

## RESEARCH ARTICLE

# Sperm in hot water: direct and indirect thermal challenges interact to impact on brown trout sperm quality

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## ABSTRACT

Climate change alters the thermal habitat of aquatic species on a global scale, generating novel environmental challenges during all life stages, including reproduction. Changes in water temperature profoundly influence the performance of ectothermic aquatic organisms. This is an especially crucial issue for migratory fish, because they traverse multiple environments in order to reproduce. In externally fertilizing migratory fish, gametes are affected by water temperature indirectly, within the reproductive organ in which they are produced during migration, as well as directly, upon release into the surrounding medium at the spawning grounds. Both direct (after release) and indirect (during production) thermal impacts on gamete quality have been investigated, but never in conjunction. Here, we assessed the cumulative influence of temperature on brown trout, *Salmo trutta*, sperm quality during sperm production (male acclimation temperature) as well as upon release (sperm activation water temperature) on two consecutive dates during the brown trout spawning season. Early in the season, warm acclimation of males reduced their fertilization probability (lower sperm velocity) when compared with cold-acclimated males, especially when the activation water temperature was also increased beyond the thermal optimum (resulting in a lower proportion of motile sperm with lower velocity). Later in the season, sperm quality was unaffected by acclimation temperature and thermal sensitivity of sperm was reduced. These results give novel insights into the complex impacts of climate change on fish sperm, with implications for the reproduction and management of hatchery and wild trout populations in future climate scenarios.

**KEY WORDS:** Computer-assisted sperm analysis, Climate change, Motility, Reproduction, Salmonid, Temperature

## INTRODUCTION

Climate change is increasing global surface temperatures at unprecedented rates (IPCC, 2013). Predictions of further temperature, precipitation and drought extremes, rising sea levels and changes to ocean circulation in the next 100 years give cause for concern regarding the persistence of terrestrial and aquatic species on a global scale (IPCC, 2014). Freshwater species face particularly acute challenges because of the cumulative effects of climate change and other anthropogenic stressors such as habitat modifications, pollution and over-exploitation, threatening their

ecosystems (IPCC, 2014). Climate change-induced decreases in suitable thermal habitats for freshwater species are already evident (Hari et al., 2006; Wenger et al., 2011a,b), forcing species to adapt or relocate in order to persist (Isaak et al., 2011; Reed et al., 2011). This is especially crucial for ectothermic species with complex life histories, such as migratory fish (Crozier et al., 2008).

Through their annual mass-spawning migrations, salmonids provide a reliable food source and crucial nutrients to otherwise sparse environments (Quinn, 2005). They are a vital component of a variety of ecosystems, and present an exceptional model for studying climate-mediated impacts on reproduction. Salmonids face novel environmental challenges as a result of the cumulative effects of climate change and habitat modification that threaten the freshwater ecosystems they navigate during their migrations, with impacts ranging from impaired locomotor ability and energy storage efficiency to potentially compromised mating and reproductive success (reviewed by Fenkes et al., 2016).

Salmonids are highly efficient swimmers (Eliason et al., 2013) and have evolved a variety of behavioural tactics and physiological mechanisms in order to respond to environmental challenges during their catabolically fuelled migrations (capital breeding; Crossin et al., 2009). For example, they avoid suboptimal river water temperatures en route through thermal refugia (Berman and Quinn, 1991; Goniea et al., 2006; High et al., 2006; Hyatt et al., 2003; Mathes et al., 2010) – delaying migratory movement in deep pools, cold water tributaries, cold alcoves and areas with groundwater seeps (collectively thermal refuges) (Caissie, 2006). However, the location and temporal stability of thermal refuges is highly variable (Dugdale et al., 2013) and migrating salmonids regularly encounter water temperatures outside their tolerance levels (Mathes et al., 2010). Thermal challenges strongly affect salmonid physiology, causing them to alter the timing of their migrations and often leading to increased pre-spawn mortality (Farrell et al., 2008). This phenomenon is predicted to be exacerbated through further climate change (Farrell, 2009). However, while the effects of increased river water temperatures on migration success and mortality of salmonids are well documented, sub-lethal effects and consequences on survivors' reproductive success upon arrival at the spawning grounds are seldom considered and are still largely unknown (Fenkes et al., 2016).

During paired matings (Esteve, 2005), salmonids release gametes into the surrounding water, where fertilization success is determined by the proximity to eggs, as well as the number and quality of the sperm released (Fitzpatrick and Liley, 2008; Gage et al., 2004; Maekawa and Onozato, 1986; Mjølnerod et al., 1998; Simmons and Fitzpatrick, 2012; Tuset et al., 2008), which is partly determined by the maturation status of the males (Lahnsteiner and Leitner, 2013). The processes of spermatogenesis and spermiation occur as distinct phases during salmonid reproductive periods, causing seasonal fluctuations in sperm quality parameters (Billard, 1986). The seasonal phasing of salmonid maturation and the close

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coordination of population-specific spawning dates are largely driven by photoperiod, while water temperature plays a modifying role and tunes the finalization of gamete maturation and spawning to locally variable thermal conditions (Pankhurst and King, 2010). Water temperature can therefore affect sperm quality of salmonids indirectly, through the thermal experience of males during maturation and sperm production (Alavi and Cosson, 2005). For example, in brown trout, Lahnsteiner and Leitner (2013) found that elevated acclimation water temperature prior to and during spawning shifted and shortened the period of peak maturation, decreased sperm velocity and the proportion of motile sperm, increased sperm DNA damage and decreased seminal fluid quality. In addition, the temperature of the activating water directly affects sperm performance upon release (Alavi and Cosson, 2005). For example, increased activation water temperature decreases initial sperm velocity and the proportion of motile cells and results in decreased hatching success of brown trout larvae (Lahnsteiner, 2012). However, the interactive effects of male acclimation and sperm activation medium temperature on sperm quality have not been investigated.

Here, we examined how increased water temperature affects brown trout reproduction by assessing both direct (sperm activation medium temperature) and indirect (male acclimation temperature) thermal effects on sperm quality. Sperm quality was tested on two consecutive dates in order to assess whether thermal effects vary during the spawning season. We hypothesized that warm acclimation together with increased activation water temperature would reduce brown trout sperm quality.

## MATERIALS AND METHODS

### Experimental setup

In October 2015, 3 year old male brown trout, *Salmo trutta* Linnaeus 1758, were obtained from Dunsop Bridge Trout Farm Ltd (Clitheroe, UK). The animals were individually PIT tagged (Biomark Inc., Boise, ID, USA) and transferred into one of two large (1800 l), circular, outdoor tanks under natural photoperiod. Tanks were aerated, water was constantly recirculated and filtered, and a low current flow was implemented to minimize stress and aggression. After a 7 day settling-in period, the water temperature was adjusted over a period of 17 days at an average rate of 0.4°C per day to arrive at a 'warm' experimental temperature of 13°C in one tank, and at the same rate but over a period of 22 days to arrive at a 'cold' experimental temperature of 8°C in the other tank. Together with a shortening photoperiod, declining water temperature triggers maturation in winter/spring-spawning salmonids (Migaud et al., 2010). For brown trout in Western Europe, river water temperatures between 1 and 8°C are optimal for egg survival, while temperatures exceeding 8°C in winter are detrimental for spawning and egg incubation (Elliott and Elliott, 2010). Our cold temperature of 8°C lies within the suggested thermal optimum for reproduction, while our warm temperature of 13°C lies outside this window, but within current climate change predictions (van Vliet et al., 2011). A detectable effect of temperature impacts within this narrow margin would therefore realistically project the possible impacts of climate change-mediated increases in river water temperatures on brown trout reproductive success. Aside from minor temperature fluctuations arising from their exposed location, the tanks were maintained at 8.4±0.6 and 12.6±0.4°C, respectively, throughout the experimental period. Trout were offered commercial trout pellets [Skretting, Trow (UK) Ltd, Northwich, UK] daily, but they ceased feeding after being subjected to declining water temperature for 3 weeks. This is natural in migrating salmonids, marking the onset

of the spawning season (Hinch et al., 2005). Pellets were then still offered occasionally, but were not taken and were therefore removed from the bottom of the tanks. Fish were dip-netted and semen (sperm and seminal fluid) expressed (see below) on three occasions. Upon cessation of feeding, males were stripped of their semen, which was discarded, and sperm used for subsequent sperm quality assessment was produced under different acclimation temperatures. Semen was then collected for sperm quality assessment at an 'early' sampling date (17 and 18 November, within 1 week of differential temperature acclimation). In commercial farming, brown trout egg fertilization is carried out around this date (P. Mart, Dunsop Bridge Trout Farm Ltd, personal communication). A final semen sample was collected further into the breeding season, at a 'later' sampling date (8 and 9 December, after 4 weeks of differential temperature acclimation).

### Semen sampling

For stripping and semen sample collection, males were lightly sedated via immersion in 0.05 g l<sup>-1</sup> tricaine methanesulfonate (MS-222), buffered with 0.05 g l<sup>-1</sup> sodium bicarbonate in holding tank water at their respective acclimation temperature. Water from their holding tanks was used to reduce stress during anaesthesia. Upon losing equilibrium, males were swiftly removed from the anaesthetic bath and placed ventral side up on a Styrofoam block covered with a wet paper towel. After drying the anal region and emptying the bladder and bowel by applying slight pressure to the region around the anal pore, semen was carefully expressed. This was achieved by applying gentle pressure to both sides of the ventral midline in a controlled motion from the pectoral fins towards the anal pore. Semen was captured directly into clean Eppendorf tubes. Samples contaminated with water, urine or faeces were discarded. Untampered samples were immediately sealed and placed into an ice-cooled container. Fish were then placed into a 400 l oxygenated recovery bath filled with holding tank water and transferred back into their holding tanks upon full recovery. On the early sampling date, semen samples were obtained from *N*=11 males in the cold tank and *N*=12 in the warm tank. On the later sampling date, *N*=8 and *N*=9 samples were obtained from cold- and warm-acclimated males, respectively. Where possible, the same males were used on the two sampling dates; individual differences in sperm quality parameters were controlled for statistically (see below).

### Ethical note

Experimental procedures were covered by a UK Home Office project licence (licence number 40/3584, licence holder H.A.S.) and were carried out under approval of the University of Manchester's ethical committee.

### Sperm quality assessment

No later than 30 min after sampling, an aliquot of semen samples from both warm- and cold-reared males was placed into each of two temperature-controlled rooms at 8 and 13°C, respectively, where they remained until analysis on the day of collection. Sperm quality assessment was carried out inside the temperature-controlled rooms in order to ensure that the microscope stage, slides and coverslips were at the required experimental temperature. Sperm from each male were activated and analysed in randomized order with distilled tap water at both 8 and 13°C. For activation, 0.5 µl of semen was transferred into a clean 1.5 ml Eppendorf tube and 1 ml of water was added and swiftly mixed. Within 3–5 s, 0.5 µl of the diluted sample was injected into the well of a multi-test microscope slide

(MP Biomedicals, LLC, Solon, OH, USA), with a coverslip over half of the depression, under a pre-focused, dark-phase microscope (DM750, Leica Microsystems GmbH, Wetzlar, Germany) at  $\times 250$  magnification. A video camera [EOS 600D, Canon (UK) Ltd, Reigate, UK] attached to the microscope recorded sperm movement at 50 frames  $s^{-1}$  from the moment of activation. Sperm quality assessment was carried out from the video recordings using automated computer-assisted sperm analysis (CASA) software [CASA\_automated plugin, www.ucs.mun.ca/~cfpurchase/CASA\_automated-files.zip; see Purchase and Earle (2012) for further documentation] for ImageJ 1.49v (32-bit). As previously established in other studies (Lahnsteiner and Kletzl, 2012; Lahnsteiner and Mansour, 2012; Rosengrave et al., 2008), we recorded the average path velocity ( $\mu m s^{-1}$ ) (Wilson-Leedy and Ingermann, 2007) as a measure of sperm swimming speed, as well as the proportion of motile cells (motile sperm count/total sperm count) across all sperm cells in the field of view every 2 s from 10 s after activation (when sample drift following injection onto the microscope slide had ceased) until 28 s after activation (when most sperm movement had ceased).

### Statistical analyses

All statistical analyses were carried out using R 3.3.1 GUI 1.68 Mavericks build (<http://www.R-project.org/>). Two general linear mixed effects models [lme4 package 1.1-12 (Bates et al., 2015) and lmerTest package 2.0-32 (<https://CRAN.R-project.org/package=lmerTest>)] were fitted individually to data from each of the two sampling dates to assess the effects of acclimation temperature and activation temperature on the log-transformed swimming speed (continuous variable) of sperm from all the males sampled (Table 1). To assess the effects on the proportion of motile cells (binomial variable), two generalized linear models [lme4 package 1.1-12 (Bates et al., 2015) and car package 2.1-3 (Fox and Weisberg, 2011)] with a binomial error distribution were fitted (Table 2). The models were fitted with acclimation temperature, activation temperature and time (s) after sperm activation as categorical fixed factors and fish ID (category) nested within log(time) (discrete variable) as random factors. Because the models

fitted for the proportion of motile cells were strongly over-dispersed, the observations in the data set were named (1–800) and observation name was fitted as an additional, non-nested, categorical random factor, which eliminated over-dispersion. For *post hoc* analyses, comparisons of least squares means [lsmeans package 2.23-5 (Lenth, 2016)] of each acclimation temperature–activation temperature combination at each time point (s) after sperm activation were calculated from the models for each of the two sampling dates. Least squares means comparisons were adjusted for unbalanced samples but not for multiple testing because of the small sample sizes (Nakagawa, 2004). *P*-values (significance level  $P < 0.05$ ) were acquired using *t*-statistics and *z*-statistics for models describing sperm swimming speed and the proportion of motile cells, respectively.

## RESULTS

### Sperm swimming speed

Early in the spawning season, brown trout sperm swimming speed was affected by acclimation temperature, activation temperature and time since sperm activation. The effect of activation temperature on sperm swimming speed varied over the time since sperm activation, as evidenced from the significant interaction between these terms (Fig. 1A, Table 1). Sperm swimming speed declined over the period post-sperm activation and differences in sperm swimming speed between acclimation/activation temperature groups were manifest in the first 16 s post-activation (Fig. 1A). Specifically, at 10–16 s post-activation, sperm from cold-reared males when activated at 8°C swam faster, whereas sperm from warm-reared males when activated at 13°C swam slower than sperm from the other treatment groups. In the same period of time since activation, cold-acclimated, 13°C-activated sperm and warm-acclimated, 8°C-activated sperm swam at similar speeds. At 18 s post-activation, sperm were faster when activated at 8°C than when activated at 13°C, whereas rearing temperature had no effect on sperm swimming speed. At 20 and 22 s post-activation, the differences in sperm swimming speed for all treatment groups

**Table 1. Factors affecting sperm swimming speed**

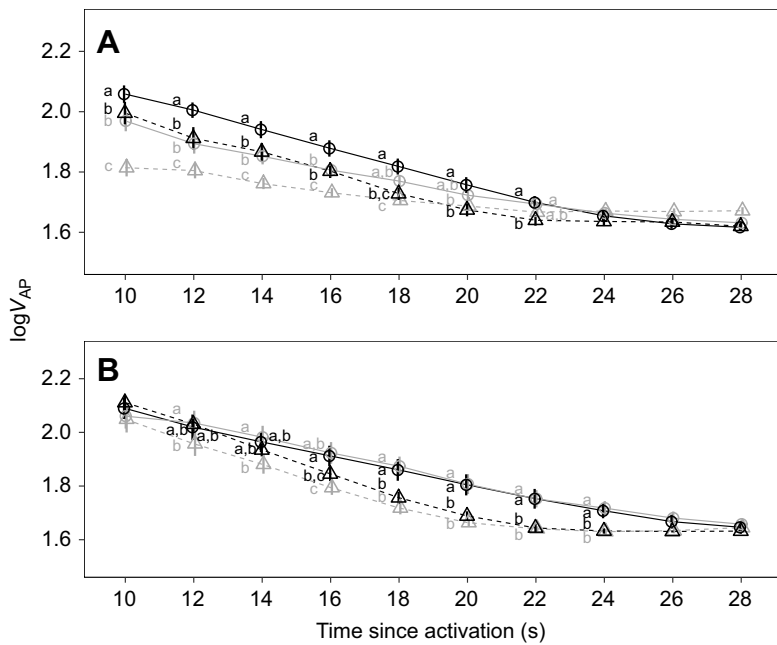
Term	Num. d.f.	Den. d.f.	<i>F</i>	<i>P</i>
Early sampling date				
Acclimation temperature ( $T_{acc}$ )*	1	21	7.096	0.015
Activation temperature ( $T_{act}$ )*	1	378	95.971	<0.0001
Time after sperm activation ( $t_{act}$ )*	9	159.34	22.935	<0.0001
$T_{acc} \times T_{act}$	1	378	0.626	0.429
$T_{acc} \times t_{act}$	9	159.34	1.794	0.073
$T_{act} \times t_{act}$ *	9	378	8.039	<0.0001
$T_{acc} \times T_{act} \times t_{act}$	9	378	1.504	0.145
Later sampling date				
Acclimation temperature ( $T_{acc}$ )	1	15	0.139	0.714
Activation temperature ( $T_{act}$ )*	1	270	158.012	<0.0001
Time after sperm activation ( $t_{act}$ )*	9	102.35	24.65	<0.0001
$T_{acc} \times T_{act}$ *	1	270	10.569	0.001
$T_{acc} \times t_{act}$	9	102.35	0.096	0.99
$T_{act} \times t_{act}$ *	9	270	7.475	<0.0001
$T_{acc} \times T_{act} \times t_{act}$	9	270	0.789	0.627

General linear mixed effects models describing the effects of male acclimation temperature (cold, 8°C,  $N=11$  for early and  $N=8$  for later sampling date; and warm, 13°C,  $N=12$  for early and  $N=9$  for later), sperm activation temperature (8 and 13°C) and time after sperm activation (10–28 s) on sperm swimming speed early and later in the spawning season. Asterisks indicate significant effects ( $P < 0.05$ ). Num., numerator; Den., denominator.

**Table 2. Factors affecting the proportion of motile cells**

Term	d.f.	$\chi^2$	<i>P</i>
Early sampling date			
(Intercept)*	1	18.703	<0.0001
Acclimation temperature ( $T_{acc}$ )	1	0.203	0.652
Activation temperature ( $T_{act}$ )*	1	9.703	<0.01
Time after sperm activation ( $t_{act}$ )*	9	73.672	<0.0001
$T_{acc} \times T_{act}$	1	0.103	0.748
$T_{acc} \times t_{act}$	9	4.37	0.885
$T_{act} \times t_{act}$	9	4.643	0.864
$T_{acc} \times T_{act} \times t_{act}$	9	4.11	0.904
Later sampling date			
(Intercept)	1	3.416	0.065
Acclimation temperature ( $T_{acc}$ )	1	0.0007	0.979
Activation temperature ( $T_{act}$ )	1	0.069	0.793
Time after sperm activation ( $t_{act}$ )	9	15.658	0.074
$T_{acc} \times T_{act}$	1	0.007	0.934
$T_{acc} \times t_{act}$	9	1.117	0.999
$T_{act} \times t_{act}$ *	9	26.378	<0.01
$T_{acc} \times T_{act} \times t_{act}$	9	2.523	0.98

Generalized linear mixed effects models describing the effects of male acclimation temperature (cold, 8°C,  $N=11$  for early and  $N=8$  for later sampling date; and warm, 13°C,  $N=12$  for early A and  $N=9$  for later), sperm activation temperature (8 and 13°C) and time after sperm activation (10–28 s) on the proportion of motile sperm cells in semen samples early and later in the spawning season. Asterisks indicate significant effects ( $P < 0.05$ ).



**Fig. 1. Thermal impact on sperm swimming speed.** Average path velocity ( $V_{AP}$ ) of sperm cells in brown trout semen sampled early (A) and later (B) in the spawning season from males acclimated to cold (8°C, black symbols and lines;  $N=11$  in A,  $N=8$  in B) and warm (13°C, grey symbols and lines;  $N=12$  in A,  $N=9$  in B) temperature, activated at 8°C (circles and solid lines) and 13°C (triangles and dashed lines) over the course of 10–28 s after sperm activation.  $V_{AP}$  ( $\mu\text{m s}^{-1}$ ) is presented on a logarithmic scale. Lines are fitted from a linear mixed effects model (Table 1); symbols and error bars show original data (means $\pm$ s.e.m.). Different letters denote significant differences ( $P<0.05$ ) between pairwise least squares means of contrasts at each sampling date.

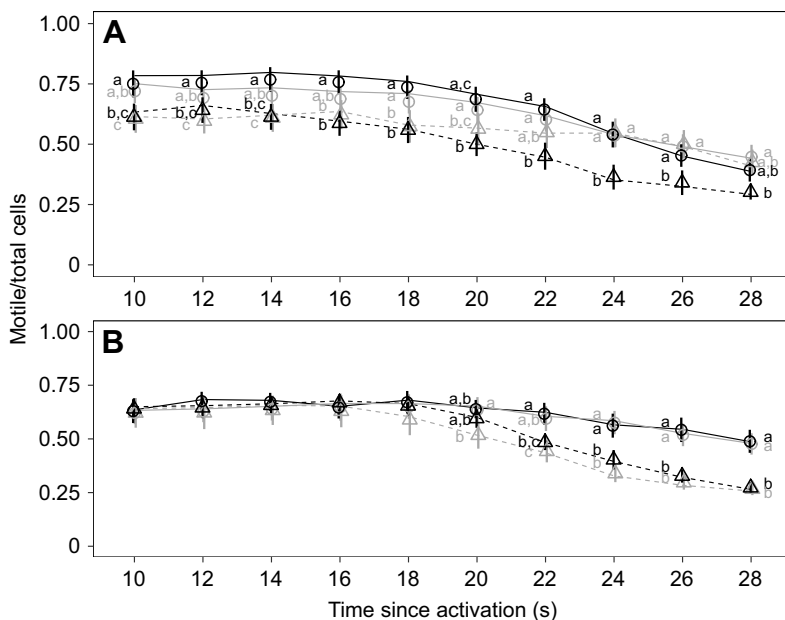
started to narrow, ceasing entirely from 24 s post-activation, when no further differences in sperm swimming speeds were detected.

Later in the spawning season, sperm swimming speed was affected by activation temperature and time since sperm activation. The effect of activation temperature varied among males subjected to the different acclimation temperatures and during the period since sperm activation, as indicated by the significant interactions between these terms (Fig. 1B, Table 1). Similar to findings for the earlier sampling date, sperm swimming speed generally declined over the time post-activation. However, sperm swimming speed did not differ between acclimation/activation temperature groups initially, as observed at the early sampling date, but instead predominantly differentiated later. Specifically, at 12 and 14 s post-activation, only sperm from warm-reared males that were activated at 8°C swam faster than when activated at 13°C. By 18 s post-activation, sperm from cold-acclimated males also swam faster at

8°C than at 13°C, while any effect of acclimation temperature had ceased. This pattern persisted up to 24 s post-activation, beyond which no further differences were detected.

#### Proportion of motile cells

Early in the spawning season, the proportion of motile cells in brown trout semen was mainly affected by activation temperature and time since sperm activation, whereas acclimation temperature had no overall effect on this sperm quality parameter (Fig. 2A, Table 2). Sperm motility declined over the period post-activation. At 10–18 s post-activation, sperm from cold-reared males activated at 8°C consistently contained a higher proportion of motile cells than sperm from both cold- and warm-reared males activated at 13°C. Between 10 and 14 s post-activation, sperm from warm-acclimated males activated at 8°C also had a higher proportion of motile cells compared with when activated at 13°C. From 18 s post-activation, acclimation



**Fig. 2. Thermal impact on sperm motility.** Proportion of motile sperm cells in brown trout semen sampled early (A) and later (B) in the spawning season from males acclimated to cold (8°C, black symbols and lines;  $N=11$  in A,  $N=8$  in B) and warm (13°C, grey symbols and lines;  $N=12$  in A,  $N=9$  in B) temperature, activated at 8°C (circles and solid lines) and 13°C (triangles and dashed lines) over the course of 10–28 s after sperm activation. Lines are fitted from a generalized linear mixed effects model (Table 2); symbols and error bars show original data (means $\pm$ s.e.m.). Different letters denote significant differences ( $P<0.05$ ) between pairwise least squares means of contrasts at each sampling date.

temperature had no detectable effect on the proportion of motile cells. From 24 s post-activation, sperm motility was similar among all treatment groups except cold-acclimated, 13°C-activated sperm, which had lower motility than the other groups.

Later in the spawning season, none of the main factors tested (acclimation temperature, activation temperature and time since sperm activation) affected the proportion of motile cells in brown trout semen samples in isolation. However, there was a variable effect of activation temperature over the period post-activation, as evidenced by a significant interaction between these terms (Fig. 2B, Table 2). While the proportion of motile cells in semen differed between activation temperatures over the whole observation period of the earlier sampling date, no differences were detected at the later date until 20 s after sperm activation (Fig. 2). Specifically, at 20 s post-activation, the proportion of motile cells differed between sperm from warm-reared males at the two activation temperatures, while acclimation temperature had no detectable effect and motility was similar for cold-acclimated male sperm at both activation temperatures. At 22 s post-activation, sperm from males at both acclimation temperatures had a higher proportion of motile cells when activated at 8°C compared with sperm from warm-reared males activated at 13°C. Cold-reared, 8°C-activated sperm also had a higher proportion of motile cells than cold-reared, 13°C-activated sperm. At 24–28 s post-activation, the proportion of motile cells differed between activation temperatures (8°C higher than 13°C) but acclimation temperature had no effect.

## DISCUSSION

The aim of this study was to test the possible detrimental effects of direct (sperm activation medium temperature) and indirect (male acclimation temperature) thermal challenges on brown trout sperm quality over the reproductive season. Consistent with our hypotheses, warm acclimation and sperm activation at increased temperature reduced sperm quality early in the spawning season. However, activation at cold temperature improved sperm performance in warm-acclimated males, albeit not to the level seen in the sperm of cold-acclimated males also activated at 8°C. The increased sperm quality of cold-acclimated males was compromised by activating sperm at warm temperatures but remained higher than that of warm-acclimated, warm-activated male sperm. These differences in sperm quality between acclimation temperature groups were no longer detected later in the season, a pattern consistent with delayed maturation of warm-acclimated males. Furthermore, while activation temperature strongly and persistently affected aspects of sperm quality of both warm- and cold-acclimated males at the early sampling date, heat sensitivity of sperm was reduced later in the spawning season.

We found that increased acclimation temperature decreased sperm velocity but not the proportion of motile cells in brown trout semen early in the spawning season, while increased activation temperature negatively affected both sperm quality parameters. Evidence that increased sperm velocity increases the competitive ability and therefore fertilizing capability of sperm exists for a number of fish species, including salmonids (Gage et al., 2004; Liljedal et al., 2008; reviewed by Simmons and Fitzpatrick, 2012). Motility, the proportion of live cells in an ejaculate, is another important factor influencing fertilization success (Hoysak and Liley, 2001; Simmons and Fitzpatrick, 2012). In addition, fertilization success is highest up to 10 s after sperm activation in salmonids and declines afterwards, making this initial time a crucial period for fertilization (Hoysak and Liley, 2001). Accordingly, our results suggest that warm acclimation and sperm activation at

increased temperature may decrease the fertilization probability of brown trout sperm at the start of the spawning season.

The effects of increased activation water temperature and elevated acclimation temperature on sperm velocity and motility have been described in a number of fish species (e.g. Billard and Cosson, 1992; Lahnsteiner, 2012; Lahnsteiner and Kletzl, 2012; Lahnsteiner and Leitner, 2013; Lahnsteiner and Mansour, 2012; Vladic and Jarvi, 1997; Williot et al., 2000; reviewed by Alavi and Cosson, 2005). Methodologies are inconsistent, however, and the effects of one thermal stimulus (acclimation or activation temperature) are routinely reported without controlling for the other. Our study is the first to measure the cumulative effects of male acclimation and sperm activation temperature simultaneously. Our results suggest that, early in the breeding season, cold-acclimated brown trout males enjoy a fertilization advantage (faster sperm) over warm-reared males, especially when the activation medium is also cool (allowing a higher proportion of sperm to swim faster). Conversely, warm-reared males have lower fertilization potential (slower sperm), especially when the activation medium temperature is also increased (reducing the proportion of motile cells and their speed). However, an upward thermal shift from cold acclimation (8°C) to 13°C upon activation reduces sperm swimming speed, while a downward shift from acclimation at warm temperature (13°C) to activation at 8°C is beneficial. This discrepancy can significantly affect the results and conclusions drawn from studies assessing thermal impacts on fish sperm quality, if one type of thermal challenge (e.g. activation temperature) is tested but the other (e.g. acclimation temperature) is not standardized or not reported. Therefore, our nuanced results highlight the importance of considering both direct and indirect thermal effects when assessing the impact of temperature on fish sperm quality.

It has been suggested that an optimal activation temperature for sperm movement exists that corresponds with species-specific local thermal conditions (Lahnsteiner and Mansour, 2012; Vladic and Jarvi, 1997). This indicates that our cold treatment of 8°C is closer to the thermal optimum for brown trout sperm than the warm treatment of 13°C. Water temperatures of rivers during upstream migration and of spawning grounds upon arrival can differ substantially because they closely follow air temperature fluctuations (Isaak et al., 2011; van Vliet et al., 2011). While migrating trout can shelter from suboptimal water temperatures in thermal refuges (High et al., 2006), spawning brown trout males are confined to the shallow, slower flowing lakes and tributaries where females choose to build their redds (Jonsson and Jonsson, 2011). Being shallower, these areas are less likely to contain thermal refuges, unless they are groundwater fed and/or at sufficiently high altitude. Therefore, an upward shift from moderate temperatures during migration to increased, suboptimal temperatures during spawning could be experienced by male salmonids, negatively affecting their sperm after release. Climatic warming has had substantial influence on river water temperatures within brown trout distributions in the past decades (Hari et al., 2006) and this trend is projected to persist, dramatically increasing river water temperatures over the next 100 years (Jonkers and Sharkey, 2016). Our results suggest that the temperature of the water in spawning tributaries (i.e. the temperature of the activation water that sperm are released into during spawning) has a stronger and more persistent effect on sperm quality than the acclimation temperature of males prior to spawning. Consequently, while a shift from increased water temperature during migration to cooler water in spawning tributaries is unlikely to occur in natural rivers, our results suggest that active protection of brown trout riverine habitat and especially thermal sheltering (e.g. through

riparian vegetation or cool water conflux) of known spawning grounds can be an effective management strategy, improving sperm quality and reproductive success of wild salmonids despite the negative impacts of increased river water temperatures en route.

Acclimation temperature had no effect on sperm swimming speed or the proportion of motile cells on our later sampling date, consistent with a delay in maturation of warm-acclimated males, as previously demonstrated for brown trout (Lahnsteiner and Leitner, 2013) and other species (Lahnsteiner and Kletzl, 2012; Manning and Kime, 1985; Pankhurst and King, 2010; Taranger et al., 2003; but see Breton and Billard, 1977). The effects of intra-testicular sperm maturation on motility parameters and the resulting changes of sperm quality over the reproductive period have been described for several fish species, yielding contrary results. Depending on the species investigated, seasonal effects on sperm quality exhibit a range of patterns, including increases, decreases and quadratic patterns with peaks in mid-season (Alavi et al., 2008, 2010; Babiak et al., 2006; Christ et al., 1996; Hjrzaee et al., 2010; Johnson, 2012; Munkittrick and Moccia, 1987; Rouxel et al., 2008; Vermeirssen et al., 2004). The inconsistency of these results is probably the result of species-specific differences in maturation patterns and the resulting fluctuations in different sperm quality parameters over the reproductive season. In salmonids, the processes of spermatogenesis and spermiation, resulting in changes in sperm quality over the reproductive period, occur as distinct phases and the release of ripe sperm cells into the sperm ducts peaks mid-season (Billard, 1986). Because individual male sperm quality was assessed repeatedly over the spawning season, testicular maturation progress could not be directly quantified in this study. However, the higher sperm swimming speed in cold-acclimated compared with warm-acclimated males at the early sampling date, but not at the later sampling date, suggests that warm-acclimated male maturation was delayed and sperm swimming speed compromised until the later sampling date (approaching mid-season), when both groups were in advanced stages of maturation.

In contrast to the early sampling date, activation temperature had no effect on the initial proportion of motile cells later in the spawning season. This suggests that, as the spawning season progresses, heat sensitivity of the sperm upon activation is reduced. Considering the substantial influence of water temperature on vital quality parameters of sperm after release, the presence of thermo-protective physiological mechanisms would be highly beneficial. This is especially relevant in salmonids, where sperm movement duration and the associated window for fertilization are extremely short (Kime et al., 2001). In the freshwater teleost *Labeo rohita*,  $Ca^{2+}$  influx and consequentially the activation and preservation of sperm movement are regulated by thermosensitive ion channels [vanilloid transient receptor potential (TRPV) channels; Majhi et al., 2013], which may confer heat resistance to the sperm cells, as previously shown for germ cells in mouse testes (Mizrak and van Dissel-Emiliani, 2008). Changes in heat sensitivity of sperm over the breeding season could be a result of the expression of TRPV channels in sperm cell membranes with advanced maturation of the male trout in our study. Mechanosensitive canonical TRP channels (TRPC channels) from the same protein family as the thermosensitive TRPV channels have been reported in rainbow trout tissue, including gonads (Patrick et al., 2010). However, to our knowledge, the presence of TRPV channels in salmonid sperm cell membranes or their potential influence on sperm movement and thermosensitivity over the course of the spawning season have not

been confirmed to date, presenting a novel avenue for future research.

## Conclusion

This study is the first to demonstrate an interplay between direct and indirect thermal challenges affecting sperm quality in brown trout on consecutive dates within the reproductive season. The results afford a novel insight into the thermal biology of fish sperm in response to climate change, with implications for the management of trout reproduction in hatcheries as well as wild populations. Similar sperm quality from males irrespective of acclimation temperature and a decrease in thermal sensitivity of sperm after release at our later sampling date suggest that the fertilization potential of trout sperm was overall increased later in the spawning season. We further show that the negative impacts of warm acclimation on sperm quality at the onset of the reproductive period are partly offset by activating sperm at cold temperatures. Controlling the water temperature at spawning grounds may therefore present an opportunity for the effective management of trout reproduction in the wild, despite some of the negative impacts of increased river water temperatures en route (i.e. acclimation temperature) due to climate change. Crucially, our nuanced results highlight the necessity to consider both direct and indirect impacts of water temperature when assessing fish sperm quality in future studies.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: M.F., J.L.F., H.A.S., R.L.N.; Formal analysis: M.F., J.L.F.; Investigation: M.F., K.O.; Resources: J.L.F., H.A.S., R.L.N.; Writing - original draft: M.F.; Writing - review & editing: M.F., J.L.F., K.O., H.A.S.; Visualization: M.F.; Supervision: J.L.F., H.A.S., R.L.N.; Project administration: H.A.S., R.L.N.; Funding acquisition: R.L.N.

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