lipid peroxidation and altered membrane properties, lipid physical state, and metabolic pathways of sperm cells have been considered to have adverse effects of oxidative damage associated with greater temperatures of the media that contains the cells (Hochacka and Somero, 1971; Shaklee et al., 1977). The degree of unsaturation in PUFAs is an important factor in maintenance of sperm stasis in an oxidative sperm cell media environment (Lenzi et al., 1996).

Polyunsaturated fatty acid contents have been associated with sperm cell membrane fluidity and structural changes (Israelachvili et al., 1980; Meizel and Turner, 1983) as well as to sperm motility (Conquer et al., 2000; Safarinejad et al., 2010; Baeza et al., 2015). Polyunsaturated fatty acids are mainly bound to phospholipids (PLs), which are identified as the most abundant lipid fraction of the sperm membrane which has significant effects on cell motility (Am-in et al., 2011). Lipids are probably utilized as an energy supply to extend the duration of sperm motility (Baeza et al., 2015) and as a result have a fundamental role in fertilization capacity (Gholami et al., 2011). Additionally, endogenous lipids, particularly phospholipid have been served as energy source for sea urchin sperm (Mohri, 1957; Mita and Yasumasu, 1983; Mita, 1991).

After consideration of the large amount of information available, there continues to be need for greater understanding of the complex interactions of temperature of the medium in which sperm reside, sperm cell motility, and sperm lipid composition of fish sperm. To our knowledge, the few studies on lipid composition of sperm relative to temperature of sperm medium have been conducted with few species, primarily mammals. The present study was designed to investigate the effects of medium temperature on carp sperm motility *in vitro* and on the associate changes in lipid composition.

2. Material and methods

2.1. Semen collection

Common carp *Cyprinus carpio* males were obtained from the fish farm of South Bohemian Research Center of Aquaculture, Vodnany, Czech Republic. Fish maintained in 4 m^3 hatchery tanks. Water flow rate was maintained at 0.2 l s^{-1}. Temperature was kept at 18–22 °C and oxygen ranged from 6 to 7 mg l^{-1}. Fish were fed daily with commercial carp pellets at 0.5% of their body weight, and the photoperiod was kept at 14 h light to 10 h dark. Five mature males of 3–4 kg body weight (4 years old) were injected with a suspension of lyophilized carp pituitary powder at 1 mg/kg body weight. Sperm samples were stripped by abdominal massage 24 h post-injection and stored in 20 ml vials on ice for no longer than 2 h before analysis.

2.2. Motility assessment

The sperm were added to 50 μl activation medium (AM) containing 10 mM Tris–HCl buffer, pH 8.0, 1 mM $CaCl_2$, and 0.125% pluronic acid (approximately 1:1000) at 4, 14, or 24 °C, on a microscope slide using the tip of a dissecting needle to combine thoroughly and obtain the appropriate cell concentration for motility analysis. Temperature ranges were chosen based on preliminary data for sperm motility in carp (Dadras et al., 2016). Sperm motility variables at each temperature were recorded at 10 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. Temperature of the AM was adjusted by a thermoblock ((Diameter size: 0.1 mm; HLC BO50/15, Germany) HLC BO50/15, Germany) and monitored during motility by copper-constantan thermocouple (Omega, L-044T, Taiwan) *via* a data logger thermometer (Omega, HH127, Taiwan). Sperm curvilinear velocity (VCL, μm/s), percentage of motile sperm after activation (motility rate, %), and duration (s) over which motility was sustained were estimated from recorded videos in triplicate for each sperm sample.

Video records were analysed by Micro-image software (Olympus Micro Image v. 4.0.1. for Windows, Japan) to estimate sperm VCL and motility rate in five successive overlapping video frames. After overlapping of frames, tracking of sperm head movements became visible, permitting calculation of VCL (Dzyuba et al., 2012).

2.3. Lipid composition

Lipid composition analysis of sperm was conducted before and after sperm activation. To assess lipid composition, 150 μl of sperm of each fish was placed in 1350 μl of AM to activate motility at 4, 14, and 24 °C until cessation of progressive movement. At the time sperm motility ceased in each temperature treatment group, sperm were centrifuged at 1000 × *g* for 5 min at 4 °C, and pellets were used for sperm lipid composition analysis. Another treatment was performed with 0.9% NaCl solution as non-activation medium (NAM) to maintain sperm in an inactivated state at each temperature. Centrifugation of sperm on which the NAM treatment was imposed occurred at the same time points as those for activated sperm samples.

2.4. Fat extraction

Lipids were extracted from the sperm samples using the methods of Hara and Radin (1978) with a slight modification for fish sperm. All chemicals and solvents were purchased from Merck (Darmstadt, Germany). Briefly, 1 g of sample was weighed and homogenised in hexane–isopropanol (3:2, v/v). The homogenate was transferred to a centrifuge tube, and 6 ml 6.67% Na_2SO_4 was added. After centrifugation for 5 min at 2737 × *g*, the upper phase of the homogenate in the centrifuge tube containing lipids was transferred into pre-weighed tubes and evaporation of the liquid occurred using nitrogen. Samples were weighed, dissolved in 2 ml hexane, and stored at −80 °C until further analyses.

2.5. Methylation and fatty acid analyses

To analyse fatty acid (FA) composition, methylation of total lipids was induced using a combination of NaOH and boron trifluoride–methanol complex as previously described (Appelqvist, 1968). Fatty acid composition was evaluated by gas chromatography (Trace Ultra FID; Thermo Scientific, Milan, Italy) on a BPX-70 50 m fused silica capillary column (id. 0.22 mm, 0.25 μm film thickness, SGE, USA) using the procedures of Sampels et al. (2005). The composition of the fatty acids when there were chromatographic peaks detected were identified by comparing sample retention times to those of the standard mixture GLC-68-A (Nu-Chek Prep, Elysian, USA) and other previously validated standards (Nu-Chek Prep, Elysian, USA; Larodan, Sweden).

2.6. Lipid class composition analysis

Major lipid classes were separated according to Olsen and Henderson (1989), with a ADC 2 system (Camag, Switzerland) using a mobile phase consisting of hexane, diethylether, and acetic acid (85:15:2, v/v/v) as described by Sampels et al. (2005). Phospholipid classes were separated and quantified according to Olsen and Henderson (1989) using a mobile phase consisting of 25 ml methyl-acetate, 25 ml isopropanol, 25 ml chloroform, 10 ml methanol, and 10 ml 0.25% KCl.

For both major lipid and phospholipid classes, plates were dipped in a solution of 3% cupric acetate in 8% phosphoric acid and were then charred for 20 min at 160 °C. Quantitative analysis of the separated lipid classes was determined by assessing the plates with a CAMAG TLC Scanner 3 (Camag, Switzerland). Identification of lipid classes was performed by comparison with a standard mix of all lipid classes (NuCheck, Prep, Elysian, USA) or single phospholipid standards (Avanti Polar Lipids, Inc., Alabama, USA) and evaluated with WinCats integration software (Camag, Switzerland).

2.7. Statistical analysis

The values of sperm velocity were assessed for distribution characteristics and homogeneity of dispersion using Shapiro–Wilk's and Levene's tests, respectively. The values for sperm velocity were normally distributed with similar dispersion values, parametric one-way ANOVA was applied and Tukey's honest significant difference test was used for comparing differences among groups. Because of the small number of observations ($n = 5$), nonparametric statistics using the Kruskal–Wallis test, followed by the multiple comparison of mean ranks for all groups, was performed for comparison of motility rate and duration of the period over which sperm motility was sustained. To compare lipid composition of motile with immotile sperm at each temperature, the nonparametric Mann–Whitney *U*-test was used. Statistical significance was considered at $P < 0.05$. All analyses and plotting were conducted using STATISTICA v. 13 software (Dell Inc., Tulsa, OK, USA).

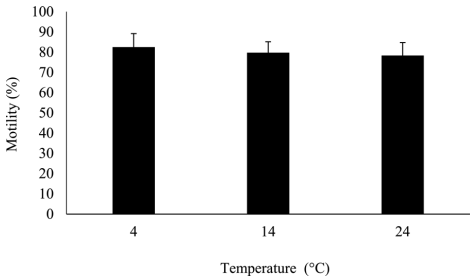

Fig. 1. Effect of temperature on motility (%) of common carp sperm. Data are mean ± SD. There were no significant differences ($P > 0.05$, Kruskal–Wallis test).

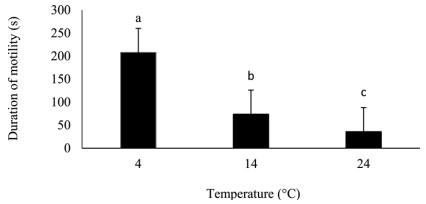

Fig. 2. Effect of temperature on duration of period over which there was sperm motility of common carp sperm. Data are mean ± SD. Values with different letters are different ($P < 0.05$, multiple comparison of mean ranks for all groups test).

3. Results

3.1. Motility analysis

The percentage of sperm with motility at 10 s post-activation was not affected by temperature (Fig. 1). The duration of sperm motility was longer at 4 °C (Fig. 2) compared with 14 and 24 °C. At 10 s post-activation, sperm had a greater VCL at 24 °C (Fig. 3) than at other temperatures.

3.2. Lipid analysis

3.2.1. Fatty acid composition

Data for the FA composition of carp sperm are included in Table 1. There were lesser proportions of n-3 PUFAs,

Table 1
Common carp sperm fatty acid composition (%) in motile (M) and immotile (IM) sperm at different *in vitro* temperatures. Data are mean ± SD. Asterisk in the same row indicates difference between motile and immotile sperm at each temperature ($P < 0.05$, Mann–Whitney *U* test).

	4 °C		14 °C		24 °C	
	M	IM	M	IM	M	IM
Saturated fatty acid						
16:0	23.7 ± 1.5	23.5 ± 0.5	25.0 ± 1.9	23.1 ± 3.4	23.9 ± 0.7	23.6 ± 2.2
18:0	8.3 ± 0.5	8.8 ± 0.3	10.0 ± 0.8	9.5 ± 0.6	8.9 ± 0.9	8.2 ± 0.6
24:0	1.3 ± 0.1	1.4 ± 0.2	1.5 ± 0.2	1.3 ± 0.1	1.3 ± 0.0	1.2 ± 0.1
Mono-unsaturated fatty acid						
16:1	2.0 ± 0.6	2.0 ± 0.2	1.4 ± 0.9	1.5 ± 0.5	1.6 ± 0.6*	3.9 ± 1.8
18:1 (n-7)	2.6 ± 0.3	2.5 ± 0.1	2.9 ± 0.2	2.5 ± 0.9	2.7 ± 0.1	3.0 ± 0.4
18:1 (n-9)	8.8 ± 3.2	7.3 ± 0.6	8.3 ± 1.5	7.6 ± 0.8	11.1 ± 8.5	9.7 ± 4.0
20:1 (n-9)	1.7 ± 0.3	1.9 ± 0.1	1.8 ± 0.5	1.8 ± 0.2	1.7 ± 0.4	1.9 ± 0.3
Poly-unsaturated fatty acid						
18:2 (n-6)	3.4 ± 0.5	3.4 ± 0.4	3.2 ± 0.5	3.3 ± 0.6	3.3 ± 0.8	3.4 ± 0.3
18:3 (n-3)	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.4	0.6 ± 0.3	0.4 ± 0.2*	0.9 ± 0.3
20:2 (n-6)	0.8 ± 0.1	0.7 ± 0.1	1.1 ± 0.4	0.8 ± 0.04	0.8 ± 0.1	0.7 ± 0.3
20:3 (n-6)	1.6 ± 1.4	0.1 ± 0.0	2.3 ± 0.7	2.8 ± 0.6	1.2 ± 1.2	1.7 ± 0.9
20:4 (n-6)	10.7 ± 0.7	11.2 ± 0.8	11.6 ± 1.3	11.4 ± 1.5	11.2 ± 1.1	9.2 ± 3.3
20:5 (n-3)	9.8 ± 1	9.3 ± 0.7	9.9 ± 0.9	9.7 ± 0.7	9.4 ± 1.0	8.4 ± 3.1
22:3 (n-3)	0.8 ± 0.1	1.0 ± 0.1	0.8 ± 08	1.0 ± 0.2	0.8 ± 0.6	0.6 ± 0.4
22:4 (n-6)	1.1 ± 0.6	1.2 ± 0.1	0.6 ± 0.1	1.0 ± 0.5	0.9 ± 0.3	0.5 ± 0.3
22:5 (n-3)	2.7 ± 0.5	2.9 ± 0.2	2.6 ± 0.2	2.5 ± 1.4	2.7 ± 0.5	1.5 ± 1.2
22:6 (n-3)	20.4 ± 2.4	22.1 ± 1.1	20.4 ± 1.2	22.9 ± 1.8	19.6 ± 1.5*	22.7 ± 1.7
$\sum$ SFA	34.2 ± 1	34.8 ± 0.5	35.9 ± 1.4	32.6 ± 3.4	33.9 ± 2.1	32.8 ± 3.3
$\sum$ MUFA	15.2 ± 3.7	13.7 ± 0.8	13.5 ± 2.6	13.2 ± 0.9	16.4 ± 6.9	15.7 ± 4.2
$\sum$ PUFA	50.3 ± 4	52.5 ± 1.5	51.2 ± 1.3	54.2 ± 3.5	49.0 ± 4.1	49.3 ± 6.0
$\sum$ n/3	33.5 ± 3.8	35.8 ± 1.3	33.6 ± 1.4	36.8 ± 2.8	32.5 ± 3.1	34.1 ± 2.4
$\sum$ n/6	17.6 ± 2.1	17.7 ± 1.3	18 ± 1.4	18.5 ± 2.4	16.9 ± 3.1	15.6 ± 4.3
n6/n3	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.5	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
SFA/UFA	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0

SFA: saturated fatty acids [16:0; 18:0; 24:0]; MUFA: mono-unsaturated fatty acids [16:1; 18:1 (n-7), 18:1 (n-9), 20:1 (n-9)]; PUFA: poly-unsaturated fatty acids [18:2 (n-6), 18:3 (n-3), 20:2 (n-6), 20:3 (n-6), 20:4 (n-6), 20:5 (n-3), 22:3 (n-3), 22:4 (n-6), 22:5 (n-3), 22:5 (n-6), 22:6 (n-3)]; Σ n/3: total omega-3 fatty acids; Σ n/6: total omega-6 fatty acids.

18:3 (n-3) and 22:6 (n-3) in motile sperm at 24 °C compared with immotile at this temperature, but no differences were observed at 4 and 14 °C. Motile sperm contained a lesser proportion of 16:1 (mono-unsaturated fatty acid) at 24 °C than immotile sperm at this temperature. The proportions of saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), and PUFAs in sperm cell membranes were not affected by temperatures.

3.2.2. Lipid class composition

There were no significant differences in total cholesterol, or in the cholesterol:PL ratio, between motile and immotile sperm at any temperature. The content of total PL was less (0.65 ± 0.11) in motile than immotile sperm at 24 °C (Fig. 4).

Five classes of PLs were identified in carp sperm: phosphatidylinositol, cardiolipin, phosphatidylethanolamine, phosphatidylcholine (PC), and phosphatidylserine (PS). Due to inadequate separation of some samples, the proportions of PC and PS were analysed in combination. At 24 °C, the combined PC and PS content was less in motile than immotile sperm (Table 2). In contrast, there were no differences in other PLs between motile and immotile sperm at the temperatures at which assessments occurred in this study (Table 2).

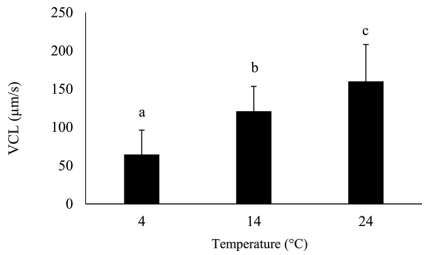

Fig. 3. Effect of temperature on common carp sperm curvilinear velocity. Data are mean ± SD. Values with different letters are different ($P < 0.05$, Tukey's HSD test).

4. Discussion

In the present study, duration of the sperm motility was longer at 4 °C compared to 14 and 24 °C. These results are consistent with previous findings (Dadras et al., 2016) in carp and rainbow trout *Oncorhynchus mykiss* (Billard and Cosson, 1988). Similar results have also been observed in burbot *Lota lota*, grayling *Thymallus thymallus*, brown trout *Salmo trutta*, and European perch *Perca fluviatilis* with duration of the period over which sperm motility is sustained at lesser temperatures being longer than what occurs at

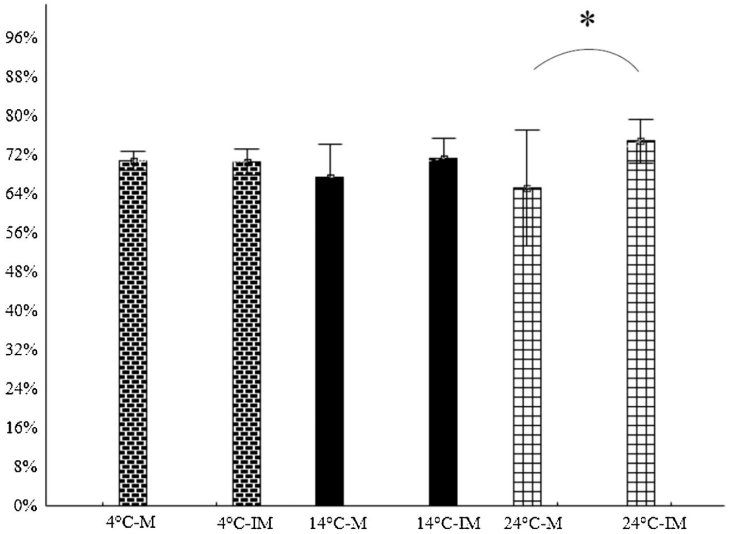

Fig. 4. Content of total phospholipids in common carp sperm. Data are mean ± SD. Asterisk indicates difference between motile (M) and immotile (IM) sperm at each temperature ($P < 0.05$, Mann–Whitney *U*-test).

Table 2
Proportions (%) of phosphatidylinositol, cardiolipin, phosphatidylethanolamine, and combined phosphatidylcholine and phosphatidylserine in common carp sperm in motile (M) and immotile (IM) sperm at different temperatures (mean values ± SD). Asterisk in the same row indicates difference between motile and immotile sperm at each temperature ($P < 0.05$, Mann–Whitney *U* test).

	4 °C		14 °C		24 °C	
	M	IM	M	IM	M	IM
PI	8.8 ± 5.9	8.3 ± 4.1	12.9 ± 7.3	12.8 ± 7.7	12.0 ± 2.2	8.4 ± 6.5
CL	4.4 ± 2.1	3.9 ± 1.4	5.3 ± 4.3	4.8 ± 1.6	6.9 ± 2.5	4.2 ± 2.0
PE	43.2 ± 8.8	39.2 ± 10.4	32.5 ± 7.9	27.6 ± 8.5	42.1 ± 3.1	32.7 ± 17.9
PC + PS	43.7 ± 9.0	48.5 ± 13.3	50.0 ± 10.2	55.2 ± 10.8	38.6 ± 6.5*	55.3 ± 16.7

PI: phosphatidylinositol; CL: cardiolipin; PE: phosphatidylethanolamine; PC + PS: combined phosphatidylcholine and phosphatidylserine.

greater temperatures (Lahnsteiner, 2011; Lahnsteiner and Mansour, 2012). The variation in intracellular cyclic adenosine monophosphate (cAMP) content during initiation of sperm motility in rainbow trout was measured at two temperatures (Cosson et al., 1995). The rate of increase in cAMP that represents the phosphorylation signal for sperm motility initiation (Morisawa, 1985) was less at 2 than at 20 °C (Cosson et al., 1995). However, in carp sperm, a direct role of cAMP is not required for the hypotonic-induced initiation of motility (Perchec Poupard et al., 1997). When brown trout sperm were placed in water, initial sperm velocity was less at temperatures ≥10 °C, while in grayling, velocity was not affected by temperature (Lahnsteiner and Mansour, 2012). In the present study, the greatest initial velocity was at 24 °C, consistent with the previous results of Lahnsteiner and Mansour (2012) where it was reported that a greater than optimal spawning temperature was associated with increased velocity within 10 s of activation in burbot sperm. The energy resources of fish sperm is limited so sperm movements cannot be sustained for long periods with this energy source, indicating that mitochondrial activity in carp sperm is insufficient to compensate for ATP utilization during the period over which motility occurs (Perchec et al., 1995). The greater sperm velocity associated with greater temperatures at the time of initiation of motility eventually affects metabolic pathways and leads to shorter durations over which motility is sustained (Purchase et al., 2010) and these findings are consistent with results reported in Senegalese sole *Solea senegalensis* and European perch (Diogo, 2010; Lahnsteiner, 2011).

In the present study, there was no difference in percentage of sperm motility with activation of motility at different temperatures. These findings are consistent with those for European perch, in which the initial motility at temperatures ranging from 4 to 16 °C was not different (Lahnsteiner, 2011). Initial motility percentage has been reported to be less at over optimal spawning temperatures in brown trout; whereas, in grayling, initial motility percentage was less at temperatures that were less than optimal (Lahnsteiner and Mansour, 2012).

The proportions of the major lipid classes were found to differ between motile and immotile sperm at 24 °C in

the present study. The proportion of phospholipids was less in the motile compared with immotile sperm at 24 °C. Interestingly, it has been shown that at higher temperature (20 °C) the level of phospholipid in sea urchin spermatozoa was decreased relative to lower temperature (0 °C), suggesting that phospholipid was easily metabolized at the higher temperature (Mita and Yasumasu, 1983).

Based on the present results, the combined proportion of PC and PS was less in motile than immotile sperm at 24 °C, suggesting a role in sperm membrane fluidity (Zaniboni et al., 2006; Wang et al., 2010) and, hence, motility (Infante and Huszagh, 1985). Phosphatidylcholine has clearly been shown a have a primary function in sperm membrane functions and potentially aids in maintaining the fertilization capacity of sperm (Evans and Settchell, 1978; Davis and Byrne, 1980) as well as conferring protection against thermal stress (Simpson et al., 1986).

In the present study, temperature had no significant effect on carp sperm SFA, which is inconsistent with previous findings where there was a decrease of SFAs in response to temperature differences in rainbow trout (Labbé and Maisse, 1996). Results of the present study revealed a lesser proportion of 18:3 (n-3) and 22:6 (n-3) at 24 °C in the motile sperm. This suggested that a decrease in the proportion of PUFAs at greater temperatures might be a source of the loss of sperm motility, which emphasizes the importance of PUFAs in fish sperm motility (Vassallo-Agius et al., 2001) similar to what has been reported in humans (Lenzi et al., 2000). Further, the proportion of 16:1 FA in motile sperm was less at 24 °C. The findings were consistent with the positive effect of the sperm head and flagella membrane unsaturated fatty acids on sperm motility of gilthead seabream *Sparus aurata* (Beirão et al., 2012). This might be attributed to modification of membrane fluidity and alteration of normal cellular functions at higher temperature.

Significant correlations exist between fatty acid composition and sperm quality variables in the European eel, *Anguilla anguilla* (Baeza et al., 2014) with a marked relationship between sperm motility and PUFA precursors, as well as between increased content of arachidonic acid and elevated sperm velocity (Baeza et al., 2014). Results of the present study are consistent with results from these previous studies, as the shortest duration of the sperm motility was observed at the greatest temperature, with differences in some PUFAs being evident in the sperm of this group. The reduction of duration in the period over which sperm motility was sustained might be influenced by the proportion of PUFAs in the sperm (Klaiwattana et al., 2016).

Temperature changes cause oxidative stress in sperm due to greater susceptibility to lipid peroxidation, as the sperm plasma membrane contains large amounts of PUFAs (Li et al., 2010; Cabrita et al., 2014; Dadras et al., 2016; Klaiwattana et al., 2016). Sperm damage induced by ROS is mediated by the oxidative actions on sperm membrane phospholipid-bound PUFAs, resulting in lipid peroxidation and loss of motility (Klaiwattana et al., 2016). Lipid peroxidation intensity was greater at 14 and 24 °C compared to 4 °C in carp sperm (Dadras et al., 2016), hence it can be inferred that reduction of PUFAs and PLs might contribute to the shorter duration of the period over which sperm motility is sustained subsequent to motility activation.

5. Conclusions

In the present study, there was a partial depletion of sperm lipids when sperm motility was activated at a greater temperature. Results also indicated that loss of some lipids and cessation of sperm motility are likely associated with detrimental actions on sperm lipid composition at greater temperatures. However, further investigation is needed to clarify the precise mechanisms involved and the relationship between sperm motility and lipid metabolism in carp sperm with various thermal conditions. The present study provides basic information for future investigations of the fertilization process of fish sperm.

Acknowledgments

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects CENAKVA (No. CZ.1.05/2.1.00/01.0024), CENAKVA II (No. LO1205 under the NPU I program), COST (No. LD14119) and by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 125/2016/Z). The authors greatly appreciate valuable help from The Lucidus Consultancy with English editing.

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CHAPTER 4

IN VITRO ACTIVATION OF EURASIAN BURBOT *LOTA LOTA* SPERMATOZOON MOTILITY IN SEMINAL FLUID AT DIFFERENT TEMPERATURES

Dadras, H., Boryshpolets, S., Golpour, A., Policar, T., .Blecha, M., Dzyuba, B., 2018. *In vitro* activation of Eurasian burbot *Lota lota* spermatozoon motility in seminal fluid at different temperatures. (Manuscript)

My share on this work was about 70%

In vitro activation of Eurasian burbot *Lota lota* spermatozoon motility in seminal fluid at different temperatures

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ABSTRACT

Factors regulating activation of spermatozoon motility in Eurasian burbot, *Lota lota*, including osmolality, calcium (Ca^{2+}) ions, and temperature were investigated. Spermatozoon motility was assessed at 4 °C and 30 °C in seminal fluid, isotonic media (with and without Ca^{2+}), and hypotonic media (with and without Ca^{2+}). Spermatozoa were activated in seminal fluid at 30 °C, which is out of the spawning temperature range, indicating that no risk of activation occurs during routine semen handling in culture. Initiation of spermatozoon motility in *L. lota* is mediated by Ca^{2+}, and sensitivity to Ca^{2+} is dependent on temperature.

Key words: *Calcium, Ion, Osmolality, Temperature*

INTRODUCTION

The burbot, *Lota lota* (L. 1758) is the only gadiform inhabiting fresh water (Scott & Crossman, 1973; Cohen et al., 1990) that spawns in midwinter in a light-limited under-ice environment (Scott and Crossman, 1973; McPhail, 1997).

Generally, spermatozoa of both freshwater and marine fish are immotile in the testis, in seminal plasma, and in solutions with osmolality similar to that of seminal plasma (Stoss, 1983; Lahnsteiner et al., 1995). Teleost spermatozoon motility is regulated by ambient conditions (Morita et al., 2003). Associations among seminal plasma composition, osmolality, and spermatozoon motility have been reported (Alavi and Cosson, 2006; Lahnsteiner, 2011). Concentration of ions, especially Ca^{2+} (Morisawa, 1985; Cosson et al., 1989) is considered a critical factor in spermatozoon motility.

Temperature of the activation medium has been shown to affect motility duration, velocity, and fertilizing ability of fish spermatozoa (Cosson et al., 1985; Billard et al., 1995; Bombardelli et al., 2013; Dadras et al., 2016).

In sperm of *L. lota*, in contrast to Salmonidae and Acipenseridae, K^+ has no effect on spermatozoon motility (Lahnsteiner et al., 1997). Lahnsteiner et al. (1997) also reported that *L. lota* sperm remained immotile in saline solution with osmolality of 400 mOsm kg^{-1}, and pH above 7.4 led to a marked decrease in motility. Apparently, in L. lota, low Ca^{2+} level and osmolality of the seminal plasma are complementary factors contributing to sperm maintenance in the quiescence state (Lahnsteiner et al., 1997). Nonetheless, several other factors might contribute to keeping sperm immotile. Zuccarelli et al. (2007) demonstrated that a combination of high K^+ level, high osmolality, and, probably, high pH is involved in keeping sperm in the quiescent state in seminal plasma in the North American burbot *L. l. maculosa*. It has also been suggested that Ca^{2+} plays a key role in the initiation of motility in *L. l. maculosa* burbot sperm (Zuccarelli et al., 2007).

Spontaneous activation of spermatozoon motility at temperatures > 5 °C was observed in *L. lota* (Lahnsteiner et al., 1997), indicating the importance of maintaining temperature at < 5 °C during spawning. On the other hand, Zuccarelli et al. (2007) found no spontaneous motility of *L. lota maculosa* spermatozoa in seminal plasma at temperatures of 2 to 10 °C. These contradictory results may suggest a species-specific phenomenon, unusual among fresh water fish species. To the best of our knowledge, the physiological processes that induce spontaneous sperm motility at high temperatures in *L. lota* have not been investigated. Information of factors controlling motility inhibition/initiation is necessary to avoid spontaneous initiation of motility during handling in artificial culture (Lahnsteiner et al., 1997).

The goal of the present study was to determine *L. lota* spermatozoon activation at 4 and 30 °C relative to $Ca2^{+}$ and osmolality.

MATERIALS AND METHODS

Semen collection

Three-year-old *L. lota* males (total length LT = 336.7±49.3 mm, mass = 255.5±111.8 g) were cultured in a circular holding tank at the University of South Bohemia, Faculty of Fisheries and Protection of Waters facility and fed roach *Rutilus rutilus* (L. 1758) and topmouth gudeon *Pseudorasbora parva* (Temminck and Schlegel, 1846) (LT = 45–60 mm). Males were treated before sperm collection with 100 mg l^{-1} MS 222 anaesthetic (exposure time 7–10 min) according to Svačina et al. (2016). Sperm was stripped by abdominal massage and stored in dry 20 ml vials on ice before and during the experiments. Sperm samples from 23 males from three successive spawning years were used to assess activation of spermatozoa in seminal fluid (SF) at 4 and 30 °C. Samples from seven males were collected for analysis of motility parameters in different media.

Motility assessment

The tested temperatures were selected based on observation of spermatozoon activation during gradually increasing temperature from 4 to 30 °C in seminal fluid. Motility activation appeared at 20 °C, and maximum motility was recorded at 30 °C. Motility of sperm at 4 °C (the temperature close to natural spawning temperature) and 30 °C was assessed in three media (approximately 1:1000 dilution) including

A) SF;
B) 10 mM Tris-HCl buffer, pH 8.5; 125 mM NaCl; and 2 mM KCl as isotonic medium containing 0.05 mM free Ca^{2+} (IM);
C) 10 mM Tris-HCl buffer, pH 8.5; 125 mM NaCl; and 2 mM KCl as isotonic medium containing 0.05 mM free Ca^{2+} + 1 mM $CaCl_2$ (IM+Ca^{2+});
D) 10 mM Tris–HCl buffer, pH 8.5 as hypotonic medium containing 0.0001 mM free Ca^{2+} (HM);
E) 10 mM Tris-HCl buffer, pH 8.5 as hypotonic medium containing 0.0001 mM free Ca^{2+} + 1 mM $CaCl_2$ (HM+Ca^{2+}).

The free Ca^{2+} concentration of isotonic and hypotonic media was calculated by shared software 'Free Ca^{2+}' (Brokaw, 1986) using total concentration values of the three components involved in Ca^{2+}equilibrium (Ca^{2+}, EGTA, and H^{+}).

To collect seminal fluid, sperm was centrifuged at 2000 × g for 20 min at 4 °C. Supernatant obtained after a second centrifugation at 1500 × g for 20 min at 4 °C was used as SF. Motility

analysis was carried out within 5 s of semen dilution with the target media on a microscope slide using the tip of a dissecting needle to mix thoroughly and obtain the appropriate cell concentration for motility analysis.

The onset of spermatozoon motility was monitored at both temperatures, and motility parameters were recorded 15 s post-activation in triplicate for each sperm sample using a CCD video camera (Sony, SSCDC50AP, Japan operating in 25 fps mode) mounted on an inverted microscope equipped with a cooling stage (Olympus IX83, United Kingdom) and a 20 x NIC contrast objective lens. Temperature of the media was adjusted by a thermoblock (HLC BO50/15, Germany) and monitored during motility by copper-constantan thermocouple (Omega, L-044T, Taiwan) via a data logger thermometer (Omega, HH127, Taiwan). Spermatozoon average path velocity (VAP μm s^{-1}), linearity (LIN, defined as: straight line velocity (VSL)/curvilinear velocity (VCL)), percentage of motile spermatozoa after activation (motility rate, %), and motility duration (s) were estimated from recorded videos. The recordings were analysed by ImageJ software and a computer-assisted sperm analyser (CASA) plugin (Wilson-Leedy and Ingermann, 2007) upgraded according to Purchase and Earle (2012).

Assessment of seminal plasma composition

Seminal fluid osmolality was measured by a Vapor Pressure Osmometer 5520 (Wescor, Logan, UT, USA) and expressed in mOsm kg^{-1}. The pH of seminal plasma was determined with an Accumet micro pH combination electrode (Fisher Scientific Co., Pittsburgh, PA, U.S.A.). Concentrations of sodium (Na^+), potassium (K^+) and chloride (Cl^-) ions were measured using potentiometry by Ion Selective Electrodes (ISE, Bayer HealthCare, Tarrytown, NY, USA). Calcium ion concentration was measured using absorption photometry applying the o-cresolphthalein complexone method (Moorehead and Biggs, 1974).

Effect of Ca^{2+} on sperm motility

To determine the minimum concentration of Ca^{2+} required for onset of motility in isotonic and hypotonic media, calcium titration (0.0001^{-1} mM for isotonic and hypotonic media as well as SF) was performed at 4 and 30 °C. Titration of ethylene glycol tetraacetic acid (EGTA) (Sigma-Aldrich St. Louis, USA) as a chelator of Ca^{2+} ion (0.0001^{-1} mM for isotonic and hypotonic media and 1-5 mM for SF) was carried out to stop motility both with and without the minimum Ca^{2+} required for motility in all studied media at 4 and 30 °C.

Statistical analysis

Because of the small number of observations (n = 7), nonparametric statistics using the Kruskal-Wallis test followed by the Mann-Whitney U-test with Bonferroni correction were performed for comparison of motility rate. All values were expressed as mean ± SD, with significance set at $P < 0.05$. All analyses and plots were conducted using the STATISTICA v. 12 (Statsoft Inc., USA).

The data of spermatozoon velocity parameters were collected from 340 000 spermatozoa. For each studied condition, velocity parameters were plotted using mean values relative to time during motility. Microsoft Excel software was used to plot the graphs.

RESULTS AND DISCUSSION

Composition of *L. lota* seminal plasma (Table 1) is similar to that reported in *L. l. maculosa* (Zuccarelli et al., 2007).

Table 1. *Composition of seminal plasma of Lota lota*

Component	Value *n=21*
pH	8.3.±0.1
Osmolality (mOsm kg^{-1})	227.9±36.8
Na^+ (mM)	108.7.±11.7
Cl^- (mM)	80.9±10.1
K^+ (mM)	20.6±2.7
Ca^{2+} (mM)	2.6±0.6

Our results showed activation of *L. lota* spermatozoa in seminal plasma at 20–30 °C, with maximum motility at 30 °C, which was outside the temperature range reported by Lahnsteiner et al. (1997). The difference of the present findings with previous studies of spermatozoon motility relative to temperature might be due to adaptation to water-body types, geographic source of fish, or individual differences. Motility was not triggered in SF containing 5 mM EGTA at 30 °C, suggesting the key role of Ca^{2+} in *L. lota* sperm activation at high temperature.

Sperm activation in isotonic medium at both tested temperatures was triggered by adding 1 mM Ca^{2+} (Table 2, medium C), emphasizing the importance of Ca^{2+} to onset of motility (Zuccarelli et al., 2007; Gonzlez-Martinez et al., 1992; Lahnsteiner et al., 1997).

We found the minimum level of Ca^{2+} required for initiation of motility at 4 °C to be significantly higher (0.25-0.75 mM) than at 30 °C (0.1–0.25 mM) (Table 2), suggesting a temperature-dependent mechanism of Ca^{2+} involvement in regulation of *L. lota* spermatozoon motility.

Table 2. *Calcium dependency of Lota lota spermatozoon motility correspond to different osmolality at 4 °C and 30 °C*

			4°C			30°C	
Osmolality	Activation solutions	Motility	Minimum EGTA (mM) determined by titration	Minimum Ca^{2+} (mM) determinated by titration	Motility	Minimum EGTA (mM) determined by titration	Minimum Ca^{2+} (mM) determinated by titration
Isotonic	A (Seminal fluid, 2.6±0.6 mM Ca^{2+})	No			Yes	5	
	B (Isotonic medium, 0.05 mM Ca^{2+})	No		0.25–0.75	No		0.10–0.25
	B (Isotonic medium, 1.05 mM Ca^{2+})	Yes			Yes		
Hypotonic	D (Hypotonic medium 0.0001 mM Ca^{2+})	Yes	0.01–0.05		No		
	E (Hypotonic medium 1.0001 mM Ca^{2+})	Yes			Could not estimate		

A) SF
B) 10 mM Tris–HCl buffer, pH 8.5; 125 mM NaCl; 2 mM KCl as isotonic medium containing 0.05 mM free Ca^{2+} (IM)
C) 10 mM Tris–HCl buffer, pH 8.5; 125 mM NaCl; 2 mM KCl as isotonic medium containing 0.05 mM free Ca^{2+} + 1 mM $CaCl_2$ (IM+Ca^{2+})
D) 10 mM Tris–HCl buffer, pH 8.5, as hypotonic medium containing 0.0001 mM free Ca^{2+} (HM)
E) 10 mM Tris–HCl buffer, pH 8.5, as hypotonic medium containing 0.0001 mM free Ca^{2+} + 1 mM Ca Cl_2 (HM+Ca^{2+})

The duration of sperm motility in isotonic medium (IM+ Ca^{2+}) at 4 °C was significantly longer (88.3±18.7 s) than that observed at 30 °C (40.2±8.2 s) [Fig. 1 (a) (b)]. The motility rate in medium C at 4 °C decreased significantly after 60 s, from 94.0±5.5% to 56.0±23.0%, while the decline in the same medium at 30 °C occurred after 25 s (78.0±8.4% to 52.0±20.5%). No change in VAP was observed at 40 s post-activation in isotonic medium with 1.05 mM $Ca2^{+}$ at 4 °C [Fig. 1 (a)], while sharp decrease in VAP was revealed immediately after initiation of motility in the same medium at 30 °C [Fig. 1 (b)], reflecting the rapid decrease of ATP content at high temperature (Perchec et al., 1995) and negative effect of higher temperatures on spermatozoon velocity (Perchec et al., 1995; Beira˜o et al. 2011; Lahnsteiner, 2012; Dadras et al., 2016). In seminal fluid and the isotonic medium with 1.05 mM Ca^{2+} at 30 °C, motility rate after 25 s decreased from 76.00±5.48% to 50.00±18.71% and from 78.0±8.4% to 52.0±20.5%, respectively. In the presence of additional Ca^{2+} (1 mM), longer motility duration (88.3±18.7 s) in IM+ Ca^{2+} compared to HM+ Ca^{2+} (61.8±5.5 s) [Fig. 1 (a)] at 4 °C might be due to osmotic shock and, consequently, hypotonic cell swelling (Perchec-Poupard et al., 1997; Cosson, 2008). In hypotonic conditions at 4 °C, Ca^{2+} in SF seems to be sufficient for motility, while at 30 °C, spermatozoa appear more vulnerable to osmotic pressure (Table 2).

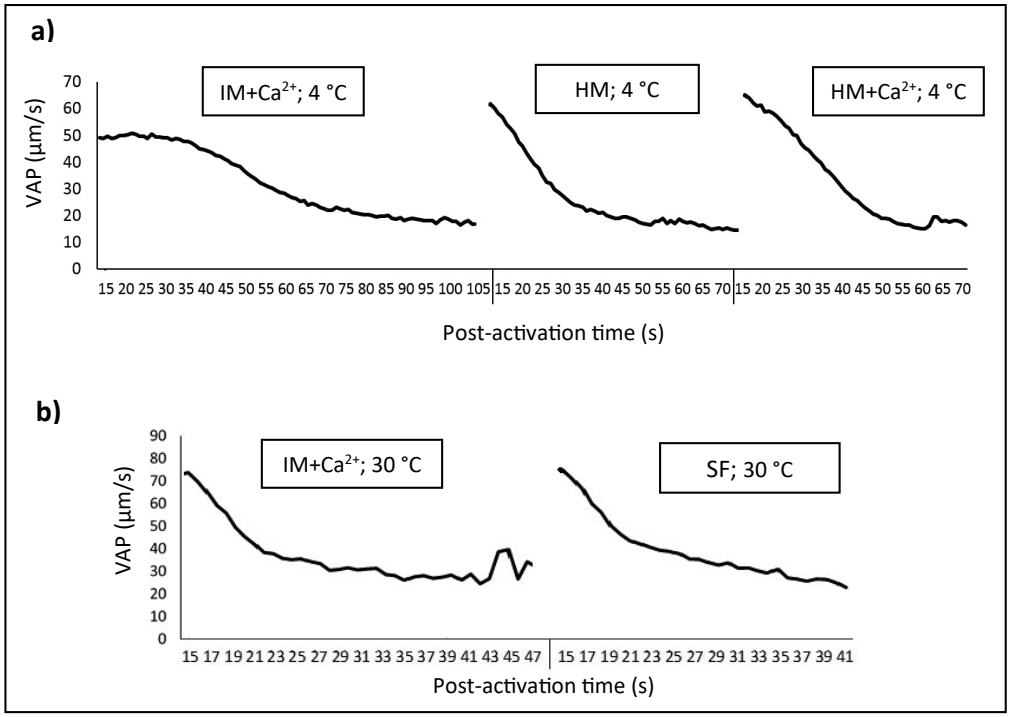

Fig. 1. *Timeline of Lota lota spermatozoon swimming velocity (VAP) in different media relative to temperature.*

a) Sperm velocity on dilution with: isotonic medium + 0.05 mM free Ca^{2+} + 1 mM Ca Cl_2 at 4 °C (IM+ Ca^{2+}; 4 °C); hypotonic medium + 0.0001 mM free Ca^{2+} at 4 °C (HM ; 4 °C); hypotonic medium + 0.0001 mM free Ca^{2+} + 1 mM Ca Cl_2 at 4 °C (HM+ Ca^{2+}; 4 °C).

b) Sperm velocity on dilution with: Sperm velocity on dilution with isotonic medium + 0.05 mM free Ca^{2+} + 1 mM Ca Cl_2 at 30 °C (IM+ Ca^{2+}; 30 °C); SF at 30 °C (SF ; 30 °C)

In the present study, sperm diluted with hypotonic medium exhibited a similar motility trend with and without addition of Ca^{2+} at 4 °C [Fig. 1 (a); Fig. 2 (a)]. Average path velocity and LIN showed similar patterns in both conditions. However, at 4 °C, motility rate decreased more rapidly, from 94.3%±7.9 to 58.6±20.4%, after 25 s in hypotonic medium with 0.0001 mM Ca^{2+} than at the same osmolality with 1.0001 mM Ca^{2+} (96.0±5.5% to 54.0±8.9% after 45 s). This might be attributed to the prominent role of intracellular Ca^{2+} in spermatozoon motility (Morisawa, 1985; Cosson et al., 1989). Lack of motility and motility of short duration (not estimated) at 30 °C was observed in hypotonic media D and E, respectively (Table 2).

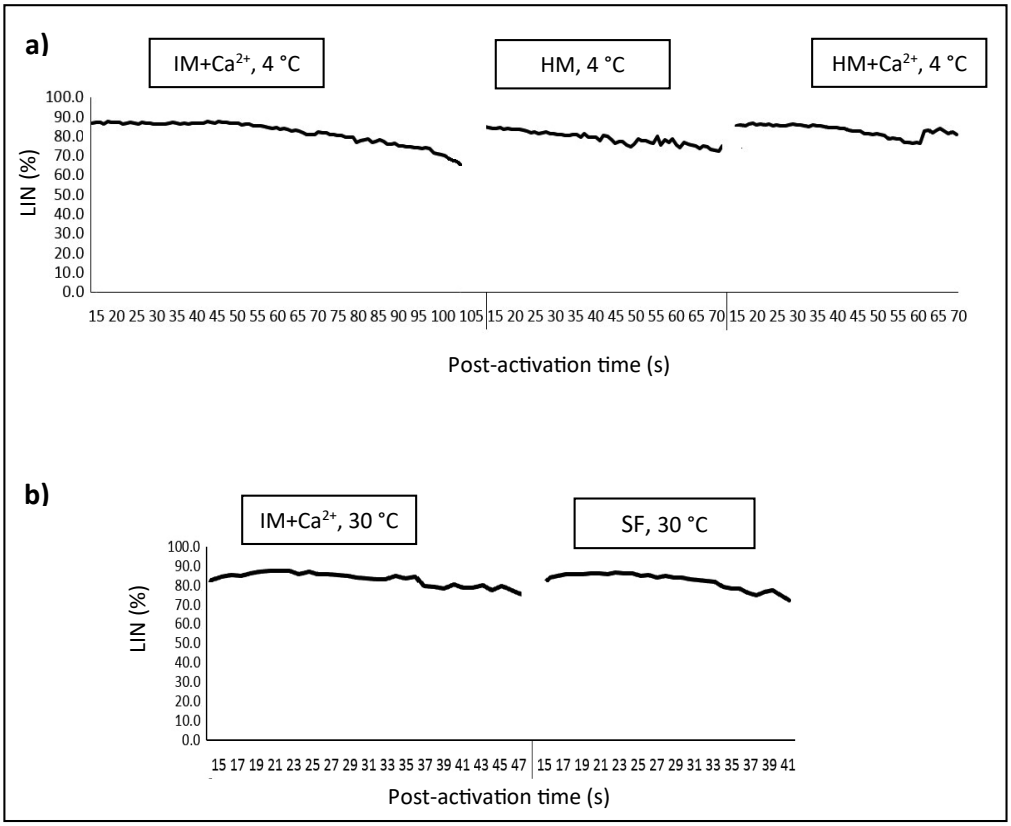

Fig. 2. *Timeline of Lota lota spermatozoon linearity (LIN) in different media relative to temperature.*
a) Sperm velocity on dilution with: isotonic medium + 0.05 mM free Ca^{2+} + 1 mM Ca Cl_2 at 4 °C (IM+ Ca^{2+}; 4 °C); hypotonic medium + 0.0001 mM free Ca^{2+} at 4 °C (HM ; 4 °C); hypotonic medium + 0.0001 mM free Ca^{2+} + 1 mM Ca Cl_2 at 4 °C (HM+ Ca^{2+}; 4 °C).
b) Sperm velocity on dilution with: Sperm velocity on dilution with isotonic medium + 0.05 mM free Ca^{2+} + 1 mM Ca Cl_2 at 30 °C (IM+ Ca^{2+}; 30 °C); SF at 30 °C (SF ; 30 °C)

Peak activation of *L. lota* spermatozoa in seminal fluid occurs at 30 °C, outside the spawning temperature range, indicating no risk of activation during semen handling. The data obtained in isotonic media show that activation of *L. lota* spermatozoa at high temperatures is likely regulated by complementary involvement of Ca^{2+}. At high temperatures, spermatozoon motility might be more sensitive to osmotic pressure and probably to Ca^{2+}, possibly related to increased membrane fluidity at high in vitro temperatures, which may present a novel avenue for future research.

ACKNOWLEDGMENTS

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic, projects CENAKVA (No. CZ.1.05/2.1.00/01.0024), CENAKVA II (No. LO1205 under the NPU I program), NAZV project QK1710310 - Utilization of new biotechnological approaches under Czech aquaculture with the aim to reach effective, high-quality and environmentally friendly fish production, project n. CZ.02.1.01./0.0/0.0/16_025/0007370 Reproductive and genetic procedures for preserving fish biodiversity and aquaculture and by the Grant Agency of the University of South Bohemia in Ceske Budejovice (125/2016/Z).

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CHAPTER 5

GENERAL DISCUSSION

Motility is an important determinant of sperm viability and fertilization ability in organisms with both internal and external fertilization (Alavi and Cosson, 2005; Cosson et al., 2008). Temperature is recognized as an environmental factor affecting sperm motility parameters (Alavi and Cosson, 2005), but the relationship of motility function to temperature has not been thoroughly studied. The basis for this research was to assess spermatozoon motility characteristics and changes of spermatozoon antioxidant enzyme activity and lipid composition outside the spawning temperature range of three commonly cultured species. With the exception of duration of motility, values of motility factors relative to temperature differed among tested species. Antioxidant enzyme response, lipid peroxidation, and changes in lipid composition were shown to differ among species at the studied temperatures.

We assessed activation of burbot spermatozoa in seminal fluid in relation to temperature, osmolality, and Ca^{2+} ions, highlighting the interaction of temperature and Ca^{2+} on spermatozoon activation in this species.

Temperature influence on spermatozoon motility variables

Sperm motility in various fish species is chiefly regulated by ion content and osmotic pressure; however, following the ejaculation of sperm into the surrounding environment, temperature plays a key role influencing motility characteristics (Vladić, 2000; Alavi and Cosson, 2005). As the metabolic rate in poikilotherm animals is correlated to temperature (Jobling, 1994), fish spermatozoon motility is expected to be considerably influenced by water temperature (Lindroth, 1947). Lindroth (1947) observed a significantly inverse relationship between the duration of swimming time for salmonid spermatozoa and water temperature. Studies subjecting spermatozoa to different ambient temperatures have shown resulting variation in motility parameters. The duration of spermatozoon motility in some freshwater teleosts including common carp (Perchec et al., 1995), hake *Merluccius merluccius* (Cosson et al., 2010), rainbow trout (Billard and Cosson, 1992), and European perch (Lahnsteiner, 2011) was prolonged at low temperatures. Spermatozoon motility rate and velocity in brown trout, burbot, and grayling *Thymallus thymallus* was significantly increased at low temperatures (Lahnsteiner and Mansour, 2012).

In the present study of common carp, rainbow trout, and sterlet, motility duration was significantly greater at 4 °C than at 14 and 24 °C. Due to the finite energy resources of fish sperm, an increase in velocity resulting from a temperature rise in the swimming solution leads to a shorter duration of motility, and conversely, lowering the temperature results in a prolonged duration of motility and reduced sperm velocity (Ginsburg, 1968; Purchase et al., 2010). These reports suggest the crucial role of ATP availability at low temperature as well as rapid depletion of ATP (Perchec et al., 1995), which reduces flagellar beat frequency, resulting in prolonged motility (Billard and Cosson, 1988; Cosson et al., 2010).

For common carp, we found no difference in initial motility percentage among the studied temperature exposures, while, at 30 s post-activation, a significantly lower motility rate was observed at 24 °C compared to 4 and 14 °C. However, at 30 s post-activation the highest swimming velocity was recorded at 14 °C. A similar trend in spermatozoon velocity was observed in rainbow trout at 14 °C. In sterlet, initial spermatozoon velocity was highest at 24 °C. Our findings of carp and sterlet regarding swimming velocity are accordance with the results of studies of Senegalese sole and European perch, which showed elevation of swimming velocity at high temperatures (Diogo, 2010; Lahnsteiner, 2011). At 10 s post-activation of burbot spermatozoa, swimming velocity was significantly greater in the range of

4 – 6 °C compared to other tested temperatures (Lahnsteiner and Mansour, 2012). However, the motility rate and velocity of brown trout were found to significantly decay at temperatures lower than 8 °C (Lahnsteiner and Mansour, 2012). In contrast, negative effects of higher temperatures on spermatozoon velocity in Senegalese sole (Beirão et al., 2011) and brown trout (Lahnsteiner, 2012) have been observed. No influence of temperature on spermatozoon velocity was reported in gilthead seabream *Sparus aurata* (Lahnsteiner and Caberlotto, 2012). These contradictory results in fish species are likely due to species-specific thermo-tolerance of the sperm (Mehlis and Bakker, 2014). Information obtained in our studies can provide an opportunity to investigate positive aspects of sperm function at target temperatures in order to enhance fertilization potential of sperm.

Effect of temperature on spermatozoon antioxidant enzymes activity

In aquatic species, environmental factors such as changes in temperature can induce oxidative stress (Lushchak, 2011). Increased ROS production causes oxidative stress in a variety of cell lines, including reproductive cells (Kazama et al., 2014). Although spermatozoa of externally fertilizing species are particularly vulnerable to oxidative stress, few studies have dealt with ROS production in fish spermatozoa (Hagedorn et al., 2012), particularly that produced by water temperatures corresponding to temperatures at or above the natural spawning temperature in common carp, brown trout, and sterlet. We conducted a series of studies of spermatozoa of teleostean and chondrostean fish species to determine the capacity of antioxidant enzymes and lipid peroxidation of spermatozoa to protect the cell against the detrimental effects of oxidative damage induced by temperature stress. Antioxidant systems protect spermatozoa against ROS, which can cause damage such as lipid peroxidation of membranes, damage to the midpiece and axoneme, and loss of motility as well as fertility (Sikka et al., 1995; Tramer et al., 1998). It is believed that generation of ROS by spermatozoa during the motility period is associated with elevation of respiration rate and concomitant rapid depletion of ATP (Lahnsteiner et al., 1999; Boryshpolets et al., 2009). This is supported by the finding in two species of sea urchin that generation of ROS in spermatozoa, associated with initiation of motility and respiration, influences motility and fertilizing potential (Kazama et al., 2014).

With increased temperature, ROS levels can increase dramatically, (Nichi et al., 2006) potentially leading to oxidative stress. In fish, studies of antioxidant enzyme activity have been focused on the detection of these enzymes in tissues (Klein et al., 2017 a,b) and not specifically in sperm. Little information is available on comparative aspects of fish sperm antioxidant enzyme activity relative to temperature. In the present study of carp spermatozoa, no significant difference was found in activity of SOD and CAT at 4, 14, and 24 °C. This agrees with the findings obtained in sheep Sertoli cells in which heat stress of 32–42 °C showed no effect on SOD activity (Salehi Nezhad et al., 2013). Our study of sterlet and rainbow trout spermatozoa revealed no difference in SOD activity at different temperatures. In contrast, SOD activity in boar spermatozoa was shown to be elevated at higher temperatures (Horky et al., 2015), which could indicate activation of protective antioxidant mechanisms against free radicals. Under summer heat stress, CAT activity in rabbit semen has been reported to demonstrate a sharp decline compared to winter (El-Tohamy et al., 2012). In our studies of sterlet and rainbow trout, spermatozoon CAT activity was higher at 4 °C compared to 14 and 24 °C, which can possibly be attributed to CAT inactivation or destruction by excess hydrogen peroxide at higher temperatures (Morgulis et al., 1926).

Lipid peroxidation is commonly used to evaluate oxidative stress in fish sperm (Li et al., 2010 a,b; Mansour et al., 2006). Considering the vulnerability of fish spermatozoa to

oxidative stress due to high quantities of PUFAs contained in the spermatozoa membrane (Pustowka et al., 2000), a complete analysis of the antioxidant capacity can provide a better understanding of how fish sperm respond to temperature stress. However, lipid peroxidation in fish spermatozoa during activation at different temperatures has seldom been evaluated. Our results revealed that TBARS content of activated common carp and rainbow trout spermatozoa varies with temperature regime; while, in sterlet, no differences were observed following activation at 4, 14, and 24 °C. The differences in TBARS level in spermatozoa of common carp and rainbow trout activated at different temperatures may be attributed to metabolic changes during motility leading to an increase in respiration rate and production of ROS (Li et al., 2010a). For instance, in the present study, TBARS content of common carp spermatozoa was significantly elevated at higher temperatures, possibly the result of reduced activity of antioxidant enzymes at higher temperatures (Lushchak and Bagnyukova, 2006).

Our findings are in agreement with studies reporting significant increase in lipid peroxidation of rabbit and mouse spermatozoa at temperatures of 37-40 °C (Alvarez and Storey, 1985). A similar increase in TBARS level of sheep Sertoli cells was reported at high temperatures (Salehi Nezhad et al., 2013), but no significant effect of temperature on TBARS levels was found in bovine epididymidis sperm stored at 4 and 34 °C.

Changes of spermatozoon lipid composition relative to temperature

Lipids in fish semen play a prominent role in supplying energy for motility (Baeza et al., 2015), and temperature stress can alter the physical properties of the membrane lipids in living cells (Losa and Murata, 2004). Among lipid classes, phospholipids have been shown to serve as the primary energy source for spermatozoon movement. Small quantities of phospholipids have been determined to act as energy sources during the spermatozoon motility period in gilthead seabream spermatozoa (Lahnsteiner and Caberlotto, 2012) as well as in many sea urchin species (Rothschild and Cleland, 1952; Mohri, 1957; Mohri, 1964; Mita and Yasumasu, 1983).

In our study of common carp, the phospholipid level was significantly lower in motile compared to immotile sperm at 24 °C. This finding is consistent with reduction of phospholipid proportion in sea urchin spermatozoa at 20 °C relative to 0 °C, indicating that phospholipids were not substantially metabolized at the lower temperature (Mita and Yasumasu, 1983). Phosphatidylcholine (PC) and phosphatidylserine (PS) belong to a class of phospholipids that play prominent roles in fluidity and motility of spermatozoa (Zaniboni et al., 2006; Wang et al., 2010; Infante and Huszagh, 1985). They are potentially involved in the fertilization capacity of sperm (Evans and Settchell, 1978; Davis and Byrne, 1980) and preserve the cell against thermal stress (Simpson et al., 1986). We observed PC and PS to be lower in motile spermatozoa relative to immotile spermatozoa at 24 °C.

Key functions of spermatozoa, including motility and cell membrane fluidity, have been associated with PUFA content (Israelachvili et al., 1980; Conquer et al., 2000; Safarinejad et al., 2010). The risk of oxidative stress in fish spermatozoa may increase with temperature rise, as the spermatozoon plasma membrane contains high quantities of PUFAs, making it susceptible to lipid peroxidation (Li et al., 2010 a, b; Cabrita et al., 2014; Klaiwattana et al., 2016). The detrimental effects of oxidative damage to sperm cells induced by high temperature of activation media include lipid peroxidation and alterations of membrane properties and metabolic pathways (Hochacka and Somero, 1971; Shaklee et al., 1977). Thus, decline in spermatozoon motility could be explained by damage induced by ROS mediated by the oxidative actions on sperm membrane phospholipid-bound PUFAs (Klaiwattana et al., 2016). Alpha lipoic acid [18:3 (n-3)] is an important PUFA considered a universal antioxidant, because it is composed of both water- and lipid-soluble antioxidants (Ibrahim et al., 2008).

We hypothesize that the decrease of 18:3 (n-3) in common carp spermatozoa seen at high temperatures might be attributable to free radical neutralization of this fatty acid to protect against detrimental effects of high temperature. The decrease of docosahexanoic acid [22:6 (n-3)], the longest chain PUFA, at high temperatures might be explained by its correlation with seminal plasma SOD-like and CAT-like activity and its inherent instability (Safarinejad, 2010).

In the present studies, lipid peroxidation in spermatozoa was significantly higher at 14 and 24 °C compared to 4 °C in common carp sperm; hence it can be suggested that reduction of some PUFAs, including 18:3 (n-3) and 22:6 (n-3), as well as of phospholipid proportion, might be linked to shorter duration of spermatozoon motility.

Role of temperature in spermatozoon activation

In the research project to explore the function of temperature as well as other key factors involved in the regulation of burbot sperm motility, osmolality and Ca^{2+} ions were studied simultaneously. Results of Lahnsteiner et al. (1997) and Zuccarelli et al. (2007) in studies of effects of temperature on the activation of burbot sperm motility in seminal fluid were contradictory. In the present study, spontaneous activation of motility in seminal fluid was observed at ~30 °C which differed from the report of >5 °C by Lahnsteiner et al. (1997).

We found addition of 5 Mm EGTA to seminal fluid to inhibit motility activation at 30 °C, indicating the critical role of Ca^{2+}, as suggested by Zuccarelli et al. (2007), and a likely complementary role of temperature in motility activation. Activation of motility in isotonic media with the addition of 1 mM Ca^{2+} at 4 °C and 30 °C may also suggest the key role of Ca^{2+} in initiating motility (Gonzlez-Martinez et al., 1992; Lahnsteiner et al., 1997). Titration of Ca^{2+} under isotonic conditions showed that the minimum level of Ca^{2+} required for initiation of motility at 4 °C was higher (0.25-0.75 mM) than at 30 °C (0.1-0.25 mM), emphasizing a temperature-dependent Ca^{2+} demand for burbot sperm motility. The level of Ca^{2+} in seminal fluid (2.6.±0.6 mM) diluted with hypotonic solution might be necessary to trigger motility at 4 °C, whereas, at 30 °C, sperm is more susceptible to osmotic pressure regardless of Ca^{2+} level.

Conclusions

We described the effect of temperature on spermatozoon motility parameters, antioxidant enzyme activity, and lipid composition of common carp, rainbow trout, and sterlet sperm, along with the role of temperature in activation of burbot sperm relative to Ca^{2+} and osmolality. The principal conclusions obtained from this study were

- Fish spermatozoon motility function is influenced by temperature in a species- specific thermo-tolerance manner in the studied species.
- Temperature changes alter the antioxidant enzyme response of sperm in the studied fish species to different degrees. The mechanisms underlying antioxidant activity of fish spermatozoa during the motile period under temperature stress are still unclear.
- Decrease in the proportion of certain lipids in common carp spermatozoa at high temperatures might be a source of a decline in spermatozoon motility rate and duration. Further research is required to define the precise physiological mechanisms involved and the relationship between spermatozoon motility and lipid metabolism in fish under different thermal conditions.
- The finding of activation of burbot sperm motility at 30 °C suggests that semen handling during standard hatchery practice is unlikely to carry a risk of spermatozoon activation. Activation of spermatozoa in burbot is likely regulated by Ca^{2+} and temperature.

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ENGLISH SUMMARY

Temperature dependency of sperm motility in different fish species

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The investigation of the fish spermatozoa motility function and enzymatic response in relation to different *in vitro* temperatures (Chapter 2) was carried out using activation of spermatozoa from taxonomically distant fish species (common carp, rainbow trout and sterlet) at 4, 14, and 24 °C. Special attention was taken to the sperm motility parameters (motility rate, duration and velocity), activity of catalase (CAT) and superoxide dismutase (SOD), and thiobarbituric acid-reactive substance (TBARS) content as marker of lipid peroxidation. Duration of motility in three aforementioned species were shown to be longest at 4 °C compared to 14 and 24 °C. In rainbow trout and carp, velocity was significantly increased at 14 °C after 10 and 30 s of spermatozoa activation respectively. While, at 60 s post-activation, the velocity of spermatozoa in sterlet was highest at 24 °C. This trend remained similar until 420 s post-activation. As results of this study, we found that there was no significant difference in SOD activity of three investigated fish species among different *in vitro* temperatures. Activity of CAT was in the highest level at 4 °C in rainbow trout and sterlet, while no difference was observed in carp spermatozoa. No similar change of TBARS content was observed in studied species. Considering our observation, temperature may affect the motility function, antioxidant enzymes response and TBARS content of sperm in studied fish species in different modes of reaction that is supposed to be explained by species-specific reaction of each species at studied temperatures.

As second aim of the present study (Chapter 3), lipid composition was analyzed in motile or immotile carp spermatozoa at different *in vitro* temperatures (4, 14 and 24 °C). The lower proportions of some fatty acids, 18:3 (n-3) and 22:6 (n-3) in motile spermatozoa were detected at 24 °C. With respect to importance of phospholipids as source of energy in fish spermatozoa movement, the total phospholipid content was lesser in motile than in immotile spermatozoa at 24 °C. At 24 °C also, phosphatidylcholine and phosphatidylserine proportions were lesser in motile than immotile spermatozoa. On the basis of these data, lipid composition of carp motile spermatozoa is affected by temperature, with decrease in proportion of some lipid compositions at higher temperature, elevation of sperm curvilinear velocity and decreased duration of the period over which motility is sustained.

The aim of the third study (Chapter 4) was to investigate role of different factors that might control the activation of spermatozoa motility, using physiological functions of osmolality, calcium (Ca^{2+}) ion and particularly temperature in burbot. Spermatozoa motility was tested at temperatures of 4 and 30 °C in following media: seminal fluid (SF), isotonic media (plus Ca^{2+} and without it) and hypotonic media (plus Ca^{2+} and without it). With regard to activation of burbot spermatozoa motility in seminal fluid at high temperature (30 °C), it seems that no risk of semen handling will occur during hatchery routine practice. The results suggest that initiation of spermatozoa motility in burbot is mediated by the simultaneous involvement of Ca^{2+} and temperature. Thus, it might be concluded that burbot spermatozoa is more sensitive to high temperature for initiation of motility relative to other freshwater fish species.

CZECH SUMMARY

Teplotní závislost motility spermatu u různých druhů ryb

Hadiseh Dadras Asyabar

Byla provedena analýza funkce motility spermatu a enzymatické odpovědi ve vztahu k rozdílným *in vitro* teplotám (Kapitola 2) za použití aktivace spermatu u taxonomicky odlišných druhů ryb (kapr obecný, pstruh duhový, jeseter malý) ve 4 °C, 14 °C a 24 °C. Významná pozornost byla věnována parametrům motility spermatu (rychlost pohybu, trvání, frekvence), aktivitě katalázy (CAT) a superoxidázy dismutázy (SOD) a látkám reagujícím s thiobarbiturovou kyselinou (TBARS) jakožto markerům peroxidace lipidů. Trvání motility u tří výše zmíněných druhů se ukázalo nejdelší ve 4 °C ve srovnání se 14 °C a 24 °C. U pstruha duhového a kapra, velocita byla signifikantně zvýšena po 10, respektive 30 s aktivace spermatu. Zatímco po 60 s post aktivace velocita spermatu byla nejvyšší ve 24 °C u jesetera malého. Tento trend zůstal podobný do 420 s postaktivace. Jakožto výsledek této studie jsme zjistili, že zde nebyl žádný signifikantní rozdíl v aktivitě SOD o všech třech analyzovaných druhů mezi různými *in vitro* teplotami. Aktivita CAT byla nejvyšší ve 4 °C u pstruha duhového a jesetera malého, zatímco u kapřího spermatu nebyl pozorován žádný rozdíl. Žádné obdobné rozdíly nebyly nalezeny u obsahu TBARS u studovaných druhů. Na základě našich pozorování můžeme říci, že teplota ovlivňuje funkci motility, odpovědi antioxidačních enzymů a obsah TBARS u spermatu studovaných druhů v odlišných způsobech reakce, která může být vysvětlována druhově specifickou reakcí každého druhu ve specifických teplotách.

Jakožto další cíl studie (Kapitola 3) byl analyzován obsah lipidů v pohyblivém a nepohyblivém kapřím spermatu za různých *in vitro* teplot (4, 14 a 24 °C). Nejnižší obsah některých mastných kyselin 18:3 (n-3) a 22:6 (n-3) u pohyblivého spermatu byl detekován ve 24 °C. Vzhledem k významu fosfolipidů jakožto zdroje energie pro pohyb spermatu, byl celkový obsah fosfolipidů nižší u pohyblivého než u nepohyblivého spermatu ve 24 °C. Poměry phosphatidylcholinu a phosphatidylserinu byly nižší u pohyblivého než u nepohyblivého spermatu. Na základě těchto dat lze říci, že obsah lipidů u pohyblivého spermatu kapra je ovlivňován teplotou se sníženým poměrem některých lipidových částí při vyšší teplotě, zvýšením velocity a sníženým trváním periody, po kterou je udržována motilita.

Cílem třetí studie (Kapitola 4) bylo zjistit roli některých faktorů, které mohou ovlivňovat aktivaci motility spermatu za využití fyziologické úlohy osmolality, vápenatých iontů (Ca^{2+}) a částečně i teploty u mníka. Motilita spermatu byla testována při teplotách 4 °C a 30 °C v následujících roztocích: seminální tekutina (SF), isotonické medium (plus Ca^{2+} nebo bez). S ohledem na aktivaci motility spermatu v seminální tekutině za vyšší teploty (30 °C) se zdá, že manipulace se spermatem během rutinní výtěrové praxe nepředstavuje žádné riziko. Výsledky ukazují, že iniciace motility spermatu je zprostředkována následným přísunem Ca^{2+} a teplotou. Proto může být učiněn závěr, že sperma mníka je mnohem citlivější k teplotě při iniciaci motility spermatu ve srovnání s ostatními sladkovodními druhy ryb.

ACKNOWLEDGEMENTS

This thesis is dedicated to my husband, Dr. Amin Golpour, who has been a constant source of support and encouragement during my Ph.D. study.

First and foremost I would like to express my deepest appreciation to my supervisor Assoc. Prof. Borys Dzyuba for his support, encouraging, personal guidance, constructive criticism and prompt feedback. Assoc. Prof. Borys Dzyuba provided a lot of academic opportunities during my study, therefore, I am very grateful to him.

My warm appreciation and gratitude are addressed to my thesis advisors, Prof. Jacky Cosson and Dr. Viktoryia Dzyuba for their enlightening guidance and inspiring instruction in the development completion of this study.

I would like to express my deepest and sincere gratitude to Prof. Otomar Linhart, Dr. Sergey Boryshpolets, Assoc. Prof. Sabine Sampels and Assoc. Prof. Tomáš Policar for their helpful suggestions and inspiration during my Ph.D. research.

I would like to thanks my colleagues, Ms. Lucie Kačerová for her academic support, Dr. Marek Rodina and Ms. Vladimíra Tučková for their excellent help and support during experiments and all of my colleagues from the Faculty of Fisheries and Protection of Waters for their technical and valuable help during experiment.

I gratefully acknowledge Dr. Catherine Labbé (INRA LPGP, Campus de Beaulieu, Rennes, France) for her hospitality, extensive discussions and personal attention during my stay in INRA institute.

Finally, my deepest gratitude goes to my parents, brother and sister for their endless love, encouragements and moral supports during my Ph.D. study.

I also appreciate the financial support from the following projects that funded parts of the research discussed in this dissertation:

- The Ministry of Education, Youth and Sports of the Czech Republic, Projects 'CENAKVA' (No. CZ.1.05/ 2.1.00/01.0024), 'CENAKVA II' (No. LO1205 under the NPU I programme);
- project n. CZ.02.1.01./0.0/0.0/16_025/0007370 Reproductive and genetic procedures;
- NAZV project QK1710310 - Utilization of new biotechnological approaches under Czech aquaculture with the aim to reach effective, high-quality and environmentally friendly fish production;
- Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 134/ 2013/Z);
- Grant Agency of the University of South Bohemia in České Budejovice (No.125/2016/Z);
- Czech Science Foundation (No. P502/15-12034S);
- COST (No. LD14119) and COST Office (Food and Agriculture COST Action FA1205: AQUAGAMETE).

LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Xin, M., Tučková, V., Rodina, M., Kholodnyy, V., **Dadras, H.,** Boryshpolets, S., Shaliutina-Kolešová, A., Linhart, O., 2018. Effects of antifreeze proteins on cryopreserved sterlet (*Acipenser ruthenus*) sperm motility variables and fertilization capacity. Animal Reproduction Science. *In press*. (IF 2017 = 1.647)

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Dadras, H., Nezami, S., Khara, H., Baradaran Noveiri, S., Shahkar, E., 2011. Relationship between sperm density, sperm pH, fertilization and hatching rates in Persian sturgeon (*Acipenser persicus* Borodin, 1897). In: Book of abstracts "International Conference on Behavioral Science, Economics, Bio-Technology & Environment Engineering", Thailand – Pattaya. October 7–8, 2011. (Lecture)

Dadras, H., Dzyuba, B., Cosson, J., Linhart, O., 2015. Comparative analysis of sperm motility of three species, *Cyprinus carpio, Oncorhynchus mykiss* and *Acipenser ruthenus* at various temperatures. In: Book of abstracts "5th International Workshop on the Biology of Fish Gametes" 7–11 September 2015, Ancona, Italy, pp. 186–188.

Dadras, H., Dzyuba, V., Cosson, J., Golpour, A., Dzyuba, B., 2016. Effect of temperature on motility and antioxidant enzymes of common carp spermatozoa. In: Book of abstracts "International Conference on Natural Science and Environment (ICNSE)", 7– 8 August 2016, Zurich, Switzerland, pp. 24–29.

Dadras, H., Boryshpolets, S., Cosson, J., Horokhovatskyi, Y., Dzyuba, B., 2017. A preliminary study of temperature effect on fertilization success in sterlet, *Acipenser ruthenus.* In: Book of abstracts "Aquaculture Europe 2017", 17–20 October 2017, Dubrovnik, Croatia, p. 253–254.

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Dzyuba, B., Sampels, S, Dzyuba, V., Silveira, AN., Kahanec, M., Silveira, RV., Kholodnyy, V., **Dadras, H.**, Rodina, M., Boryshpolets, S., 2017. Spermatozoon structure, lipid composition and motility in relation to internal fertilization in freshwater stingray *Potamotrygon motoro*. In: Book of abstracts "6th International Workshop on the Biology of Fish Gametes", 4–7 Spetember 2017, Vodňany, Czech Republic, p. 100.

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TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Hadiseh Dadras Asyabar
Research department	2014–2018 – Laboratory of Reproductive Physiology, FFWP
Supervisor	Assoc. Prof. Borys Dzyuba
Period	27th October 2014 until 19th September 2018

Ph.D. courses	**Year**
English language (IELTS)	2014
Fish reproduction	2015
Pond aquaculture	2015
Applied hydrobiology	2015
Basic of scientific communication	2016
Ichthyology and fish taxonomy	2016

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2015 2016 2017 2018

International conferences	**Year**
Dadras, H., Dzyuba, B., Cosson, J., Linhart, O., 2015. Comparative analysis of sperm motility of three species, *Cyprinus carpio, Oncorhynchus mykiss* and *Acipenser ruthenus* at various temperatures. In: Book of abstracts "5th International Workshop on the Biology of Fish Gametes" 7–11 September 2015, Ancona, Italy, pp. 186–188.	2015
Dadras, H., Dzyuba, V., Cosson, J., Golpour, A., Dzyuba, B., 2016. Effect of temperature on motility and antioxidant enzymes of common carp spermatozoa. In: Book of abstracts "International Conference on Natural Science and Environment (ICNSE)", 7– 8 August 2016, Zurich, Switzerland, pp. 24–29.	2016
Dadras, H., Boryshpolets, S., Cosson, J., Horokhovatskyi, Y., Dzyuba, B., 2017. A preliminary study of temperature effect on fertilization success in sterlet, *Acipenser ruthenus*. In: Book of abstracts "Aquaculture Europe 2017", 17–20 October 2017, Dubrovnik, Croatia, p. 253–254.	2017
Dadras, H., Sample, S., Policar, T., Blecha, M., Dzyuba, B., 2017. Changes of spermatozoa lipid composition in relation to thermo-activation process in burbot, *Lota lota*. In: Book of abstracts "6th International Workshop on the Biology of Fish Gametes", 4–7 September 2017, Vodňany, Czech Republic, p. 99.	2017
Dadras, H., Dzyuba, V., Golpour, A., Dzyuba, B., 201**8**. Antioxidant response of sterlet *Acipenser ruthenus* and rainbow trout *Oncorhynchus mykiss* spermatozoa to different *in vitro* temperatures. In: Book of abstracts: "IMPRESS conference, Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation", 17–20 June 2018, Arendal, Norway.	2018

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Catherine Labbé, Laboratory of Fish Physiology and Genomics INRA institute, France (3 months)	2016
Catherine Labbé, Laboratory of Fish Physiology and Genomics INRA institute, France (4 months)	2017

CURRICULUM VITAE

PERSONAL INFORMATION

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EDUCATION

2014 – 2018	Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic
2008–2010	M.Sc., in Fisheries, Azad University of Lahijan, Iran
2003–2007	B.Sc., in Fisheries, Azad University of Lahijan, Iran

TRAINING

13.01–16.01. 2015	3rd Aquagamete training school on techniques in reproductive biology and cryobanking, University of Algarve in Faro, Portugal

RESPONSIBLE LEADER OF PROJECTS

International summer school 2016: The *in vitro* effect of various temperatures on motility, membrane lipid composition, morphology and antioxidant enzymes activity of fish spermatozoon

RESEARCH STAY AND COLLABORATIONS

01.09–30. 11 2016	Dr. Catherine Labbé, Institute National de la Recherche Agronomique, INRA institute, Rennes, France
01.05–31.08.2017	Dr. Catherine Labbé, Institute National de la Recherche Agronomique, INRA institute, Rennes, France