Thermal reaction norms in sperm performance of Atlantic cod (*Gadus morhua*)

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ABSTRACT

Phenotypic plasticity occurs when a genotype produces variable phenotypes under different environments; the shapes of such responses are known as norms of reaction. The genetic scale that reaction norms can be determined is restricted by the experimental unit that can be exposed to variable environments. This has limited their description beyond the family level in higher organisms, thus hindering our understanding of differences in plasticity at the scale of the individual. Using a three year common-garden experiment we quantify reaction norms in sperm performance of individual genotypes within different families of Atlantic cod (*Gadus morhua*). Cod sperm showed phenotypic plasticity in swimming performance across temperatures (3, 6, 11, 21^{o}C) but the pattern of the response depended upon how long sperm had been swimming (30, 60, 120, 180 s). Sperm generally swam fastest at intermediate temperatures when first assessed at 30 s post-activation. However, a significant genotype×environment interaction was present, indicating inter-individual differences in phenotypic plasticity. To our knowledge this is the first study to describe variable sperm performance across environmental conditions as a reaction norm. The results have potential theoretical, conservation and aquaculture implications.

INTRODUCTION

The capacity for individuals and populations to respond to spatial and temporal environmental fluctuation is paramount to species persistence. While populations can evolve through time, individuals cannot undergo genetic change and their reproductive success can be severely jeopardized under suboptimal conditions. Individual fitness will decline with environmental change if the optimal expression of an important trait is rigid and requires specific conditions. Within this context, it has long

been recognized that traits with flexible expression may enable long-term existence under unpredictable environments (Baldwin 1896; Morgan 1896). How large-scale environmental perturbations, such as climate change, affect species persistence will depend to some degree on the ability of individuals and populations to respond and adapt to such changes.

Phenotypic plasticity occurs when a given genotype produces variable phenotypes under different environments (Schlichting and Pigliucci 1998; West-Eberhard 2003). Phenotypes may represent continuous (e.g., colour intensity, growth rate) or discrete (e.g., colour morphs, alternative maturation strategies) traits, and plasticity can be adaptive, maladaptive, or non-adaptive (neutral) depending on the trait and context (reviewed by Ghalambor *et al.* 2007). Plasticity can be represented as a reaction norm (also known as a norm of reaction), which serves to quantify and visualize the linear or nonlinear shape of the phenotypic response to the environment (Woltereck 1909; Schlichting and Pigliucci 1998).

In addition to mean trait values, reaction norms themselves may be under selection (Scheiner 1993; Hutchings 2004), as the optimum phenotypic expression of a given trait may vary under unpredictable environments. Arguments have been made that reaction norms both hinder and promote adaptation (Price *et al.* 2003; Ghalambor *et al.* 2007). For example, if adaptive phenotypic plasticity shifts mean trait expression to optimal levels in each environment, the genotype is protected from selection and evolution does not occur. In contrast, adaptive plasticity may enable trait expression to move towards, but not reach, the optimum in a different environment. This allows individuals to persist, thus enabling directional selection to further pull the trait towards optimality. Additionally, non-adaptive plasticity can promote rapid evolutionary change if novel phenotypes are produced at extreme

environments through the release of cryptic genetic variation (Queitsch *et al.* 2002; Rutherford 2000, 2003; Ghalambor *et al.* 2007).

Reaction norms vary temporally and spatially within species, and although genetic differences have been examined in limited detail they are likely ubiquitous. Examples include physiological traits such as growth rate in caterpillars (Kingsolver *et al.* 2006) or energy allocation in fish (Purchase and Brown 2001), morphological traits like colour patterns in flies (Ottenheim *et al.* 1996) and behavioural traits such as reproductive signaling rates in moths (Jia *et al.* 2000). The resolution at which genetic differences in reaction norms persist is generally unknown, but can occur at the population level on finer scales than revealed by selectively neutral DNA markers (Hutchings *et al.* 2007). Genetic differences in reaction norms among populations would indicate how individuals, on average, differentially respond to environmental variation. They can also vary among full- or half-sibling families, as indicated by studies on plants (e.g., Agrawal 2001; Waller *et al.* 2008), invertebrates (e.g., Kingsolver *et al.* 2006; Engqvist 2008), and a very small number of vertebrates (Beacham and Murray 1985; Yamahira *et al.* 2007).

Sexually reproducing animals with external fertilization have sperm that are ideal for studying reaction norms. Our search of the literature (March 2009) revealed no published work on sperm quality framed in this context, but see Engqvist (2008) for a study on sperm quantity in scorpion flies. There are three major advantages of studying reaction norms of sperm quality. First, for many species, males provide nothing to the next generation except sperm, and the genetic material within, to fertilize eggs. In such cases, fertilization ability is the ultimate expression of male quality. Sperm have measureable characteristics, such as swimming velocity, that are often tightly linked to fertilization success. Therefore, studies can be conducted on traits that not only directly relate to fitness, but represent the compilation of a series of life processes that may have taken decades to achieve. Secondly, single cells

are likely more sensitive to environmental variation than the whole organism and thus reactions norms can be determined on finer scales. Unlike many other types of cells, it is relatively easy to assess the impact of the environment on sperm. Lastly, although by definition phenotypic plasticity is a function of the genotype, in reality it is not possible to compare genotypes in many cases. For most sexually reproducing animals, the family level is the finest genetic scale upon which reaction norms can be studied, and in such cases environmental gradients are tested *among-siblings* (i.e. among closely related genotypes). Although individual sperm from an ejaculate share only 50% of the haploid genetic material that they contribute to the next generation, sperm swimming "behaviour" is thought to be under diploid control of the father (Parker 1993; Parker and Begon 1993; Haig and Bergstrom 1995; Bernasconi et al. 2004). Therefore, if different sperm from a single male are subjected to a variety of controlled environments, reaction norms can be genetically controlled in a way that is somewhat analogous to clonal organisms (see Scheiner 1993). Such studies are powerful as they are capable of testing environmental gradients *within-siblings* (i.e. within genotypes or intra-individual), which fits within the theoretical construct of phenotypic plasticity.

We undertook a two-stage, three year common-garden experiment to investigate reactions norms of sperm quality in a marine fish. Using three families of Atlantic cod (*Gadus morhua*) we addressed three hypotheses: (a) there is phenotypic plasticity in sperm performance with temperature, and that a potential response to temperature depends on how long sperm have been swimming, (b) there is genetic variability in the thermal reaction norm, and (c) extreme environments promote novel phenotypes. Our results document reaction norms of individual genotypes, and have potential theoretical, conservation and aquaculture implications.

METHODS

Study organism

Atlantic cod are widely distributed on both sides of the North Atlantic Ocean, but recent evidence indicates that populations show much more homing to spawning sites than previously believed (Robichaud and Rose 2001; Svedang *et al.* 2007). Males provide nothing but sperm to reproduction and although cod are known to form dense spawning aggregations (if males lek they may help choose spawning sites), details of spawning behaviour have not been documented in the wild. In captivity, research has shown that there is male display, male-male competition, female mate selection, and males which sneak fertilizations (Brawn 1961; Hutchings *et al.* 1999). Sperm competition is intense, and multiple paternity of a single batch of eggs does occur in tank spawnings (Hutchings *et al.* 1999; Rakitin *et al.* 1999a, 2001; Herlin *et al.* 2008). Under sperm competition, most egg fertilizations likely occur within seconds of gamete release in natural situations and sperm swimming speed is therefore expected to be linked to fitness. However, why cod sperm can remain viable for an unusually long time (Trippel and Morgan 1994) is unknown.

General framework of experiment design

An efficient way of disentangling environmental from genetic influences on phenotypic variation is to use "common-garden" experiments. In such studies, individuals from different groups (e.g., populations, families) are kept under identical environmental conditions. Phenotypic differences among groups would therefore indicate a genetic, and if positively related to fitness, adaptive basis. If multiple environments are used, observed phenotypic differences in such experiments can result from three sources: the environment, the genotype, or their interaction. A significant G×E interaction would

indicate differences among groups in how the phenotypic trait(s) responds to a range of environments, and thus indicates genetic variation in phenotypic plasticity. Common-garden experiments are regularly undertaken with short-lived organisms such as annual plants, but are rare in long-lived vertebrates due to practical complexities and expense.

Common-garden Phase I

Source of fish

Wild cod were captured from the Bay of Fundy (Canada) in 2004 and brought to the Fisheries and Oceans Canada Biological Station in St. Andrews, New Brunswick (45° N, 67° W). These fish were kept under ambient temperature and lighting conditions and fed a mixture of squid and mackerel. In February and March of 2005 gametes were stripped from these fish and different full-sib families were created from *in vitro* fertilizations. Standard aquaculture protocols were used for embryo incubation (~ 6^{o}C) and larval rearing (~ 10^{o}C). Each family was initially kept in a different incubator and larval rearing tank, but was subjected to the same conditions in a common room. At about 6 months in age, each juvenile was implanted with a passive integrated transponder (PIT) tag and placed in a common tank (families combined) in September of 2005. These fish were raised in a flow-through seawater system under ambient photoperiod and salinity and fed marine grower pelletized diet (EWOS Canada; Surrey, British Columbia). Temperature was maintained between 2-4^{o}C leading up to and during the spawning season in 2008.

Each individually tagged cod was assessed on January 9 2008 to determine total body length (±0.1 cm) and weight (±1 g) prior to commencement of the spawning season at three years of age. The sex of each fish and its maturation status were determined using a small sample of gametes obtained by

cannulation. Four brothers (analogous to individual genotypes and hereafter referred to as individuals) were chosen from each of three families for the experiment (Figure 1). Specific individuals were selected in order to minimize size differences among families (Table 1), as the swimming ability of cod sperm may be influenced by some measures of fish "size" (see discussion for references).

Collection of semen for sperm experiments

The assessment of sperm was conducted between February 25 and March 8 2008. One individual was assessed for each family per day (three fish per day, four experimental days). The target fish were anesthetized with MS-222 in order to minimize stress during handling and stripping. The external urogenital pore was wiped dry, and semen was obtained by applying slight pressure on the abdomen. To help avoid seawater, urine, and feces contamination the first 1-2 mL of ejaculate was discarded in a standardized manner. Approximately 10 mL of ejaculate was then collected in a 50 mL sterilized dry Pyrex beaker and immediately covered with aluminum foil and temporarily placed in a cooler at 6^{o}C. Samples were then held in a 5-7^{o}C environmental chamber until sperm assessment.

Spermatocrit (defined as the ratio of packed white cells to the total volume of semen × 100%) was used to estimate spermatozoa density (Rakitin *et al.* 1999b). Semen from each male was drawn into three microhaematocrit capillary tubes (75 mm length, 1.1-1.2 mm internal diameter). One end of each tube was sealed with critoseal and they were centrifuged for 10 min at 7500 rpm. The mean of the three measurements per male created a single datum (Table 1) that was used for statistical analysis.

Common-garden Phase II

Sperm exposure to temperature

Four test temperatures were used (3±1, 6±1, 11±1, 21±1^{o}C) in sperm assessment, which were chosen based on a compromise between a wide comparison range and the limits of our facility. Cod will naturally spawn at 3 and 6^{o}C but do not reproduce at 21^{o}C as they never experience this temperature in the wild. The experiment was conducted in a series of four temperature-controlled rooms. The microscope, activating medium, slides, coverslips, and pipette tips were acclimated to room temperature before sperm activation. Semen from each male was transferred from the storage beaker (~ 6^{o}C) into plastic tubes and placed in the test room for 15-20 min before activity trials commenced (preliminary experiments showed that semen slowly reached test temperature over this time period). Each semen sample was tested at the four temperatures (repeated measures). The test order of the temperatures was randomized among the four days (one individual from a given family on each day): Day 1 = 21, 11, 3, 6^{o}C; Day 2 = 3, 11, 21, 6; Day 3 = 11, 6, 21, 3; Day 4 = 6, 3, 11, 21. Temperature of the swimming sperm was continually and precisely monitored during the experiment using an infrared temperature gun aimed at the edge of the slide coverslip.

All sperm activity tests were completed within 12 h of semen collection. Rouxel *et al.* (2008) reported that a significant decrease in Atlantic cod sperm motility doesn't occur until after 168 h of storage at 4^{o}C. Therefore, we feel confident that the maximum of 12 h delay in sperm activation post collection had a minimal affect on sperm quality in our experiment. Moreover, the study was designed such that any effect would not bias comparisons among temperatures or families, but would appear as variability among individuals within a family (see discussion).

Sperm activation procedure

Fish sperm is inactive *in vivo*, and remains inactive until in contact with water of appropriate chemistry. The activating medium was composed of 32 ppt seawater plus 1% v/w bovine serum albumin (BSA). BSA was added to prevent sperm from sticking to the glass slides (Rouxel *et al.* 2008). Air was bubbled into the activating medium to maintain dissolved oxygen levels at saturation. Repeated measurements indicated the pH ranged from 7.18-7.20.

Sperm activity was induced by pipetting 0.5 µl of semen into a plastic tube containing 300 µl of activating medium. The semen and activating medium were shaken for 5 s to homogenize the sample. We pipetted 40 µl of this dilution into a well of a 10-well multi-test glass slide (MP Biomedicals) and put a coverslip in place.

Sperm from many fish species are active for very short periods of time. When there is sperm competition, as in cod, swimming speed should influence fertilization ability more than swimming longevity (Rudolfsen *et al.* 2006), and most fertilizations should occur within seconds. In our experiment, sperm from the same semen sample and activation procedure were assessed at 30, 60, 120, 180 s (± 5) post-activation (Figure 1). The entire sperm activation and assessment process was repeated completely multiple times (Figure 1). Any replicate that deviated outside of the ± 1^{o}C target temperature or contained sperm that were obviously drifting was discarded, and is not included in the numbers reported. A minimum of three replicates were used for each fish at each temperature in the subsequent analysis (Figure 1). These procedural replications gave very similar results (Figure 2) and were averaged (see below) for statistical purposes.

Sperm assessment

Sperm activity was captured using a compound microscope (400X magnification) equipped with

a 40X negative phase objective and Basler camera (Model A312fc) attached to a personal computer via a FireWire connector. Images were evaluated in real time using an Integrated Semen Analysis System version 1.0.17 (ISAS; Projectes i Serveis R+D S.L.). The system was set to capture images at 25 frames·s^{-1}.

The software estimated several potentially useful characteristics of sperm activity: average velocity on the point-to-point track followed by the cell (curvilinear velocity, VCL), average velocity of the straight line between the start and end points (linear velocity, VSL), and average velocity of the smoothed cell path (average path velocity, VAP). These are all likely correlated and one must be careful not to test the same hypothesis multiple times using different metrics. Following convention for thermal reaction norms (Angilletta 2009) we refer to sperm quality as a function of temperate as sperm performance.

Sperm activity was captured at the targeted times post-activation and digitally stored. Each sperm recording was manually checked for quality control after the experiment was completed. Sperm tracks were removed from further analysis if the software incorrectly combined crossing tracks of multiple sperm, split the track of a single sperm, or if a sperm swam out of the field of view before adequately being assessed. Accurate tracks were present for 11,075 swimming sperm. Following Tuset *et al.* (2008) cod sperm with VSL $\leq$ 4 μm·s^{-1} were not considered to be exhibiting progressive motility and were excluded (only 1 of the 11,075 sperm met this criterion). We did not apply the cutoff used by Rudolfsen *et al.* (2005, 2008), where cod sperm with VAP < 20 μm·s^{-1} and/or VSL <10 μm·s^{-1} were considered to be static. This would have removed 1483 sperm from our analysis, in a manner that was clearly related to temperature and time post-activation. Sperm that had been swimming at fast velocities often had slowed below this cutoff by 120 s at high temperatures. A total of 11,074 swimming sperm

were used in subsequent analyses.

Statistics

Statistics were conducted using SAS-9.1 and Minitab-15. Significance was set at $\alpha = 0.05$ and assumptions of parametric statistics were checked by examining model residuals.

Fish characteristics

We tried to select specific fish to eliminate fish size as a source of variability. The three cod families were compared for fish characteristics using one-way ANOVA (analysis of variance). The dependent variables were total length, body weight, Fulton's condition factor, and spermatocrit (Table 1). Bonferroni corrections were not required as families did not significantly differ in any parameter at $\alpha = 0.05$.

Sperm analysis

Variance components were examined for random factors with a fully nested ANOVA (Families, Individuals, Replicates, Sperm) to determine repeatability of the experimental procedure at each time post-activation for each temperature (Figure 2). When averaged across times and temperatures, 84% of the variability in sperm swimming velocity (VCL) was due to variability among sperm cells within a procedural replicate (the activation and assessment process). This was followed by 9% for differences among individuals within a family, 4% among families, and 3% among procedural replicates (Figure 2). Our experimental procedure for activating and documenting sperm swimming velocity was thus highly repeatable.

Swimming velocities of individual sperm cells were highly variable and likely non-normal in distribution. Therefore the median velocity among sperm was calculated instead of the mean within each

replicated sperm activation procedure. Procedural replicates (at least three) were averaged in order to produce one datum for each fish (n=12), per time post-activation (n=4), per temperature (n=4). Different goals were addressed using different measures of sperm performance. (1) Faster swimming sperm are expected to be more likely to fertilize eggs, and thus sperm quality should be related to initial velocity (Rudolfsen *et al*. 2006). Sperm swimming trajectories are not necessarily expected to be linear, especially given there were no female cues to potentially guide sperm (Urbach *et al*. 2005). Therefore swimming velocities over the actual track followed by the sperm were used as a measure of "quality" (VCL). (2) To investigate how temperature and time influence variability among individual sperm cells within an ejaculate, the coefficient of variation (%) of sperm swimming velocity (VCL) was calculated within each procedural replicate (as opposed to the median), and then averaged across replicates. During the experiment we noticed that sperm swimming "behaviour" seemed to be different at 3^{o}C than at the other temperatures. *Post hoc*, we sought to (3) determine how viscosity might influence sperm propulsion using the wobble index (WOB), which is the oscillation percentage of the real track with respect to the average track (calculated by the software used as VAP/VCL, ISAS 2008). The wobble index measures the side-to-side motion of the sperm head per unit of distance traveled. High values are sperm that are swimming with less head motion, i.e. they require less tail beats to travel the same distance.

Sperm performance was analyzed using a mixed-model nested repeated-measures balanced ANOVA (Figure 1), using Proc GLM in SAS. The main independent variables were (a) family (random); (b) individual (random) nested within family; (c) temperature (fixed, repeated-measures); and (d) time (fixed; repeated-measures). Appropriate interaction terms were also included and were

individually assigned to obtain correct error terms for within-subjects (repeated-measures) tests (Table 2).

RESULTS

The common-tank raised cod attained a body length of 45-50 cm after nearly three years of growth (Table 1). Spermatocrit was much more variable among males than were metrics of body size (coefficient of variation: total length=5.7%, body weight=19.6%, Fulton's condition=7.5%, spermatocrit=31.7%). However, the three families were not significantly different in body length, weight, condition or spermatocrit (Table 1). Moreover, among the 12 fish used, there was no significant correlation between VCL and fish characteristics (fish length, $r = 0.083$, $p = 0.798$; body weight, $r = -0.071$, $p = 0.826$; Fulton's condition, $r = -0.471$, $p = 0.123$; spermatocrit, $r = -0.546$, $p = 0.066$).

There was phenotypic plasticity in sperm swimming velocity to temperature (Figure 3) at all times post-activation. The average response across individuals and families in Figure 3 negates the description as a "reaction norm" in the strictest sense of the term (no longer the response of a specific genotype), although we will refer to it as such for continuity. The thermal reaction norms were generally dome shaped but were more linear when sperm had been swimming for longer periods of time (Figure 3). Thus, the relationship between swimming velocity and temperature depended upon how long the sperm had been swimming (Temperature×Time interaction, Table 2). The breakdown of the interaction can be seen graphically in Figure 3. The decline in sperm swimming velocity with time post-activation occurred more rapidly at warmer temperatures (Figure 3). At 30 s post-activation, peak swimming velocities occurred at 11°C, however peak velocities occurred at colder temperatures if sperm had been swimming for longer periods; 6°C for 60 and 120 s, and 3°C for 180 s (Figure 3). At the times tested,

sperm generally swam faster at 3 than 21°C. Whether sperm would have swum faster at 21°C immediately after activation is unknown (sperm were first assessed 30 seconds post-activation).

The analysis showed a significant Genotype×Environment interaction (genetic differences in phenotypic plasticity). Unexpectedly, thermal reaction norms of sperm swimming velocity differed more among individuals within a family than among families (Figure 2, Table 2). This result is based on a nested analysis indicating where most of the variation exists (see discussion). Reaction norms are shown for each of the 12 individuals in Figure 4. The full analysis accounts for repeated-measures of temperature and time post-activation, families, individuals within families, and appropriate interactions (Table 2). There was no overall statistically significant difference among the three families, or in how they responded to temperature and time (Table 2). However, there were substantial differences among individuals and this depended on both time post-activation and temperature (significant Temp×Genotype(Family) interaction, Table 2). For example, at 30 s post-activation, swimming velocities of three individuals from Family B increased from 6 to 11°C, but sharply decreased for the other individual, while temperature had much less affect on sperm from three of the four individuals from Family C (Figure 4). Variability among individuals was highest at 3°C and lowest at 21°C.

Individual sperm from the same animal (i.e., genotype) responded differently to environmental variation. The coefficient of variation in sperm swimming velocity was affected by time post-activation and temperature, but not their interaction (Figure 5, Table 2). Variability among sperm cells within ejaculates did not significantly differ among families, but did vary among individuals and this depended on both time post-activation and temperature (Table 2).

Wobble was significantly influenced by both temperature and time post-activation and their interaction (Table 2). The nature of the interaction can be seen in Figure 6. Sperm exhibited much more

side-to-side swimming motion at 3^{o}C than the other temperatures, but there was significant variability among individuals (Table 2, Figure 6). There was less of an affect from time than temperature, but on average sperm that had been swimming for longer periods tended to show slightly more side-to-side motion.

DISCUSSION

Atlantic cod sperm exhibited phenotypic plasticity in swimming performance across temperatures; however the description of the reaction norm is dramatically different depending on how long sperm have been swimming when being assessed. Thus, the shape of phenotypic plasticity to one environment (temperature) is dependent on another environment (time). There was a statistically significant G×E interaction at one hierarchical level, but not another. Unexpectedly, differences in thermal reaction norms of sperm performance were greater among individuals within a family than among families (discussed below). To our knowledge this is the first study on sperm to describe performance as a reaction norm to variable environments. Additionally, we found no published study on a sexually reproducing animal that explicitly stated that reaction norms were documented for individual genotypes, which could be due to no previous research or a lack of use of the terminology.

Selection can act on genotypic differences in phenotypic plasticity and therefore populations may evolve new reaction norms under changing conditions (Gotthard and Nylin 1995). Current climate trends are predicted to produce increases in ocean temperatures into the foreseeable future and although cod spawn seasonally at specific water temperatures, thermal limits for successful reproduction are unknown. If populations change their distribution to avoid warm water and occupy new spawning sites, the local retention of eggs and larvae by currents may be affected in ways that alter current patterns of

recruitment and productivity. However, phenotypic plasticity in many traits (including sperm swimming ability) would potentially enable successful reproduction over a wide range of temperatures at traditional spawning sites. Moreover, variation in such reaction norms may enable further persistence under warm conditions through genetic assimilation (Price *et al.* 2003; Ghalambor *et al.* 2007).

Our experiment was designed to investigate genetic differences in phenotypic plasticity, under controlled environments, at two levels; within- and among-families. Unexpectedly, our results show greater variability among individuals within a family than among families. The reason for this is unknown. In our opinion, our experiment had more control than would be typical with a species like cod. For example, the source fish were collected at the same time/place and experienced the same captive conditions before being used in artificial fertilizations. The F_1 offspring were raised for three years under common conditions, during which different sperm presumably experienced common developmental conditions within their father. Finally, sperm from different fathers were treated to the same environmental conditions during assessment. Some possible explanations for greater variability among individuals than families need to be considered. Cod are batch spawners and male sperm quality may potentially change throughout a spawning season (Rouxel *et al.* 2008). In our study, sperm from a given fish were assessed on one day. Within a family, if some individuals were at peak seasonal performance and some others at pre- or post-peak performance, individual variability in sperm quality associated with spawn timing may have been enough to mask relatively higher sperm variability among families in the nested analysis (although it seems more likely that individuals within a family would be synchronized in their spawning cycles than fish from different families). Additionally, sperm from all fish could not be assessed at the same time, so we chose to control most carefully for temperature and family comparisons. As a result, the different individuals within a family were not assessed on the same

day. However, there is no obvious reason for this to have introduced variability. For example, the water used in sperm activation on all days was obtained from a common container that was kept in a cold room at 6^{o}C and air was bubbled into the activation medium to ensure oxygen saturation.

Temperature influences fish swimming performance through both physiological and physical mechanisms. High temperature may increase metabolism but is strongly inversely related to viscosity. The swimming behaviour of ~55 cm African lungfish (*Protopterus annectens*) was affected at higher viscosities (mud) because less distance was travelled per tail beat, i.e. the stride length decreased (Horner and Jayne 2008). At the millimeter scale, Fuiman and Batty (1997) separated mechanical from physiological effects of temperature on the swimming behaviour of larval herring (*Clupea harengus*) and found that smaller individuals were much more susceptible to the effects of viscosity than larger larvae. Due to their extremely small size, viscosity is expected to have a large effect on sperm. Kupriyanova and Havenhand (2005) showed that about half of the decline in polychaete sperm swimming velocity with temperature could be attributed to viscosity. In our study, sperm generally swam with more side-to-side motion at the lowest temperature (highest viscosity), but there was also more variable among individual fish at 3^{o}C. Given reductions in stride length, sperm must use more tail beats to cover the same distance, which results in more side-to-side motion over the swimming track. However we cannot separate physiological and mechanical effects of temperature. Moreover, the kinematic viscosity for seawater at the test temperatures would be 3^{o}C = 1.66 X 10^{-6} $m^2 \cdot s^{-1}$, 6 = 1.52 X 10^{-6}, 11 = 1.32 X 10^{-6}, 21 = 1.03 X 10^{-6}, and thus the proportional change in wobble at the lowest temperature cannot be explained by an equivalent proportional change in viscosity.

The production of novel phenotypes may be expected in extreme or stressful environments due to the release of cryptic genetic variation (Queitsch *et al.* 2002; Rutherford 2000, 2003; Ghalambor *et al.*

2007). We tested this hypothesis with 11,074 sperm from 12 individual Atlantic cod. If novel phenotypes are produced under extreme environments we would predict higher variability among individual fish in those situations. Variability among individual fish in sperm swimming velocity and wobble tended to be greatest at the coldest temperature but was most uniform at the highest temperature and thus the test is inconclusive. At 3^{o}C our coldest test temperature is closer to preferred cod spawning temperatures than 21^{o}C. Such results might be explained by sperm "burning out" at 21^{o}C before our first assessment at 30 s post-activation. Further research should be directed to testing this hypothesis.

Our study builds on several others that have used common garden experiments to investigate reaction norms of Atlantic cod in relation to temperature. When two populations were grown under similar conditions, the higher latitude population had faster growth rates, but this was independent of temperature (Purchase and Brown 2000). Purchase and Brown (2001) showed genetic differences among populations in how relative liver weight (major energy store) responded to changing water temperatures. Research on three populations of northwest Atlantic cod showed differences in phenotypic plasticity in body shape to constant rearing temperatures and food supply (Marcil *et al.* 2006). Hutchings *et al.* (2007) found genetic differences in life history reaction norms of cod, where warm-water populations were more sensitive to food and cold-water population were more sensitive to temperature. This growing body of literature is highlighting important genetic differences among populations; however our study is the first to investigate individual variation in phenotypic plasticity.

Two types of studies have investigated the influence of temperature on sperm. One has researched the effect of maintaining adults at different temperatures before the collection of semen, and then comparing sperm at common temperatures. Shrimp (*Litopenaeus vannamei*) kept at lower temperatures produced more sperm and had higher proportions of normal sperm than those at high

418 temperatures (Perez-Velazquez *et al.* 2001). Carp (*Cyprinus carpio*) kept at cold and warm temperatures
419 produced sperm with different chemical composition, but they did not differ in proportion motile or in
420 how long sperm remained motile (Emri *et al.* 1998). Other studies have compared sperm performance
421 under different temperatures of the sperm activating medium. Vladić and Järvi (1997) investigated
422 sperm longevity in two species of fish although they did not place the results into the context of reaction
423 norms. Sperm swam for longer time periods in colder water for both species, but the response to
424 temperature was more sensitive for Atlantic salmon (*Salmo salar*) than for brown trout (*Salmo trutta*).
425 See Alavi and Cosson (2005) for a review of both types of studies for three groups of fishes. Our study
426 is unique, in that we compare variability within- and among-families in different environments.
427 Our research contributes to a growing body of literature on cod semen. In most fish species that
428 have been investigated (largely freshwater species) sperm swim for short amounts of time, with
429 longevity generally being less than 1 min. In contrast, Atlantic cod sperm swim for long periods and can
430 achieve up to 50% fertilization success after 1 h under artificial conditions (Trippel and Morgan, 1994).
431 However, due to finite energy stores, faster swimming sperm are predicted to swim for shorter periods
432 of time (shorter longevity), and thus longevity may be inversely related to fitness within species,
433 especially those under intense sperm competition like cod. Whether the longevity of cod sperm is
434 adaptive under natural situations is unknown. The proportion of cells that are motile has repeatedly been
435 shown to have no effect on cod fertilization ability (Trippel and Neilson 1992; Rakitin *et al.* 1999a;
436 Rudolfsen *et al.* 2008). However, higher sperm densities increase fertilization success, both when
437 artificially pipetted (Butts *et al.* 2009) and when occurring naturally in variable ejaculates (spermatocrit,
438 Rakitin *et al.* 1999a). Faster swimming sperm likely fertilize more eggs (Rudolfsen *et al.* 2008;
439 Skjæraasen *et al.* 2009) although this is not certain (Trippel and Neilson 1992; Rudolfsen *et al.* 2005),

and evaluation of swimming speed is variable depending upon the presence of maternal cues (Litvak and Trippel 1998). Sperm quality changes through the spawning season (Rouxel *et al.* 2008), and seems to be influenced by male condition (relative weight) but not absolute size (Rakitin *et al.* 1999a; Tuset *et al.* 2008). Percent of motile cells and swimming speeds are influenced by salinity (Livak and Trippel 1998).

Here we demonstrated that temperature influences sperm swimming velocity but that the magnitude and direction of the response depends on individual variability and the time post-activation, which may be of practical importance to aquaculture operations. This also indicates that caution should be taken when drawing conclusions about potential lack of genetic differences in phenotypic plasticity in studies that use limited numbers of environments or genotypes. Additionally, the reason for the relatively high amount of variability among sperm cells within an ejaculate (as compared to among replicates, individuals, families) is unknown, but may help explain why sperm quality is hard to predict from fish characteristics. Our cod had been maintained at the same temperature, and thus the effect of temperature on sperm performance of fish that have been acclimated to different conditions remains unknown.

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464

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592

FIGURE LEGENDS

Figure 1: Design of the "Phase II" common garden experiment. Shown is the hierarchical structure for a single temperature. There were three families of cod, each containing four individuals. Sperm were obtained from one brother from each family at the same time on a given day. The sperm activation procedure was repeated at least three times for each fish, and the movement of different individual sperm was determined at four time periods per activation. This entire process was repeated for the four manipulated temperatures each day, whose order of testing was randomized among days (different brothers used on different days).

Figure 2: Results of 16 fully-nested ANOVAs (4 temperatures and 4 time periods) separating variance components for sperm swimming velocity (VCL) among families, individuals within-families, procedural replicates within-individuals, sperm cells within-replicates. Most of the variability for a given temperature and time post-activation was among cells within a sperm activation procedure and assessment (average = 84%). On average only 3% of the variability was among replicated activation procedures.

Figure 3: Average "reaction norms" of sperm curvilinear swimming velocity (VCL) at four test temperatures. Swimming velocities at assigned elapsed time periods post sperm activation are shown as different lines. Values shown are means among individuals and families (N = 12 fish). Note that thermal reaction norms are more linear for sperm that have been swimming longer.

Figure 4: Reaction norms of sperm swimming velocity at four test temperatures. Swimming velocities at assigned elapsed time periods since sperm activation are shown as different panels. Values shown are individual genotype averages among procedural replicates. Solid lines = Family A, dotted lines = Family B, dashed lines = Family C. There is a significant G×E interaction.

Figure 5: Mean coefficient of variation (among replicates) of sperm curvilinear swimming velocity (within replicates) at four test temperatures. Swimming velocities at assigned elapsed time periods since sperm activation are shown as different panels. Values shown are individual genotype averages among procedural replicates. Solid lines = Family A, dotted lines = Family B, dashed lines = Family C. There is a significant G×E interaction.

Figure 6: Reaction norms of sperm swimming behaviour at four test temperatures. The mean wobble index is plotted on the y-axis; note that higher values are sperm swimming with less side-to-side head motion. The different time periods since sperm activation are shown as different panels. Values shown are individual genotype averages among procedural replicates. Solid lines = Family A, dotted lines = Family B, dashed lines = Family C. There is a significant G×E interaction.

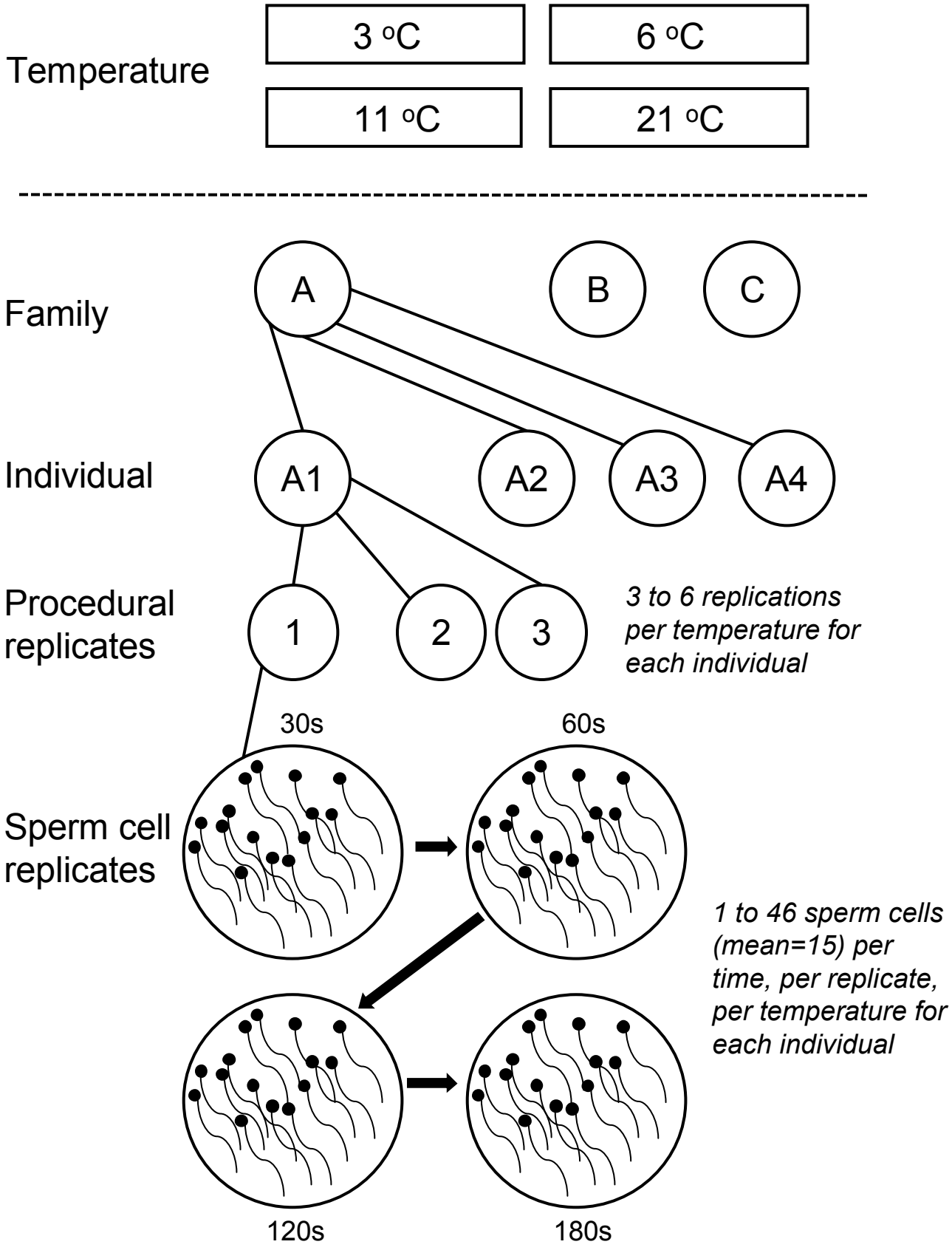

Figure 1

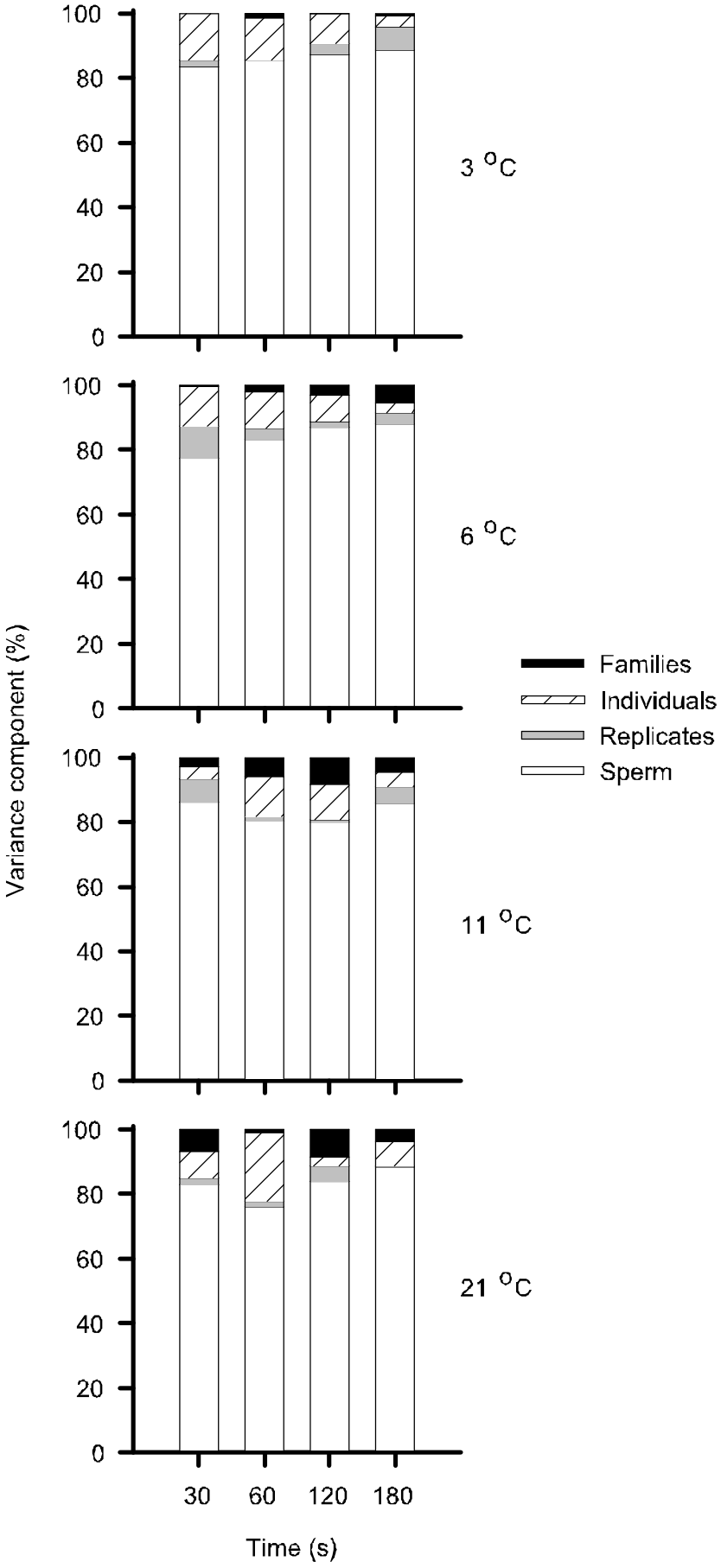
Variance component (%)
100
80
60
40
20
0
3 °C
6 °C
11 °C
21 °C
Families
Individuals
Replicates
Sperm
30
60
120
180
Time (s)

Figure 2

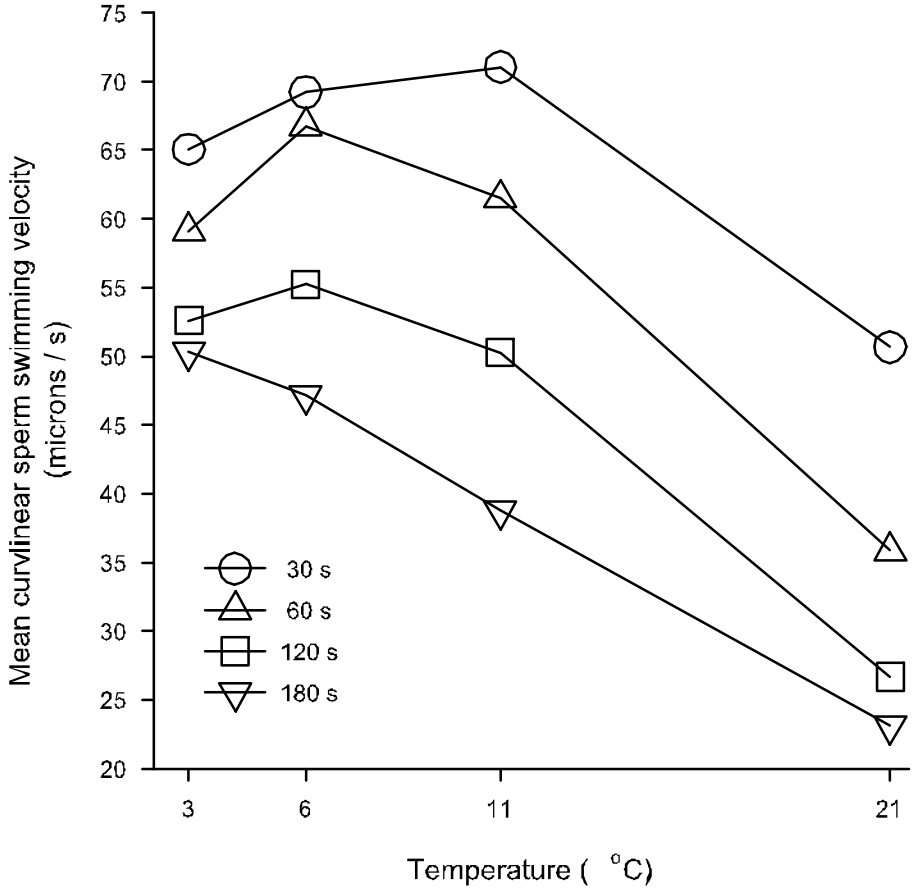
Mean curvilinear sperm swimming velocity
(microns / s)
75
70
65
60
55
50
45
40
35
30
25
20
30 s
60 s
120 s
180 s
3
6
11
21
Temperature (°C)

Figure 3

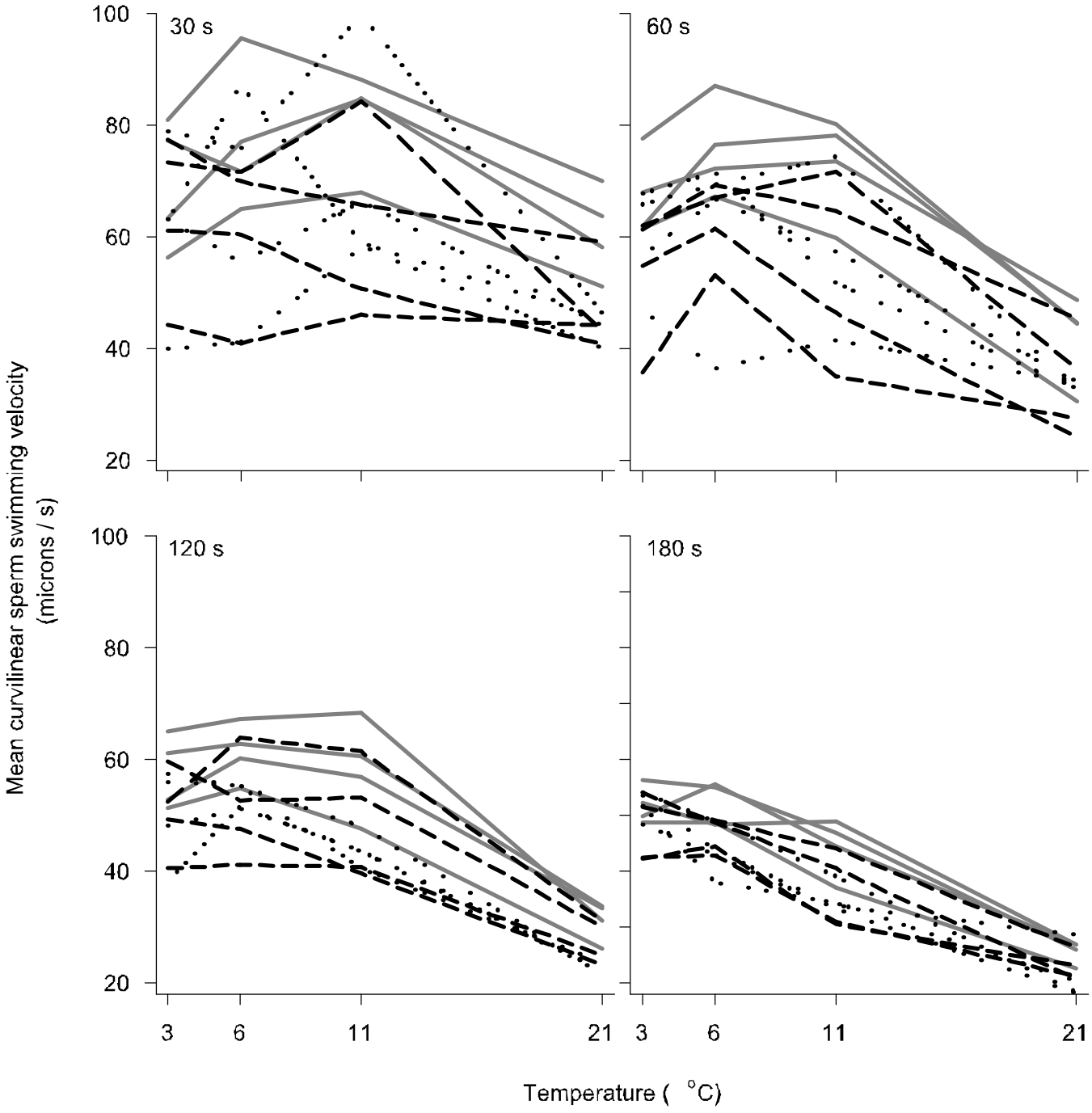
30 s
60 s
120 s
180 s
100
80
60
40
20
3
6
11
21
Mean curvilinear sperm swimming velocity (microns / s)
Temperature (°C)

Figure 4

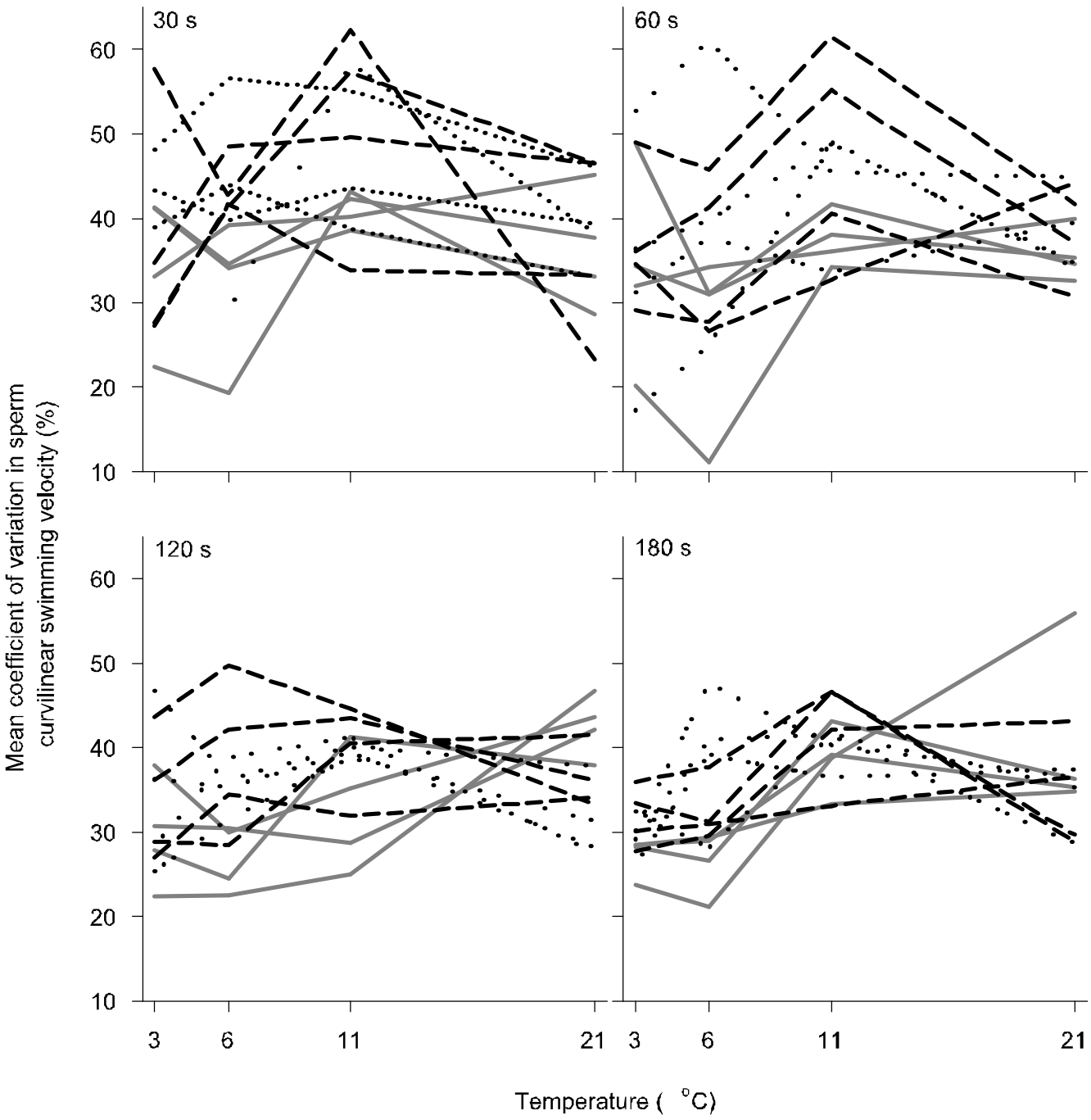
30 s
60 s
120 s
180 s
Mean coefficient of variation in sperm curvilinear swimming velocity (%)
10
20
30
40
50
60
3
6
11
21
Temperature (°C)

Figure 5

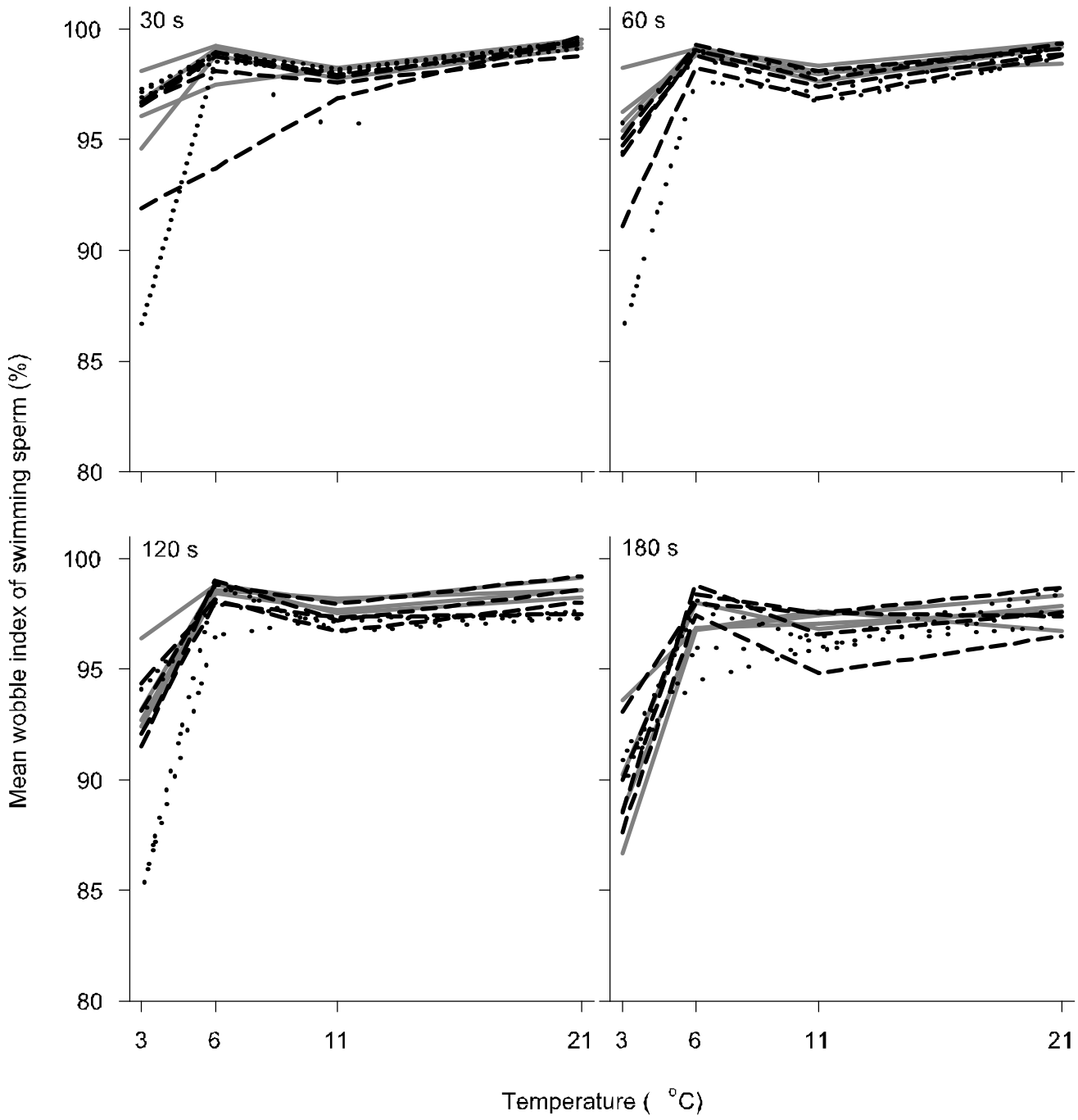
30 s
60 s
120 s
180 s
100
95
90
85
80
3
6
11
21
Mean wobble index of swimming sperm (%)
Temperature (°C)

Figure 6

Table 1: Characteristics of each individual genotype (i.e. brother) from full-sib families of Atlantic cod used in the sperm analyses.

Family[c]	Individual	Total length (cm)	Body weight (g)	Fulton's condition[a]	Spermatocrit (%)	Mean VCL ($\mu m \cdot s^{-1}$)[b]
	A1	51.7	1575	1.14	34.3	59.1
	A2	46.4	1230	1.23	34.0	58.2
A	A3	45.8	1075	1.12	26.3	65.0
	A4	42.3	920	1.22	41.0	49.7
	Mean $\pm$ S.D.	46.6 $\pm$ 3.9	1200 $\pm$ 280	1.18 $\pm$ 0.06	33.9 $\pm$ 6.0	58.0 $\pm$ 6.3
	B1	47.0	1335	1.29	39.7	42.0
	B2	48.7	1365	1.18	32.7	48.6
B	B3	51.7	1830	1.32	37.0	55.3
	B4	48.2	1275	1.14	59.0	47.6
	Mean $\pm$ S.D.	48.9 $\pm$ 2.0	1451 $\pm$ 255	1.23 $\pm$ 0.09	42.1 $\pm$ 11.6	48.2 $\pm$ 5.7
	C1	47.1	1260	1.21	40.7	38.2
	C2	45.5	1100	1.17	35.0	54.2
C	C3	44.7	1020	1.14	11.3	55.7
	C4	46.6	1445	1.43	49.3	43.7
	Mean $\pm$ S.D.	46.0 $\pm$ 1.1	1206 $\pm$ 188	1.24 $\pm$ 0.13	34.1 $\pm$ 16.3	48.0 $\pm$ 8.5

[a] Fulton's condition factor is equal to $100(\text{Weight} \cdot \text{Length}^{-3})$ and represents relative weight for a given length.

[b] Mean VCL is sperm curvilinear velocity averaged across replicates, temperatures and times.

[c] Families were not significantly different in any of the general parameters (One-way ANOVAS: $P_{length}=0.290$, $P_{weight}=0.301$, $P_{condition}=0.635$, $P_{spermatocrit}=0.568$); see Table 2 for statistics on VCL.

Table 2: Mixed-model nested repeated-measures balanced ANOVA for comparing sperm performance of Atlantic cod.

				Sperm swimming performance		
				Sperm velocity	*Sperm variability*	*Sperm wobble*
Source[a]	Term	d.f.	Error	Median VCL ($\mu m \cdot s^{-1}$)	Coefficient of variation VCL (%)	VAP/VCL (%)
				F, p[b]	F, p[b]	F, p[b]
1	Family	2	2	2.76, 0.116	2.00, 0.191	1.60, 0.254
2	Genotype(Family)	9	11	28.28, <0.001	7.11, <0.001	7.57, <0.001
3	Temp	3	5	86.25, <0.001	6.91, 0.001	123.23, <0.001
4	Family×Temp	6	5	0.77, 0.603	2.01, 0.099	0.55, 0.767
5	Temp×Genotype(Family)	27	11	2.76, <0.001	2.60, 0.001	2.39, 0.001
6	Time	3	8	60.63, <0.001	5.57, 0.004	44.41, <0.001
7	Family×Time	6	8	1.00, 0.448	0.62, 0.709	1.80, 0.138
8	Time×Genotype(Family)	27	11	3.20, <0.001	1.98, 0.010	1.32, 0.173
9	Temp×Time	9	11	6.90, <0.001	1.35, 0.223	6.01, <0.001
10	Family×Temp×Time	18	11	0.89, 0.587	0.83, 0.657	1.18, 0.301
11	Error	81				
	TOTAL	191				

[a] "Source" is a code for the different terms, "d.f." is degrees of freedom, "Error" refers to which "Source" is used in the denominator of the F-test, and "Temp" is temperature. Genotype refers to an individual fish and is use to match standard convention for assessing G×E interactions. The term Temperature×Time×Genotype(Family) is not included in the model; this appears as error (Source 11) and is the correct error term for testing five of the other terms. Sperm velocity (VCL) is the mean among procedural replicates of the median among sperm cells within a procedural replicate. Sperm variability is the arsine transformed mean among procedural replicates of the coefficient of variation in velocity (VCL) among sperm cells within a procedural replicate. Wobble is the oscillation index (high values have less wobble) and has been arsine transformed.

[b] The models explained 96.1%, 82.0%, and 94.3% of the variance among fish respectively.