Population differences in early development in grayling (*Thymallus thymallus*): a common garden experiment.

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Forord/preface

Denne oppgaven har blitt skrevet ved Universitetet i Oslo i perioden august 2007 – mai 2009. Arbeidet er en del av et større prosjekt kalt ”The early stages of adaptive evolution: the speed of evolution” ved senter for evolusjonær og økologisk syntese (CEES).

Det er mange mennesker som nå fortjener takk for hjelp og støtte. Først og fremst en stor takk til min veileder Asbjørn Vøllestad som hele veien har vært tilgjengelig, og som har bidratt med uvurderlig faglig veiledning. Gjennomlesninger, konstruktiv kritikk, gode råd og oppmuntring fra deg har, i tillegg til å inspirere, gjort arbeidet enklere og mer strukturert.

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Jo, Kristian og Maria skal ha takk for gjennomlesning og kommentarer til manuskriptet. Disse og Unni fortjener en ekstra stor takk for å ha holdt det gående med quiz, pølsefest og private feltkurs helt siden vår spede biologi-barndom på Blindern for snart fem år siden. Alle mine medstudenter skal ha en stor takk for å ha skapt et veldig godt faglig og sosialt studiemiljø som har gjort arbeidet langt lettere og, ikke minst, morsommere!

Familie og venner generelt fortjener mange takk for støtte og sårt tiltrengte distraksjoner. Far, Kirsti og Hanne takkes for å ha hjulpet til med korrekturlesing. Sist, men ikke minst, en kjempetakk til Hanne. Det er hundrevis av ting å takke for, men særlig takk for all hjelp og støtte når oppgaven har krevd sitt, og takk for rundhåndethet med bakverk av alle slag!

Takk til alle som har bidratt til å gjøre denne prosessen lærerik, morsom og givende!

Gaute,
Blindern, mai 2009

Contents

Abstract

Following colonization of new habitats and subsequent strong selection, adaptation to environmental conditions might be expected to be rapid. In a mountainous lake in Norway, Lesjaskogsvatnet, more than 20 distinct spawning demes of grayling have been established since the lake was colonized, less than 25 generations ago. The demes spawn in tributaries consistently exhibiting either cold or warm temperature conditions. I conducted a common garden experiment to investigate if differences in early developmental characters had been established between four demes in response to the different nursery environments the grayling embryos and larvae experience. Different subsets of individuals from the four demes were subjected to three different treatment temperatures to test for temperature effects. Traits related to timing of development (i.e. eye pigmentation and hatching) as well as growth and energy consumption (i.e. length and yolk sack size) were measured for individuals from daily samples from all demes in all temperatures. I found no differences in the timing of early developmental traits. However, traits related to larval growth and energy consumption showed significant variation. Such variation most likely reflects genetic differences; however the observed variation did not unambiguously correspond with predictions of countergradient variation or local adaptation. I conclude that the observed differences in growth related traits between demes are most probably a result of both directional and random processes influencing evolutionary change.

1. Introduction

Eco-devo, or ecological developmental biology, is a research field receiving an increasing amount of attention. Within this line of research one tries to understand how ecological processes influence the development of individual organisms (Gilbert, 2001; Sultan, 2007). There are multiple environmental factors that may influence the development of an individual (e.g. Arnott et al., 2006; Gagliano et al., 2007): temperature, photoperiod, diet, dissolved oxygen content, population density; the list goes on. For ectotherms in general, temperature is considered to be by far the most important external factor for regulating gene expression, controlling much of the variation seen in embryonic ontogenetic rates in fishes (Kamler, 2002). The environmental conditions experienced by a population may be impacted by anthropogenic or natural influences. Consequently, individuals of an affected population may need to modify ontogenic traits and/or the timing of development.

Modification of traits, or modification of the timing of traits, may happen by means of phenotypic plasticity, or by rapid evolution. Phenotypic plasticity is defined as the ability of a single genotype to develop different phenotypes under different environmental conditions (Stearns, 1992). Norms of reaction are commonly used to illustrate the phenotypic plasticity of genotypes (see figure 1). If the scope for phenotypic plasticity is not great enough to accommodate a change in the environment, performance may be altered further through adaptation. Such adaptation can come about in two ways (Yamahira & Conover, 2002). In several studies researchers have discovered that organisms living under suboptimal conditions actually achieve faster growth than conspecifics occupying more benign habitats (e.g. Conover & Present, 1990; Nicieza et al. 1994; Arendt & Wilson, 1999; Laugen et al., 2003; Oufiero & Angiletta, 2006). This phenomenon has been termed countergradient variation (illustrated in figure 1b and c). Countergradient variation is defined as "...a geographical pattern of genotypes (with respect to environments) in which genetic influences on a trait oppose environmental influences..." (Conover & Schultz, 1995). The alternative way for a population to adaptively alter the shape of its reaction norm is through evolutionary adaptation to local conditions. When comparing two populations occupying habitats of differing character which have achieved adaptation through this process, their reaction norms for a trait associated with this adaptation will have differing slopes: populations are expected to exhibit optimized performance within the environment to which they are adapted (as illustrated in figure 1d). Non-parallel norms of reaction illustrates what is known as genotype

x environment (G x E) interaction; different genotypes express differential interactions with the environment (figure 1c and d).

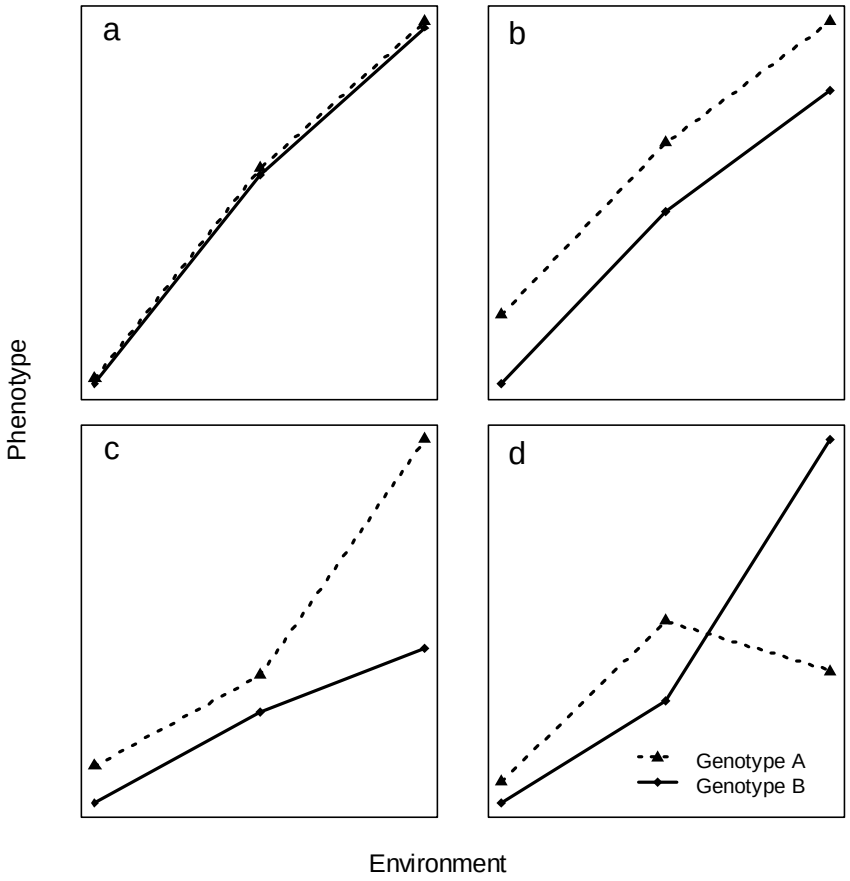


Figure 1: Variation in reaction norms (modified from Sultan, 2007). A reaction norm illustrates the capacity of a genotype to respond by altering its phenotype under changing environmental conditions. Two different genotypes may have exactly matching phenotypes (as in a). Genotypes can have parallel reaction norms with a shift resulting in one genotype outperforming the other at all environments (as in b). If different genotypes respond differently to different environmental conditions we have what is called genotype-environment (GxE) interactions. GxE interactions may result in reaction norms where one genotype outperforms the other at all environments, while the environment exerts differential influence on the performance of the two (as in c). Alternatively, GxE interactions may give rise to crossing reaction norms and varying rank order of genotypes in different environments (as in d).

A change in the population reaction norm requires genetic change; either through stochastic processes like genetic drift, or through adaptive evolution. Today it is realized that ecological and evolutionary dynamics do not necessarily operate on vastly different timescales (Hairston et al., 2005). There are a mounting number of studies displaying evidence lending support to the theory of rapid evolution (e.g. Gíslason et al., 1999; Groman & Pellmyr, 2000; Haugen & Vøllestad, 2001; Hendry et al., 2000). Among the most important factors contributing to rapid evolution is colonization of novel habitats, and population subdivision due to heterogeneity of the habitat (Reznick & Ghalambor, 2001). For

evolution to take place, genetic variation must be present (e.g. Dobzhansky, 1941; Simpson, 1953). For evolution to be considered adaptive it is required that observed changes in genotypic frequencies result from organisms being more "fit" leaving behind more copies of their genes than what individuals that are less "fit" do. Adaptive evolution comes about through direct response to natural selection (e.g. Futuyma, 2005). However, evolutionary change can also be caused by stochastic processes altering the genotypic frequencies in a population at random. Such random genetic change is commonly referred to as genetic drift (e.g. Lande, 1976). A founder effect is the random genetic drift following a bottleneck where a new population is established by a very small number of individuals from a larger population (e.g. Wright, 1931; Futuyma, 2005).

What happens during early life history of an individual fish tends to have a large impact on physiology and performance later in life (Stearns, 1992; Fuiman & Werner, 2002; Macqueen et al., 2007). In salmonids, thermal adaptation is thought to be important, especially during the egg-stage and subsequent phase following emergence of the fry (Jensen et al., 2008). For fish larvae in general it must be stressed that size matters (Billerbeck et al., 2001), and Miller and colleagues (1988) mention several aspects of early life history in which this view is likely to be applicable. This rule has been termed the "bigger-is-better"-hypothesis; for fish larvae performance is size-dependent to a stronger degree than it is age-dependent, or dependent on any other measure of biological time (Hare & Cowen, 1997; Sogard, 1997; Fuiman & Werner, 2002). Schultz and colleagues (1998) investigated the existence of size selective winter mortality as a mechanism for observed compensatory growth rate in Atlantic silverside (*Menidia menidia*) juveniles. Atlantic silversides from high latitude populations grow faster in their first growth season than do conspecifics from lower latitudes. Schultz and colleagues concluded that this was, at least partly, an effect of size dependent winter mortality: smaller individuals did not survive as long as larger individuals under simulated winter conditions.

The European grayling (*Thymallus thymallus*) is one of four species included in the genus *Thymallus* in the Salmonidae family (Koskinen et al., 2000). Being a spring spawner, it is quite unique among salmonids (Northcote, 1995). Grayling can live in lakes or rivers, but reproduction is usually restricted to running water. The species is distributed, in suitable habitats, over most of northern Europe (Northcote, 1995). The population studied here resides in a mountainous lake in central Norway called Lesjaskogsvatnet (Figure 2). In a phylogeographical investigation Koskinen and colleagues (2000) concluded that it is highly

plausible that Lesjaskogsvatnet was colonized by very few grayling individuals from the river Gudbrandsdalslågen. This likely happened during the late 1880s (Haugen & Vøllestad, 2001). Knowledge about the time, and nature, of the colonization of Lesjaskogsvatnet by grayling makes the system highly interesting for studying the speed of evolution. Since the colonization, the grayling have established more than 20 demes spawning in separate tributaries (Barson et al., 2009). Several studies focusing on evolutionary aspects of the grayling population of Lesjaskogsvatnet and its surrounding area have been, and are being, conducted (e.g. life history evolution: Haugen & Vøllestad, 2001; egg size divergence: Gregersen et al., 2008; isolation mechanisms: Barson et al., 2009).

Here, adaptive evolution of larval grayling, as a response to diverging environmental selection pressures, is under investigation. The tributaries utilized for reproduction by the Lesjaskogsvatnet grayling was classified by Gregersen et al. (2008) as either large-and-cold (LC) or small-and-warm (SW), depending on certain habitat characteristics. To contrast the development of larval grayling from different demes a common-garden experiment was conducted. In the experiment, individuals from four tributaries (two LC, two SW) were subjected to three experimental temperatures and studied from fertilization to swim-up. Swim-up refers to emergence of the larvae from the gravel (Crisp, 1988), here it was taken to be when most of the larvae in a container (see figure 5) where swimming freely in the water column and had exhausted most of their yolk sac. Daily measurements of two classes of traits were obtained from all demes at all treatment temperatures throughout the experimental period: 1) Developmental traits: eyeing (defined as the first appearance of eye pigment detectable through the egg membranes) and hatching, and 2) growth related traits: length and yolk sac area at time.

Haugen and Vøllestad (2000) conducted a study of several traits related to growth and development of grayling in the same area, but on a different scale. They investigated differences between the Lesjaskogsvatnet population and two other populations derived from Lesjaskogsvatnet grayling. In their study, they discovered apparent adaptations for many growth related traits. Hatching, on the other hand, exhibited much less variation, and no signs of local adaptation. When looking at different grayling demes within Lesjaskogsvatnet I am interested in looking for signs of genetic differences. Have the grayling responded to the differing environmental conditions by adaptation? Alternatively, the grayling could have a plastic response common to individuals of all demes. If this is the case, reaction norms for all

demes should appear more or less identical (as in figure 1a). If reaction norms are differing this is indicative of genetic effects. The appearance of the reaction norms could inform us of the nature of selection pressures causing the adaptations. If adaptation is caused by temperature *per se* we should observe reaction norms with differing slopes (they may even be crossing as in figure 1d). This would be indicative of local adaptation as a result of G x E interactions; fitness is maximized under native environmental conditions, but reduced elsewhere (Yamahira & Conover, 2002). In a mountainous lake like Lesjaskogsvatnet the growth season is very short. For north-temperate fishes the first winter is usually a period of energy deficit (Post & Parkinson, 2001). If adaptation is caused by the short growth season constraining developmental time we should observe a shift in the intercept of reaction norms (as in figure 1b and c). The selection pressure will be most stringent for the demes with the shortest growth season (i.e. LC demes). Individuals from these demes are expected to compensate for the shorter growth season with a higher general capacity for growth (Yamahira & Conover, 2002). This should give rise to elevated reaction norms compared to demes with less stringent selection (i.e. countergradient variation). Any genetic effect observed might also be caused by random genetic drift. Through the colonization process, the grayling of Lesjaskogsvatnet is thought to have gone through a severe bottleneck. This is expected to reduce genetic variability and hence impede response to selection (e.g. Lande, 1988; Willi et al., 2006). Potentially genetic drift could also complicate the interpretation of results since the outcome of evolution by genetic drift cannot be predicted (e.g. Lande, 1976).

2. Materials and methods

2.1. The study organism

In southern Norway, the natural distribution of grayling is limited to the Glomma river system to which the grayling probably migrated from Lake Vänern in Sweden shortly after the retreat of the land ice covering this area during the Weichsel glaciation (Koskinen et al., 2000). In a biogeographical study, Koskinen and colleagues (2000) discovered that all grayling in Lesjaskogvatnet were of the same haplotype group. The grayling in Gudbrandsdalslågen, which was found to constitute the basis for the founding population of the Lesjaskogsvatnet grayling, had individuals from two haplotype groups. In fact, the haplotype group found to be most common in Gudbrandsdalslågen was not found in Lesjaskogsvatnet at all. The results of Koskinen et al. (2000) are in concordance with historical records. These tell us that grayling was able to colonize Lesjaskogsvatnet during a short period of time by a man-made canal, between the upper reaches of Gudbrandsdalslågen and the lake, during the late 1880s (less than 25 generations ago). The subsequent closing of the canal made further immigration of grayling difficult, but emigration out of the lake is still possible (Haugen & Vøllestad, 2001).

For grayling, age at first reproduction is variable depending on growth rate (Northcote, 1995): some populations have individuals reproducing for the first time as two years old, while in other populations sexual maturity is not reached until 5-6 years of age. Once mature, grayling normally spawn every spring (Northcote, 1995; Kristiansen & Døving, 1996). Lake-living grayling usually spawn in running water, and they return to their natal stream with a high degree of precision prior to reproducing (Kristiansen & Døving, 1996). Spawning usually takes place at temperatures between 4 °C and 7 °C, although it is known to sometimes occur at temperatures up to 15 °C (Northcote, 1995). Kristiansen and Døving (1996) found that grayling in Lake Mjøsa, situated in southern central Norway, ascended into tributaries to spawn once temperatures reached 5°C. This 5°C temperature threshold seem to be the case in Lesjaskogsvatnet grayling, but there is also a component of time contributing to the likelihood of spawning (Barson et al., 2009). Fertilized eggs hatch after approximately 130-140 degree days (defined as number of days multiplied with mean temperature) and the larvae remain buried under a few centimetres of gravel until the yolk sac is consumed (Haugen & Vøllestad, 2000). At this stage the larvae emerge from the gravel (swim-up). According to Bardonnet and Gaudin (1991), emergence takes place after 276-320 degree days post fertilization. The

larvae then spend 1-1.5 months in their natal tributary before migratin or drifting passively downstream to enter the sympatric phase in the lake (Haugen & Vøllestad, 2000). The survival and development of eggs and larvae are highly dependent upon temperature. Jungwirth and Winkler (1984) found that survival during embryonal development was highest between 6 °C and 13.5°C, mortality was 100% at temperatures above 16°C. Within the viable range, developmental duration was negatively correlated with temperature.

2.2. The study area

Lesjaskogsvatnet (UTM 32V 471706 6897665; area: 4.3 km^2) is a shallow lake situated 611 meters above sea level in the north-western part of Oppland county, in central Norway (see figure 2). It is divided into three basins, and two fairly large rivers, Gudbrandsdalslågen and Rauma, drain out of the south-eastern and north-western end of the lake, respectively. All along the northern and southern sides of the lake there are several small tributaries draining into the lake. These are the locations for grayling reproduction as well as being nursery habitats for the eggs and larvae. During spring time, the slope on the northern side of the lake receives considerably more sunlight than the shaded southern slope. The northern slope is also less steep than the southern slope. As a result of these and other qualitatively different characteristics of the different tributaries, tributaries can generally be classified as either large-and-cold (LC) or small-and-warm (SW) (Gregersen et al., 2008). Generally, spring ice-out commences at an earlier stage in SW tributaries than in LC tributaries, and SW tributaries have a higher temperature than LC tributaries through spring. Furthermore, LC tributaries typically have higher stream velocity and are wider than SW tributaries (Bærum, 2008). For this study four tributaries (two LC and two SW) were used (see figure 2 and table 1).

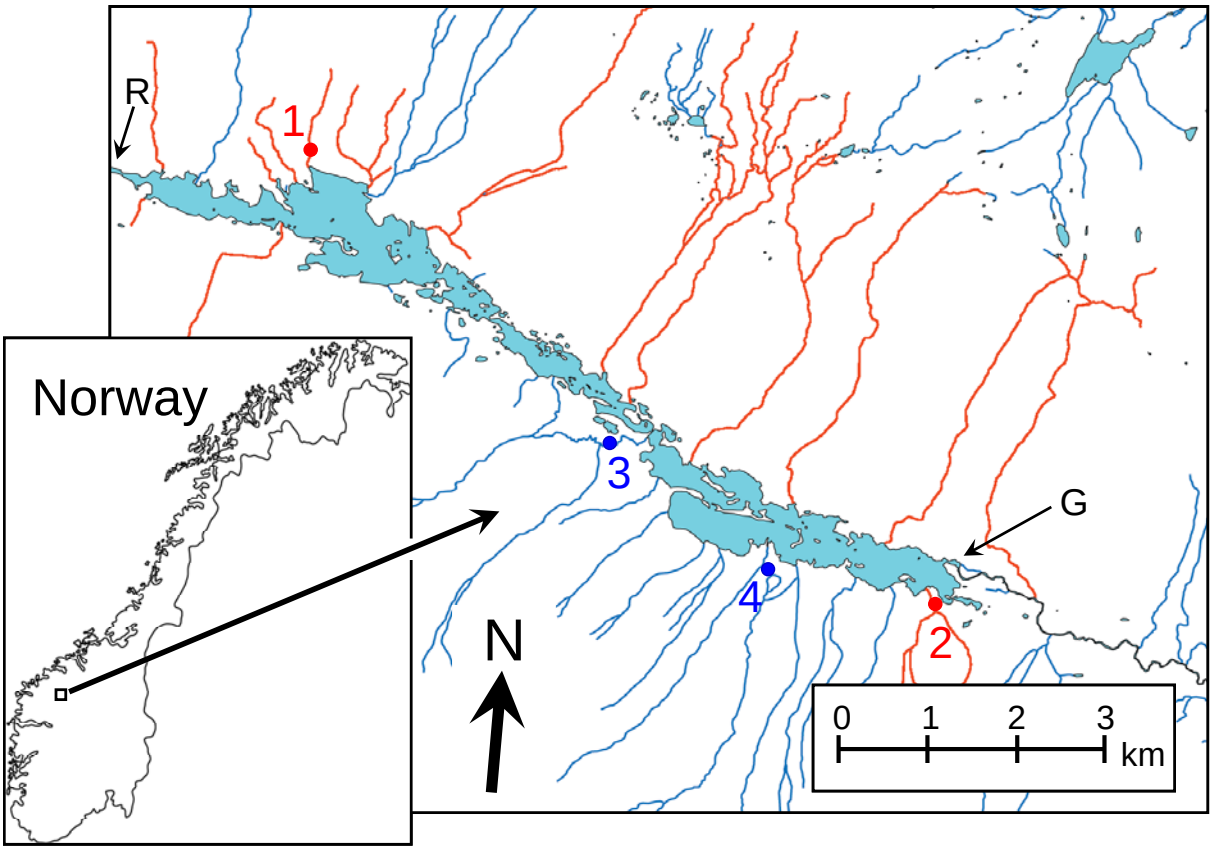


Figure 2: Map of the lake Lesjaskogsvatnet. Tributaries colored blue are defined as large and cold (LC) tributaries while the tributaries colored red are defined as small and warm (SW) tributaries (Gregersen et al. 2008). 1= Steinbekken, 2= Sandbekken, 3 = Valåe, 4 = Hyrjon, R = Rauma outlet and G = Gudbrandsdalslågen outlet. For further details see text.

Table 1: Environmental characteristics for the four spawning tributaries included in this thesis (modified from Gregersen et al. 2008)

Tributary	**Tributary type**	**Mean June temperature (°C)**	**Tributary width (m)**
Steinbekken	Small/warm	6.0	0.75
Sandbekken	Small/warm	6.0	1
Hyrjon	Large/cold	3.4	5
Valåe	Large/cold	2.9	6

2.3. Field sampling and experimental design

Adult spawning grayling were intercepted during their migration and captured using traps and fyke nets. This was done in the start of June 2007 in SW streams, and in the end of June the same year in LC streams. The traps were checked twice daily and captured fish were transferred to a holding pen upstream. Once capturing was complete, adult fish were transferred from the holding pen to a tank containing benzocaine (concentration 50-200 mg/L)

for sedation. After sedation each fish was weighed and fork length was measured (see table 2) before the fish was stripped of gametes and put in a tank filled with fresh water to recover. After this procedure, all fish were released upstream of the capture site. Gametes were stored in small plastic zip-lock bags with oxygen added and kept cool on ice for transport by car to the fish holding facility at the Veterinary Institute of Norway, located in Oslo. Transportation took approximately five hours and was carried out on the 11th and 22nd of June 2007 for warm stream demes and cold stream demes respectively.

Table 2: Descriptive data (number of fish, per tributary and sex; weight and length) for the fish captured and stripped for gametes during field sampling.

Deme	**Number**	**Sex (N)**	**Weight (g) (Mean ± SD)**	**Length (mm) (Mean ± SD)**
Steinbekken	36	♀: 20	226.8 ± 130.3	287.6 ± 56.4
		♂: 16	337.6 ± 89.3	333.25 ± 28.2
Sandbekken	28	♀: 17	187.2 ± 85.6	268.6 ± 11.5
		♂: 11	263.9 ± 100.7	305.6 ± 35.2
Hyrjon	7	♀: 4	158.8 ± 90.4	254.8 ± 30.6
		♂: 3	244.0 ± 61.2	299.3 ± 22.1
Valåe	44	♀: 20	236.4 ± 106.8	284.4 ± 51.7
		♂: 24	298.7 ± 82.2	327.0 ± 41.9

Once in Oslo, the eggs were pooled deme wise: an equal volume of eggs from each female was measured up and mixed carefully. The eggs from each deme were then split into a number of batches equal to the number of males from that deme (see table 2). To avoid sperm competition, each batch was subsequently fertilized with sperm from one male. After approximately five minutes of contact with the sperm the eggs were rinsed with fresh water. Following fertilization, the batches of eggs from each deme were mixed together again before they were partitioned into three treatment groups. Each group was then split into two replicates. Three separate experimental tanks containing water of three different target temperatures (see figure 3) were set up. 6 °C, 8 °C and 10 °C were chosen as target temperatures. These temperatures were chosen, partly to represent cold, medium and warm temperatures experienced by developing grayling larvae in nature (e.g. Jungwirth and Winkler, 1984), and partly because of limitations of our capability to manipulate temperatures further due to technical constraints.

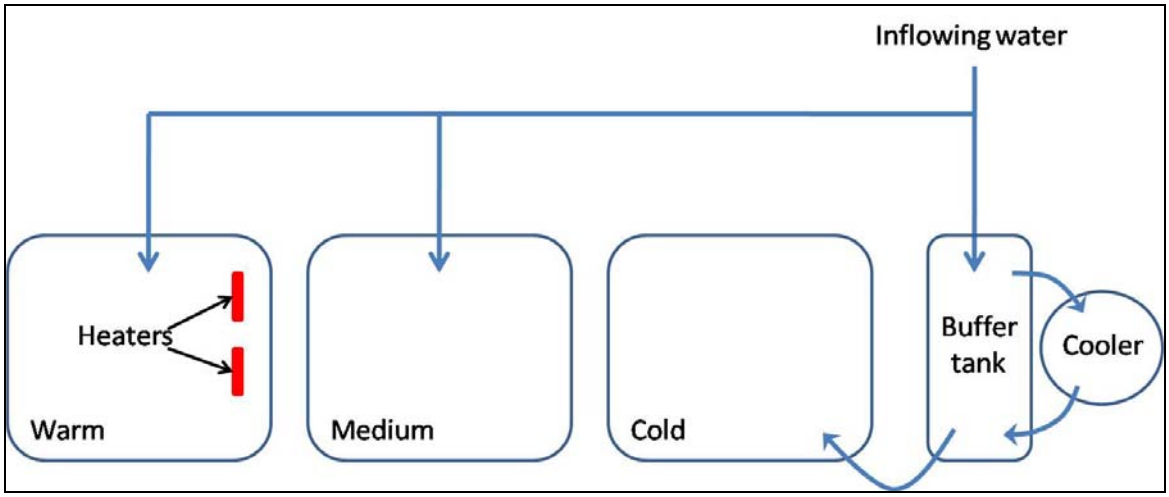


Figure 3: Schematic representation of the experimental set-up. Three separate treatment tanks, each containing numerous containers for holding eggs (see figure 5), were used.

Inflowing water was comprised of activated-charcoal filtered Oslo city tap water from the lake Maridalsvannet. The temperature of the inflowing water was approximately 8°C, and was thus used in the medium temperature treatment tank without much need for manipulation of temperature. However, to adjust for any increase in water temperature due to influence from the ambient temperature in the room, the amount of inflowing water was adjusted whenever necessary. Two standard aquarium heaters were used to maintain the target 10°C in the warm temperature treatment tank. A different set-up was used to maintain a stable 6°C temperature in the cold temperature treatment tank: the inflowing water was directed into a buffer tank. Water in the buffer tank was kept as cold as possible using a cooler. Cool water was then pumped from the buffer tank into the cold temperature treatment tank, and the precise adjustment of temperature was done by adjusting the amount of inflowing cool water. Temperatures were registered using temperature loggers (HOBO), and manual measurements were taken daily in order to make necessary adjustments. In the warm treatment tank we were able to keep the mean temperature very close to the desired 10 °C without any overall trend towards increase or decrease in temperature during the experiment, although a slightly humped pattern can be seen (see figure 4). Both in the medium and the cold treatment the temperature increased during the experimental period. This was partly caused by a slight increase in the temperature of the inflowing water during the course of the experimental period. Also contributing to this trend was an increasing room temperature at the experimental facility; heat released by the water cooler added to this effect.

Lowess, or locally weighted scatterplot smoother, was used to fit trend lines to the mean temperature data illustrated in figure 4. This is a non-parametric technique used to apply a smooth curve through a set of data points. It fits one polynomial regression for each observation, emphasizing explanatory variable values (here date) close to the points at which the response (temperature) is estimated (Fox, 1997).

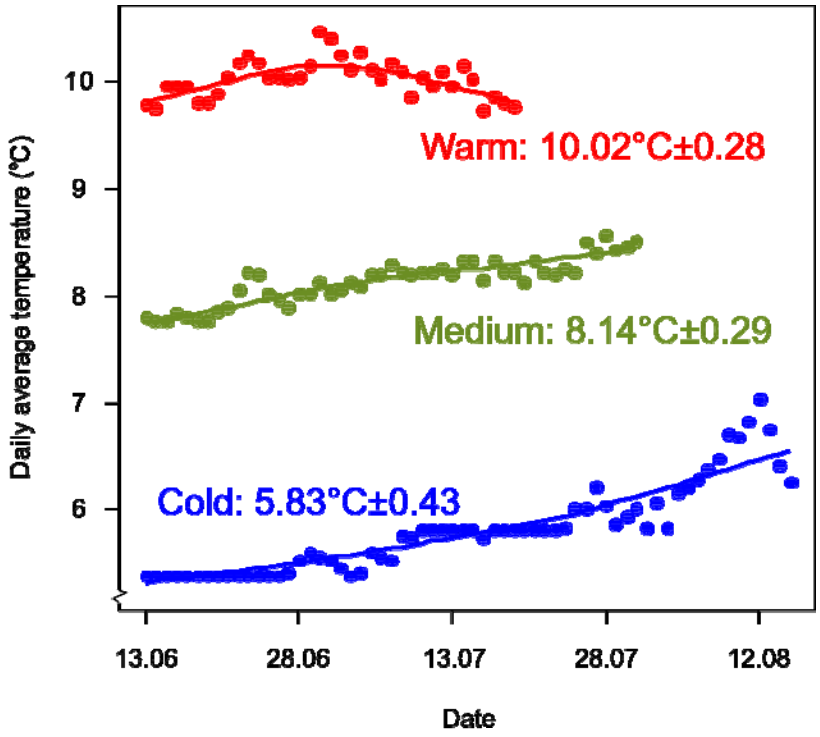


Figure 4: Plot illustrating variation in daily mean temperature (as measured by temperature loggers) in the three treatment tanks. Lowess curves added as trend lines (see text). Temperatures (mean ± SD) in °C for each treatment indicated in figure.

Fertilized eggs were placed in containers as illustrated in figure 5, and the containers were put in the treatment tanks (see Haugen & Vøllestad, 2000). In the complete set-up, each of the three tanks contained two replicate containers from each deme. Only two replicates were used, mainly because of a restricted number of eggs available from some demes (mainly Hyrjon, see table 2). Also, tank capacity-constraints and the laboriousness of the sampling procedure (see section 2.4) contributed to making it very difficult to obtain the number of replicates desirable from a theoretical point of view (e.g. Crawley, 2005).

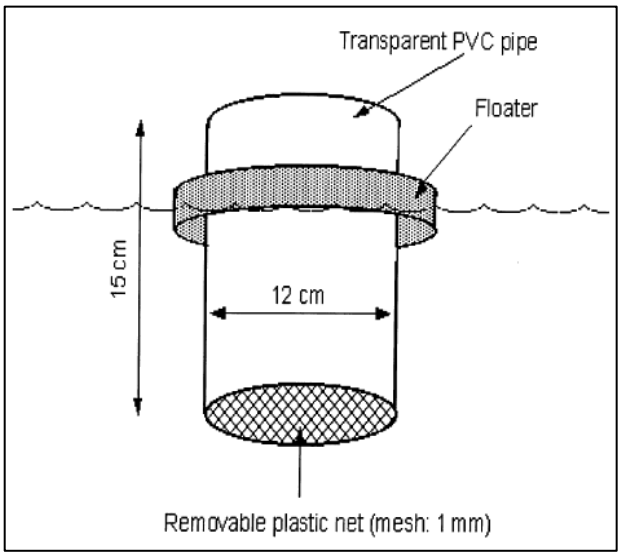


Figure 5: Containers used in the common garden experiments. Containers were allowed to drift freely in the tanks and the plastic net in the bottom allowed for exchange of water.

2.4. Data acquisition and analysis

Each day following fertilization approximately five individuals (eggs or larvae) were sampled from each deme in each treatment, alternating between the two replicates (see table 3). Sampling was done by haphazardly picking individuals out of the containers with a suction balloon attached to a glass tube. The sampled eggs/larvae were carefully freed from water before fixation in buffered formalin in order to optimally retain their physical characters (see e.g. Kruse & Dalley, 1990). Sampling was carried out every day over the 65 day experimental period with the exception of one day at which sampling was impossible due to technical problems.

Table 3: Number of eggs/larvae sampled during the common-garden experiment. Numbers given as total number of eggs/larvae sampled per deme, but also split up by treatment and replicate as well as summed up to grand totals.

Deme	**Total N**	**Treatment**					
		Warm		**Medium**		**Cold**	
		Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
Steinbekken	534	56	50	81	71	146	130
Sandbekken	533	58	51	81	70	142	131
Hyrjon	471	60	51	93	76	99	92
Valåe	604	67	56	105	87	153	136
Sum	2142	241	208	360	304	540	489

In the lab, all of the daily samples were visually inspected to check for eggs with eye pigment and for hatched individuals. Numbers of eyed and hatched individuals per sample were registered. Photographs of eggs and larvae were obtained using a Leica DC300 digital camera mounted on a Leica MZ8 stereo microscope, and connected to a computer running IrfanView version 3.99 (available at: http://www.irfanview.com/). A sample of 120 eggs (5 eggs from each deme in each treatment for the first two days after fertilization) was photographed. Initial egg size was estimated from these photographs as mean egg diameter measured in pixels using UTHSCSA Image Tool version 3.0 (available at: http://ddsdx.uthscsa.edu/dig/itdesc.html). The measure was converted to metric units by calibrating the programme using a photograph of a microscope scale slide at the relevant magnification. Out of the 2142 individuals sampled (see table 3), 717 had hatched and these where measured for length and yolk sac area. All larvae were photographed twice; usually once with the left side facing the camera, and once with the right side facing the camera. Two photographs were taken to be able to do two separate measurements of each of the continuous traits on every individual. To mitigate potential measuring error, the mean of the two measurements was used in the analysis. Based on a random subsample of 100 individuals there was a mean deviation of 1.06% between the two length measurements done on each individual. The corresponding number for yolk sac area estimates was 7.77%. On certain occasions larvae were fixed in awkward positions and were too brittle to straighten out. If so, photographs in three positions (i.e. side facing up, back facing up and/or yolk sac facing up) were obtained in order to get the measurements needed. Like egg diameter larval length was measured in pixels using UTHSCSA Image Tool, and converted as explained over. Length was measured from the tip of the cranium to the visible end of the notochord along the notochord (see figure 6). ImageJ version 1.38 (Available at: http://rsbweb.nih.gov/ij/download.html) was used to obtain a measurement of the yolk sac area. This was done by fitting an ellipse to a polygon drawn along the edge of the yolk sac. The program then returns the longest and shortest possible axis of the fitted ellipse (see figure 6). Axis lengths obtained from the two photographs of each individual were averaged after converting to metric, by dividing axis length in pixels on the number of pixels per mm obtained by measuring the microscope scale slide photographs (see above). The resulting estimates were used to estimate each individual's yolk sac area using the formula for the area of an ellipse (πab; a and b being one-half of the ellipse's major and minor axes, respectively).

Six individuals were excluded from the statistical analysis due to obvious disfigurement or severe damage during handling making any measurements uncertain. In four individuals the yolk sac was damaged or completely removed due to handling, making measurements only on length possible. One individual had an intact yolk sac, but a damaged body, making only measurement of the yolk sac area possible.

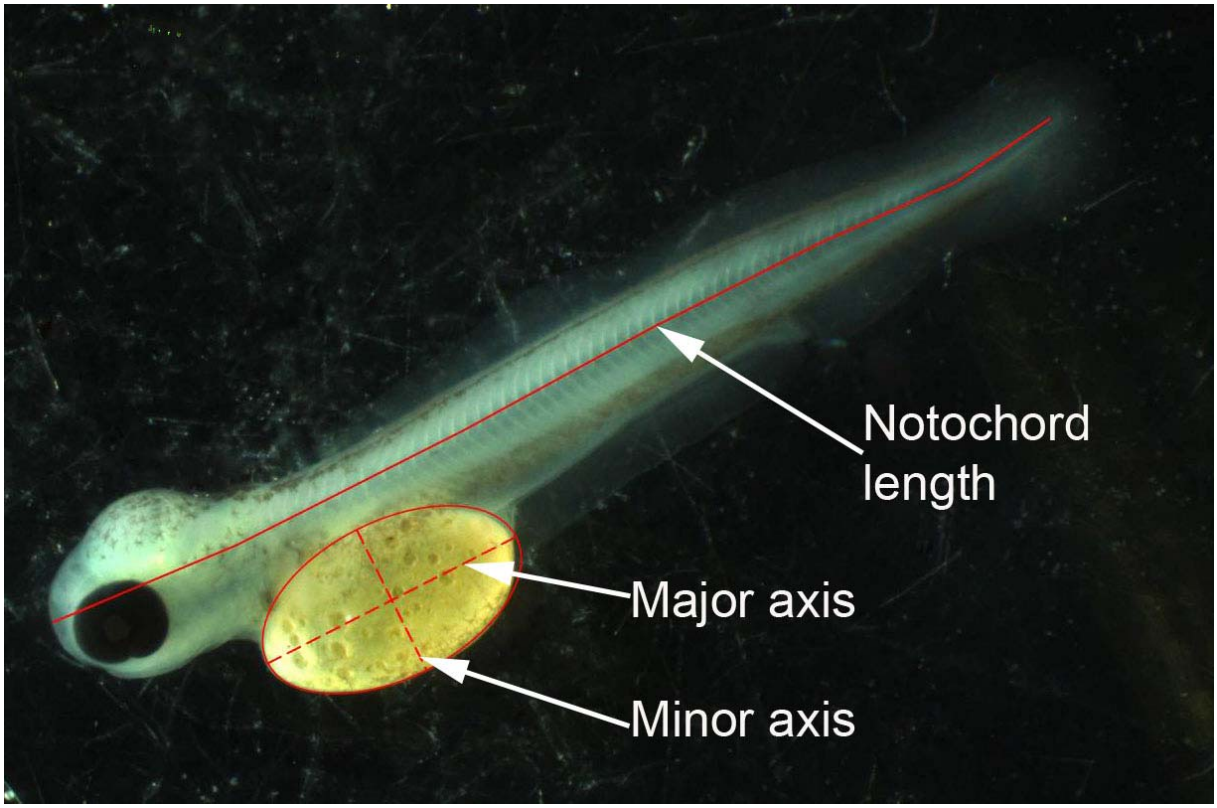


Figure 6: Illustration showing morphometric measurements obtained from each photo taken of the larvae. This photo depicts a larva from Hyrjon at 182 °d (warm treatment).

A one-way ANOVA on egg diameter with deme as effect was carried out, and a Tukey-Kramer HSD (honestly significant difference) test was used to compare deme means. Because of a small sample size, the effect of treatment could not be included.

Continuous response variables were analyzed using linear mixed effect models (LME, Pinheiro and Bates 2000), fitted by means of the restricted maximum likelihood (REML) method. In the LME models fitted (see appendix figure 1 and 2), the random effect of replicate was nested under deme and treatment. Both the response variable in question (larval length or yolk sac area) and the continuous covariate (degreedays (°d); a measure of time estimated as accumulated mean daily temperature) were logarithmically transformed to reduce heteroscedasticity. Individual linear regressions of length/yolk sac area on degreedays

were fitted for each deme in every temperature (see appendix table 2 and 3). Slopes from these regressions were used as estimates of growth- and yolk absorption-rates in visualizations (see figure 10 and 11). Full factorial models were used when analyzing the data (see equation 1).

$$Y_{ijk} = ß_0 + ß_1 °d_{ijk} + ß_2 Deme_j + ß_3 Treat_i + ß_4 Deme_j *°d_{ijk} + ß_5 Treat_i *Deme_j + ß_6 Treat_i *°d_{ijk} + ß_7 Deme_j *Treat_i *°d_{ijk} + b_{(ij)k} + \varepsilon_{ijk} \quad \text{(Eq.1)}$$

Here, "*Y*" is the response variable, ßs correspond to fixed parameters under estimation and $b_{(ij)k}$ corresponds to the random effect of replicate (k) nested under treatment level (i) and deme (j). Variables are provided in italics where "°*d*" indicates degreedays (ln-transformed), "*Deme*" is self-explanatory, while "*Treat*" means treatment. ε_{ijk} represent the between-individual variance (i.e. the residual variance) which is assumed to be normally distributed within replicates, and for a given link function. An asterisk indicates that we are dealing with an interaction term. In order to check for possible first-order temporal autocorrelation violations, Durbin-Watson tests were conducted for the fitted LME models.

Binary response variables were analyzed by means of logistic regressions (using logit-link function). For these, no transformations of predictor variables were necessary. Due to loss of degrees of freedom, these data could not be analyzed using generalized linear mixed effect models (GLME) with replicate as a random effect. Individual logistic regressions were fitted for each deme in every treatment, and inverse predictions (inverse logit) were carried out on these in order to obtain estimates for visualization (see figure 8 and 9).

All statistical analysis of the data was carried out using the statistical software JMP 5.0 (SAS Institute Inc., Cary, NC, USA). Figures were created using R v2.8.0 (available at http://www.r-project.org/).

3. Results

3.1. Initial egg size

Mean initial egg size differed significantly among demes (see figure 7, one-way ANOVA: $F_{3, 116} = 5.440$, $P = 0.0016$). However the explanatory power of this model was low ($R^2 = 0.123$). The highest mean value of initial egg size was obtained from eggs from Steinbekken. The smallest mean value was the one from eggs from Sandbekken.

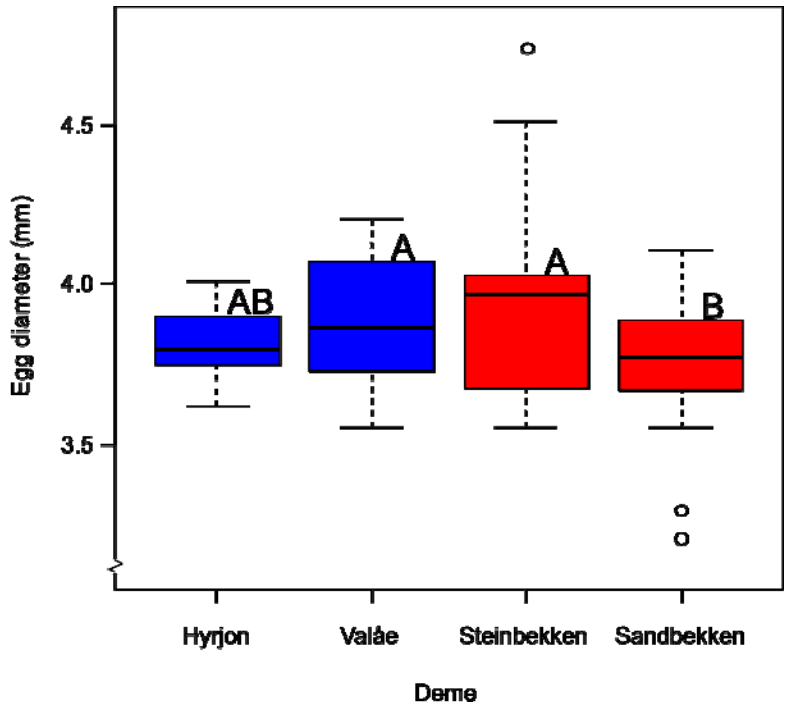


Figure 7: Boxplot of egg size distributions for the four grayling demes under investigation. The thick central black lines of the boxes indicate median, the upper and lower margins of the boxes indicate the third and first quartile respectively. Whiskers indicate minimum/maximum values of egg diameter not including observations more than 1.5 times the interquartile range above the third quartile or below the first quartile. Observations outside this range are defined as outliers (indicated by open circles). Lettering represents the results from a Tukey-Kramer HSD test. Demes which do not share the same letters are taken to be significantly different from one another. Color coding: blue indicates LC demes, red indicates SW demes.

3.2. Eye development

Timing of eye development (judged by the onset of pigmentation) was significantly influenced by temperature (see table 4). A highly significant degree days*treatment interaction indicated that a non-additive effect of altering the treatment temperature was present. Notably, the number of degree days until eye pigmentation dropped in the warm treatment temperature (see figure 8). There was no significant deme-effect, and none of the interaction-terms containing deme were statistically significant. There was a substantial amount of variation in the obtained estimates (see figure 8 and appendix table 1). Since there was no support in the data for any deme-effect, treatment wise mean numbers of degree days for 50% probability of developing pigmented eyes were estimated (see figure 8). This was done to illustrate the general trend in the data (i.e. increasing the temperature from 8 °C to 10 °C led to a shortening of the time to eye pigmentation).

Table 4: Summary test statistics for a logistic model with binomial error distribution, with presence/absence of eye pigment as nominal response variable. R^2: 0.750. N=2142. Parameter estimates available in appendix table 1. Layout details: °d is the degree days-covariate, asterisk in a model term indicates interaction effect, Df indicates degrees of freedom for the relevant model term, P-values taken to be significant (P-value<0.05) are highlighted in red.

Model term	**Df**	**Wald χ^2**	**P-value**
°d	1	119.99	<0.001
Deme	3	4.73	0.192
Treatment	2	27.02	<0.001
Deme*°d	3	3.35	0.342
Treatm*Deme	6	12.10	0.059
°d*Treatm	2	23.73	<0.001
Treatm*Deme*°d	6	5.68	0.459

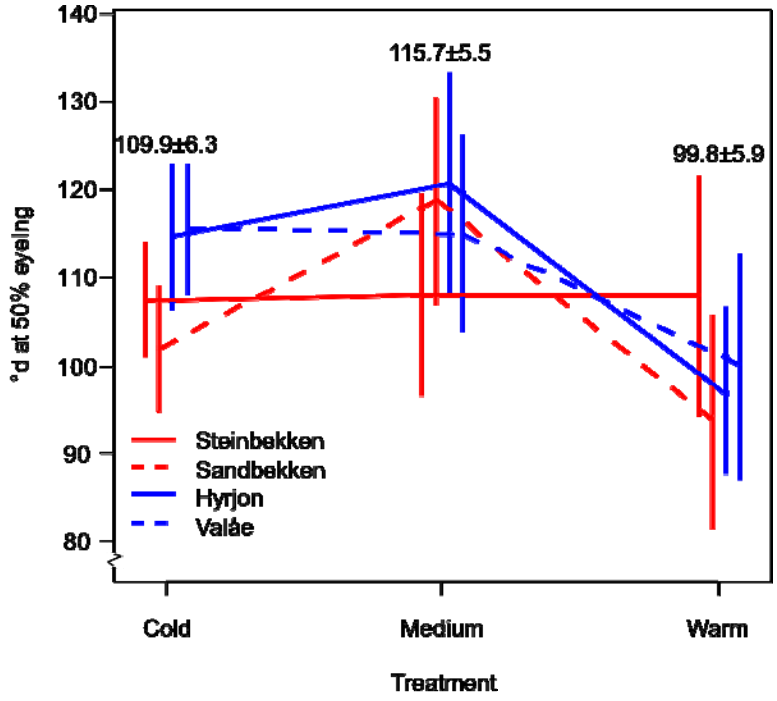


Figure 8: Reaction norms depicting degree days at 50% probability of having a pigmented eye at the three experimental treatment temperatures for the four demes (based on model estimates by means of inverse prediction). Vertical bars indicate 95% confidence limits. The numbers inside the figure frame represent treatment wise mean numbers of degree days at 50% probability of having a pigmented eye ± 95% confidence limits. Color coding: blue indicates LC demes, red indicates SW demes.

3.3. Hatching

Timing of hatching was significantly influenced by temperature (see table 5). As for eyeing, the number of degree days required for hatching was inversely correlated with treatment temperature (see figure 9). No significant deme-effect was found, and no interaction term containing deme was significant. Also as for eyeing the variation in the obtained estimates for hatching was large (see figure 9 and appendix table 1). Because of the lack of deme effects, treatment wise means for degree days at 50% probability of hatching were estimated (see figure 9). This was done in order to illustrate the general trend in the data, and to make quantitative predictions about growth and energy-consumption prior to hatching.

Table 5: Summary test statistics for a logistic model with binomial error distribution with hatching as nominal response variable. R^2: 0.850. N=2142. Parameter estimates available in appendix table 1. (For further details on layout see table 4)

Model term	Df	Wald χ^2	P-value
°d	1	73.64	<0.001
Deme	3	1.04	0.791
Treatment	2	6.81	0.033
Deme*°d	3	0.58	0.900
Treatm*Deme	6	7.25	0.298
°d*Treatm	2	12.27	0.002
Treatm*Deme*°d	6	6.47	0.372

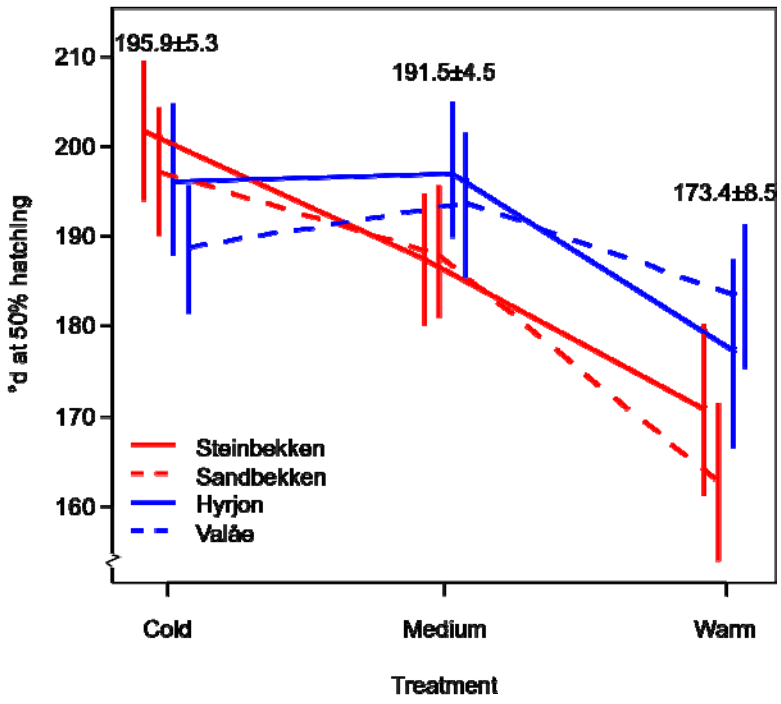


Figure 9: Reaction norms illustrating degree days at 50% probability of hatching at the three experimental treatment temperatures for the four demes studied (based on model estimates by means of inverse prediction). Vertical bars indicate 95% confidence limits. The numbers inside the figure frame represent treatment wise mean numbers of degree days at 50% probability of hatching ± 95% confidence limits. Color coding: blue indicates LC demes, red indicates SW demes.

3.4. Length growth through time

Growth rates clearly differed among populations and treatments, this was apparent because of the significant three-way interaction (see table 6). There appeared to be a general trend of positive correlation between growth rate and temperature (see figure 10a). The applied LME model further revealed significant effects on growth, as measured by larval length, by all other model terms except for the treatment*deme interaction. At the coldest treatment

temperature, growth rates for LC deme individuals were somewhat higher than those for SW deme individuals (see figure 10a). At the remaining temperatures there was no apparent deme-type related pattern in the rank order of growth rates. Grayling from all demes attained their highest growth rate in the warm treatment temperature. Larval lengths at hatching were estimated using deme wise means for degree days at 50% probability of hatching (see figure 9). Deme reaction norms for larval length at hatching appeared to have differing slopes and intercepts (see figure 10b). Individuals from all demes except Sandbekken attained the largest length at hatching in the medium treatment temperature.

Table 6: Summary test statistics for a linear mixed model with ln larval length as response variable. Replicate is random effect. Adjusted R^2: 0.760. N=710. Autocorrelation coefficient: 0.110, Durbin-Watson test statistic: 1.550 (Durbin-Watson test). Parameter estimates available in appendix table 1. Square brackets indicate nesting (for further details on layout see table 4).

Model term	**Df**	**Sum of squares**	**F-ratio**	**P-value**
ln°d	1	2.571	1190.14	<0.001
Deme	3	0.160	24.62	<0.001
Treatment	2	0.982	227.27	<0.001
Deme*ln°D	3	0.034	5.21	0.002
Treatm*Deme	6	0.028	2.15	0.122
ln°D*Treatm	2	0.086	19.95	<0.001
Treatm*Deme*ln°D	6	0.031	2.42	0.026
Replicate [Deme,Treat]	12	0.004		
Error	674	1.456		
Total	709	6.393		

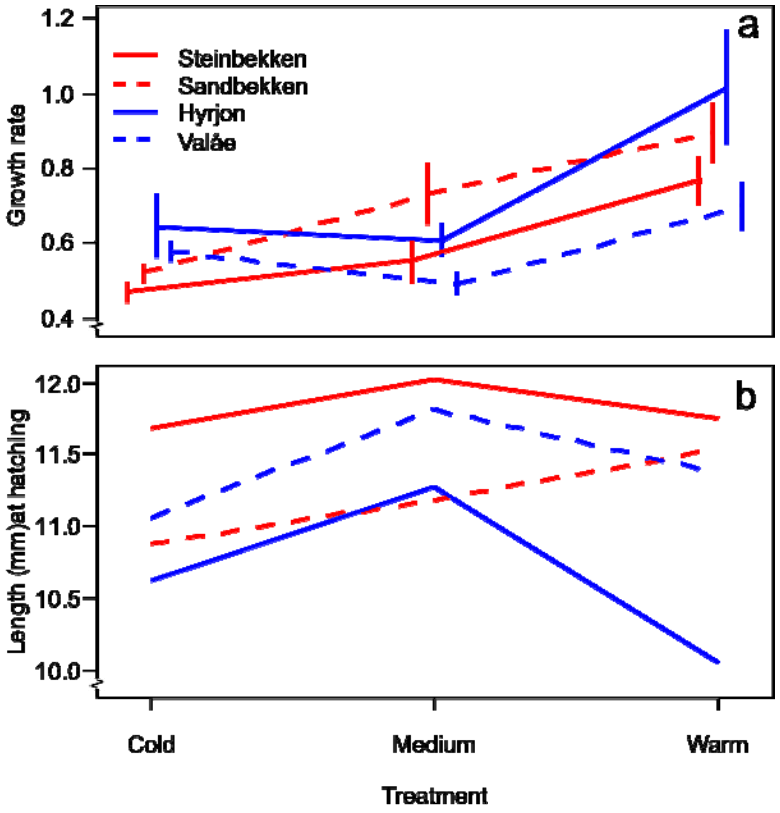


Figure 10: a) Reaction norms illustrating the effect of the different experimental treatment temperatures on growth rate estimates (slope of deme wise linear regressions of ln length on degreedays for each temperature) for the four demes studied. Vertical bars indicate standard errors. b) Reaction norms illustrating the effect of treatment temperature on back-transformed estimates of length at hatching (as estimated using deme wise means for degree days at 50% probability of hatching at the different treatment temperatures (see figure 9)). Color coding: blue indicates LC demes, red indicates SW demes.

3.5. Yolk sac size

In the analysis of yolk sac area, the same effects came out as statistically significant as in the analysis of length growth (see table 7). The significant three-way interaction term indicated that the reaction norms depicted in figure 11 were in fact dissimilar. For LC deme grayling, yolk consumption rate appeared to be highest in the medium treatment temperature (see figure 11a). The highest energy consumption rate for SW deme individuals was found at the highest treatment temperature. For yolk sac size at hatching, estimates were made using deme wise means for degree days at 50% probability for hatching (see figure 9). Reaction norms for individuals from LC demes had comparable slopes. Grayling embryos from LC demes seemed to invest more nutrition prior to hatching in the warm and cold treatment than they did in the medium treatment (see figure 11b). SW deme individuals seemed to exhibit an inverse

relationship between treatment temperature and pre-hatching nutrient investment, which was maximized at warm temperatures.

Table 7: Summary test statistics for a linear mixed model with ln Yolk sac area as response variable. Replicate is random effect. Adjusted R^2: 0.713. N=707. Autocorrelation coefficient: 0.215, Durbin-Watson test statistic: 1.337 (Durbin-Watson test). Parameter estimates available in appendix table 1. Square brackets indicate nesting (for further details on layout see table 4).

Model term	Df	Sum of sq	F-ratio	P-value
ln°d	1	50.139	503.15	<0.001
Deme	3	7.320	24.49	<0.001
Treatment	2	3.964	19.89	0.002
Deme*ln°D	3	4.138	13.84	<0.001
Treatm*Deme	6	0.833	1.39	0.294
ln°D*Treatm	2	1.394	6.99	0.001
Treatm*Deme*ln°D	6	2.718	4.55	0.002
Replicate [Deme,Treat]	12	1.929		
Error	671	66.864		
Total	706	244.909		

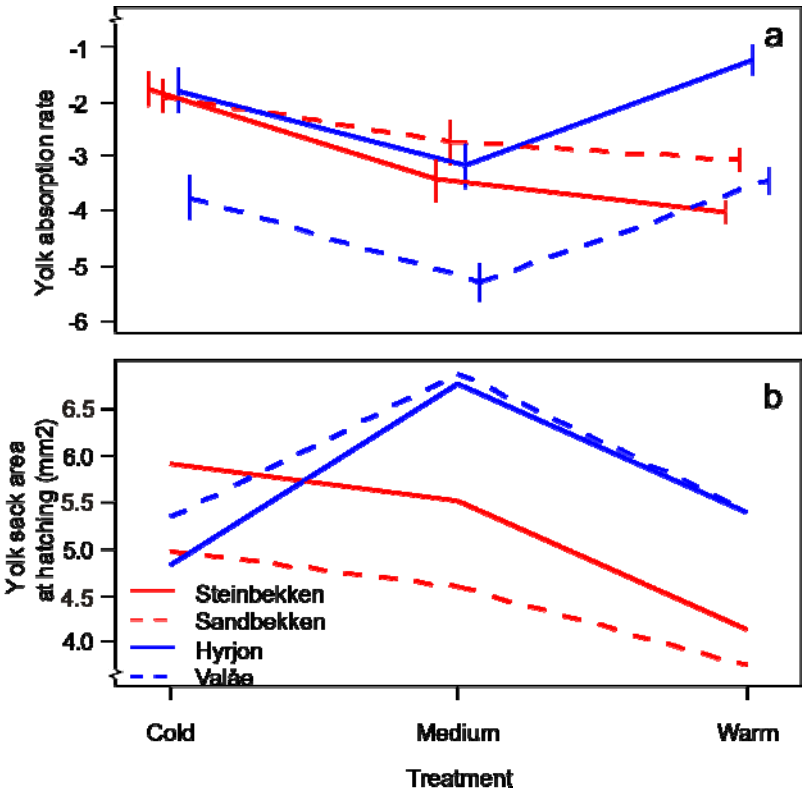


Figure 11: a) Reaction norms illustrating the effect of the different treatment temperatures on energy consumption rate estimates (slope of deme wise linear regressions of yolk sac area on degreedays for each temperature) for the four demes studied. Vertical bars indicate standard errors. b) Reaction norms illustrating the effect of treatment temperature on back-transformed estimates of yolk sac area at hatching (as calculated using deme wise means for degree days at 50% probability of hatching at the different treatment temperatures (see figure 9)). Color coding: blue indicates LC demes, red indicates SW demes.

4. Discussion

The results presented in this study demonstrate genetic differences in growth related traits between grayling from four demes in Lesjaskogsvatnet. Furthermore, the grayling from different demes respond differently to changes in developmental temperature, with respect to growth and energy usage. This is indicated by differences in reaction norms for these traits (see figure 10 and 11). Among deme variation in growth-related traits is likely to be genetically based since the results were obtained from a common garden experiment where non-genetic influences are kept under control. However, the data does not conclusively support any clear pattern of adaptation of growth related traits to the conditions experienced by the grayling (i.e. countergradient variation or local adaptation). In contrast to growth related traits, the timing of developmental traits among the demes is invariant. Temperature significantly influenced all the traits studied.

4.1. Timing of developmental traits

Highly significant temperature effects on the investigated developmental traits (hatching and eyeing) indicate that development commences at a faster pace at higher temperatures. This is in line with earlier research on the field (e.g. Jungwirth & Winkler, 1984; Beacham & Murray, 1985; Elliott et al., 1987; Haugen & Vøllestad, 2000). Jungwirth and Winkler (1984) found that the time to 50% probability of hatching by grayling was 248.4, 233.6 and 217 degreedays at 6 °C, 8 °C and 10 °C respectively. In this study, mean time to 50% hatching was 195.9, 191.5 and 173.4 degreedays in the cold (6 °C), medium (8 °C) and warm (10 °C) treatment temperatures respectively (see figure 9). The discrepancies are quite striking. Jungwirth and Winkler's (1984) estimates for time to hatching are approximately 21%, 18% and 20% higher than estimates from this study at 6 °C, 8 °C and 10 °C respectively. Humpesch (1985) conducted a study of hatching success and embryonic development in six salmonid species. In his study, time to 50% probability of hatching for grayling was 255, 200 and 165 degreedays at 5 °C, 10 °C and 15 °C respectively. For the 10 °C temperature, the estimate of developmental time from Humpesch's study is approximately 13% higher than the estimate from this study. In a study with methodology comparable to this study, Haugen and Vøllestad (2000) subjected grayling from Lesjaskogsvatnet, and adjacent populations derived from Lesjaskogsvatnet, to three treatment temperatures (7.9 ± 0.1 °C, 9.1 ± 0.2 °C and 11.2 ± 0.3 °C). Time to 50% probability of hatching in their study was approximately 190, 182 and

168 degreedays at their cold, medium and warm temperature respectively. Although identical temperatures were not used, the obtained estimates from this study and Haugen and Vøllestad's (2000) study are highly comparable. For the one, almost identical, treatment temperature used (approximately 8 °C) estimates from the two studies deviate by 0.78%. The warm treatment temperature of this study is intermediate between the medium and warm temperature from Haugen and Vøllestad's (2000) study. The same is the case for estimates obtained from the treatment temperatures in question. The estimates from Humpesch's (1985) and especially Jungwirth and Winkler's (1984) study are clearly differing from the results of the two studies on grayling from the Lesjaskogsvatnet region. These discrepancies might be caused by non-identical experimental designs. Another possibility is that we could be dealing with grayling with other adaptations. The grayling in Humpesch's (1985) and Jungwirth and Winkler's (1984) studies came from rivers in central continental Europe. It is conceivable that these fish might have adapted to differing environmental conditions by altering their developmental rates.

Without countergradient variation for developmental traits, the fact that grayling from all demes in this study develop faster in higher temperatures gives grayling from SW demes a further head-start: not only are they spawned earlier than LC deme grayling; since they experience higher spring temperatures their early development also commences at a higher rate. Therefore, they are predicted to hatch sooner after fertilization than LC deme grayling. This could give rise to stronger selection for catch-up growth among LC deme grayling.

No significant among deme variation was detected for timing of developmental traits. Some studies highlight the potential for local adaptation of developmental timing in salmonids: for instance, Pacific salmon appear to exhibit substantial interpopulational variation in timing of hatching (e.g. Beacham & Murray, 1988; Konecki et al., 1995; Granath et al., 2004). Furthermore, in a study of six species of salmonids, Humpesch (1985) found that the intraspecific differences in hatching success were highest in grayling and rainbow trout (*Oncorhyncus mykiss*). One reason why adaptation of developmental traits could not be detected in this study might be because of the population's history: the Lesjaskogsvatnet grayling population probably went through a severe bottleneck during the colonization process. Thus, it is quite likely that the demes studied here were established by a very low number of founders. Moreover, Barson and colleagues (2009) found evidence that might indicate additional bottlenecks since the founding event. Bottlenecking and subsequent genetic drift might result in reduced genetic variation and a reduced potential for adaptation

(e.g. Lande, 1988; Willi et al., 2006). If genetic variation was indeed low for these developmental traits there may not have been much material for the divergent selection pressures of the different tributaries to act upon. Thus, divergent evolution of the developmental schedule between demes may have been impossible. Furthermore, the demes of Lesjaskogsvatnet are not completely isolated (Barson et al., 2009), and gene flow between demes may further reduce the graylings' capability to adapt to local conditions.

The apparent lack of among deme differences in developmental rates could also have a more trivial explanation. The data on developmental traits exhibit large variability (see figures 8 and 9). This might be attributed to daily samples containing too few individuals. Another possibility is that it is caused by the nature of the sampling technique. Although sampling of individuals was done as haphazardly as possible, there is no guarantee that I got an actual, representative sample. Especially at developmental transitions, such as hatching, obtaining a good sample is difficult. For instance, hatched individuals might have lumped together, increasing the probability of over- or underrepresentation of hatched individuals in the sample.

Because of the nature of the experimental setup, it is feasible that timing of hatching was not solely a function of genetic makeup and temperature: in fishes, rupturing of the protective envelopes surrounding the embryo results of partial enzymatic lysis and muscular movement (Schoots et al., 1982). In this experiment, hatching enzyme, quite likely, leaked into the water during hatching. If leaked hatching enzyme came into contact with unhatched eggs from other demes, the timing of their subsequent hatching might have been influenced. On the other hand, no among deme variation was detected for the timing of eyeing. First detection of eyeing was, on average, possible 70.1 degree days before hatching. It is therefore highly unlikely that it is influenced by enzymes secreted by other individuals in the treatment tanks. The fact that timing of eyeing seems to be conserved across demes lends support to the notion that this might also be the case for timing of hatching. Crisp (1988) found that the relationship between time-to-eyeing and time-to-hatching for salmonids can be approximated by a linear regression. The relative time-interval between the phenomena thus seems to be fixed. For data on Atlantic salmon a linear regression accounts for 94% of the variation in the data (Crisp, 1988). Crisps study adds to the credibility of my data: the data obtained here likely represents the factual time of development for the demes studied, and are probably not the result of a flaw in the experimental design.

There has been much discussion about whether or not timing of hatching is positively correlated with egg size (Kamler, 2002 and references therein). Kamler (2002) suggests that yolk deficit rarely occurs in an egg with a developing embryo; thus ontogenic rate prior to hatching is not predicted to be influenced by egg size. Although initial egg sizes differed among demes, timing of developmental traits did not, thus no such correlation seems to exist here. This is in concordance with the results of Haugen and Vøllestad (2000) who found that "egg size did not covary with day at hatching" for the grayling in their study. Moreover, Beacham and Murray (1985) did not find any size-related maternal effects on hatching and emergence timing in Chum salmon (*Oncorhyncus nerka*). Also, in this study mean initial egg diameters did not differ by more than 0.19 mm (4.9% of grand mean initial egg size) between demes. Studies inferring egg size as an important determinant of incubation time are often concerned with interspecific, rather than intraspecific, relationships and variability in egg size is usually much higher (e.g. Ware, 1975; Teletchea et al., 2009).

4.2. Growth and energy use

I found, genetically based, among deme variation for both traits studied related to growth and energy use. These traits were also significantly influenced by temperature. This is in line with the known fact that both growth rate and yolk consumption rate increases with temperature within the tolerable range of temperature (Kamler, 2008 and references therein). Significant three-way interactions indicate that the different demes respond differently to the treatment temperatures. In their study, Haugen and Vøllestad (2000) also discovered significant interactions of this type.

Growth rate estimates for LC deme individuals may, to some extent, be interpreted as indicative of local adaptation. Larvae from these demes grow faster than SW deme individuals under experimental conditions mimicking LC deme habitats (i.e. the cold treatment temperature). The full picture emerging from the data, however, does not seem to correspond with predictions for local adaptation or countergradient variation: at the medium and warm treatment temperatures, the rank order of demes appears not to follow a pattern that can be ascribed to conditions in their natal tributaries. Furthermore, LC deme individuals do better in the higher treatment temperatures than they do in the coldest one. There is a general trend in all demes for growth rates to increase with temperature, much in line with results from comparable studies (Reviewed by Kamler, 2008). Interestingly, norms of reaction for

larval length at hatching reveal a somewhat different pattern (see figure 10b). The reaction norm for length at hatching for Sandbekken individuals fits the bill for local adaptation: these embryos grow the most prior to hatching in the warmest treatment temperature and the least in the coldest. The two LC demes seem to maximize pre-hatching growth at 8°C, possibly reflecting a temperature constraint reducing growth prior to hatching at lower temperatures. Steinbekken grayling appear to outperform all other demes at every temperature in a co-gradient fashion. Individuals from Steinbekken also seem not to be highly influenced by temperature when it comes to growth prior to hatching. Similarly, length at hatching for the warm-adapted population in Haugen and Vøllestad's (2000) study appear to be less affected by temperature than the other populations.

Estimates of yolk sac absorption rates could be taken to illustrate local adaption constrained by temperature: SW deme individuals display a positive correlation between yolk sac consumption and temperature, while larvae from LC demes have the highest rate of consumption at the medium temperature. Also, estimates of yolk sac area at hatching for SW deme grayling seem to lend some support to evolution of local adaptation to maximize pre-hatching yolk sac consumption. LC deme larvae seem not to have achieved much local adaptation with respect to this trait, as they perform about equally well in the warm and cold treatment temperatures. Furthermore, they do not do any better than SW deme individuals in the cold treatment temperature. An alternative explanation could focus on selective pressures not having caused alteration in the actual speed of energy consumption, but rather in the efficiency of energy conversion. In such a scenario, LC deme individuals could have adapted by growing more per unit of yolk sac consumed. If conversion efficiencies were equal in all demes we would have a neutral expectation that the energetic costs of growth would give rise to the following pattern: the larvae attaining the longest body length in a certain treatment temperature should be the ones possessing the smallest yolk sac. When looking at larval length and yolk sac area at hatching (figure 10b and 11b respectively) in the cold treatment temperature, the emerging pattern is exactly opposite. Steinbekken individuals seem to have the most efficient conversion, contradictory to any prediction for adaptation by LC deme individuals. As treatment temperature increases the pattern seems to approach the neutral expectation of similar conversion efficiency. The overall pattern gives the impression of differential pre-hatching conversion efficiency among the demes.

The differences in traits related to growth and energy utilization do not, unambiguously correspond with predictions for local adaptation, and certainly not

countergradient variation. Any lack of adaptation might be caused by evolutionary constraints, resulting from the history of the Lesjaskogsvatnet grayling. It is possible that the demes under investigation have not had equal opportunities for environmental adaptation. If the founders of some demes had higher genetic variability than others for traits involved in such adaptation, this might give rise to a pattern of reaction norms that is complex and difficult to interpret. Also, if leaked hatching enzyme has influenced timing of hatching, this might potentially have altered the growth- and yolk sac consumption trajectories for the affected individuals. Such alterations could obscure underlying tendencies for local adaptation or countergradient variation.

The apparent mismatch with predictions from theory on local adaptation and countergradient variation might also be explained in terms of what kind of selection pressure is operating. What if size is not maximized to increase fitness? What if bigger is actually not better? Some researchers have indeed criticized the "bigger-is-better" hypothesis, and even suggested that smaller might be better (e.g. Litvak & Leggett, 1992; Pepin et al., 1997; Lund et al., 2003; Carlson et al., 2008). Lund and colleagues (2003) found that winter mortality was not size-dependent in a study of first-year survival in three separate populations of brown trout. In their study the reasons for this were believed to be related to severity of winter conditions and mortality rates: survival is more likely to be size-dependent when conditions are moderate. Size-dependent survival is less likely under extreme environmental conditions since mortality in such instances is high for all size-classes (Sogard, 1997). Carlson and colleagues (2008) argue that self-sustaining populations should be adapted to their environment, and also the predictable, seasonal variation within it. They present empirical data on stream-dwelling brown trout (*Salmo trutta*) supporting this view. However, their data show that, although smaller is in general better, bigger is actually better for the smallest individuals. Thus, although winter conditions are harsh in the Lesjaskogsvatnet area, the grayling are expected to have adapted to this. If they have, we should expect some degree of size-dependent survival. Furthermore, judging by Carlson and colleagues' (2008) own results, large size should be favorable for small fish like the ones studied here.

Other researchers have also pointed to different selective agents than temperature or season length causing observed size patterns. Two studies on two different salmon species infer predation as a possible selective agent inducing adaptive alteration of timing of emergence and size at emergence (Brännäs, 1995; Sundström et al., 2005). In the absence of predators it might pay off to develop fast and emerge early. If predators are present this might

alter the optimal time for emergence. When fry are able to emerge before the predator arrives it pays off to emerge early to gain habitat experience (Brännäs, 1995). If, however, the predator is already present, emerging late increases survival (Brännäs, 1995). Differing predation pressures between demes in Lesjaskogsvatnet could lead to adaptive responses in terms of altered developmental rates. Since the timing of developmental traits did not differ between demes in this study, it seems as if this has not been an important factor for influencing the pattern of development and subsequent growth.

In a study of the vulnerability to predation of capelin (*Mallotus villosus*) yolk sac larvae, the researchers found that being smaller at a certain age might increase survival probability (Litvak & Legget, 1992). The Lesjaskogsvatnet fish community consists of grayling, brown trout and European minnow (*Phoxinus phoxinus*) (Gammelsrud, 1982). It is conceivable that brown trout, and perhaps even grayling, might prey on grayling larvae upon emergence. If such predation is size-selective for large individuals, it is possible that it could influence the shape of an individual's optimal growth trajectory. At some stage, however, all individuals must pass through the size-range preferred by the predator. Therefore, if altering the growth trajectory in response to predation should have any adaptive value, there must be some temporal variation in predation risk. I find it unlikely that there will be much temporal variation in predation pressure during the critical time for the growth and development of grayling larvae in Lesjaskogsvatnet. Growth season being short, predators, if at all present, should be a constant threat during the summer months.

In his master thesis, Krogstad (2008) conducted a reciprocal transplant experiment on grayling eggs from a small number of Lesjaskogsvatnet tributaries. He investigated certain early developmental traits, and some of the results he obtained are fairly comparable to certain results in this study. He emphasized maternal effects as a possible cause for the observed variation. Possible implications of maternal effects in fish are reviewed by Heath and Blouw (1998). If such effects are present, and if they are physiological side effects and not consequences of adaptive evolution, they have the potential of confounding results in studies like this one. Unfortunately, egg size related maternal effects could not be incorporated into the models in this study due to incompatibility with model structure. In this study initial egg sizes were significantly different among demes. The Tukey-Kramer HSD test could only assert that mean initial egg size was truly smaller in Sandbekken than in both Steinbekken and Valåe. The latter two were also the demes with the largest mature females (see table 2). At hatching, the same two demes had the longest individuals in the cold and medium treatment

temperature. In the warm treatment temperature, Sandbekken individuals seem to have "caught up". For yolk sac size, an individual's condition at hatching seems to be more closely related to which deme it belongs to than to its initial egg size. Generally, egg size is thought to correlate positively with yolk absorption rate (Kamler, 2008). Potentially, this could make individuals from large eggs capable of (over)compensating by absorbing extra yolk prior to hatching. This could, potentially, further complicate this kind of informal inference from the visualizations of the results. When looking at yolk absorption rates, Valåe stands out as the deme with the highest rate in the two coldest temperatures. The other three demes cluster together at these temperatures. At the warm treatment temperature, Hyrjon stands out with the lowest rate while the other three demes cluster quite closely. All in all there is no clear pattern of maternal effects on yolk absorption rate. Moreover, as mentioned, the differences in egg diameter between demes are not very large. Nonetheless, maternal effects cannot be formally ruled out as a possible confounding factor in this study.

Obedzinski and Letcher (2004) conducted a common garden study on the growth and development of different Atlantic salmon (*Salmo salar*) populations. They emphasize the necessity of examining multiple traits through several life stage transitions to conclusively address questions concerning developmental differences and trade-offs. It is possible that, had we continued our experiment for a longer time period, different results would have been obtained. Grayling larvae persist in their natal stream for up to 1-1.5 months before entering the sympatric stage in the lake (Haugen & Vøllestad, 2001). Stream-specific selection pressures within this timeframe could alter the growth trajectories of the larvae from the different demes in a fashion consistent with the predictions of local adaptation. If the time constraint of a short growth season is a selective agent for LC demes any compensatory growth could also take place within this time window. Also, since phenotypes usually reflect optimization of a whole suite of traits (Niewiarowski & Angiletta, 2008), it cannot be ruled out that growth could trade off with some important trait(s) not considered here.

4.3. Concluding remarks and future research

In summary, while the timing of developmental traits appear to be invariant among demes, evidence of significant among deme variation in continuous developmental traits was found. All investigated traits were significantly influenced by temperature. The lack of variation among demes in relation to timing of hatching might possibly be caused by hatching enzyme

from early hatching individuals leaking into the water and influencing the timing of hatching of other individuals. On the other hand, genetic constraints imposed by a severe bottleneck event during colonization could also give rise to a conserved developmental schedule throughout the Lesjaskogsvatnet grayling population. The genetic differences in growth-related traits are not easily interpreted. Parts of the variation could be taken to reflect some degree of local adaption. However, it seems probable that adaptation might have been somewhat constrained by lack of genetic variation, possibly due to a bottleneck event and subsequent genetic drift. Tradeoffs with other traits, or adaptation to some environmental parameter not considered here might also be conceivable. I conclude that evolutionary changes in growth-related traits have probably come about as a result of several processes, both directional and random of nature. However, one must bear in mind the possible effects of some confounding factors: maternal effects, flaws in the sampling scheme and enzymatic influence of development are all potential sources of error.

There are a number of measures that could be taken to avoid the possible caveats of this study in future experiments: 1) Separation of individuals in individual containers prior to hatching could ameliorate the potential problem of leaked hatching enzyme influencing the timing of hatching of other individuals. 2) Lengthening the experimental period to include the time from swim-up to the sympatric stage, could shed light on whether developmental timing and growth trajectories differ between demes after swim-up. 3) More developmental traits could be included to find possible trade-offs not accounted for in this study. 4) Further measures might possibly be taken to assure the capture of approximately equal numbers of mature individuals in all demes included in the experiment. 5) More environmental variables could be registered to account for more of the residual variation, and possibly uncover alternative selective agents, which may or may not be correlated with temperature. 6) In an attempt to try to completely rule out maternal effects as source of error, f_2 offspring of captive fish could be used in an equivalent study.

References

Arendt J.D. and Wilson D.S. 1999. Countergradient selection for rapid growth in pumpkinseed sunfish: disentangling ecological and evolutionary effects. Ecology 80: 2793-2798.

Arnott S.A., Chiba S and Conover D.O. 2006. Evolution of intrinsic growth rate: metabolic costs drive trade-offs between growth and swimming performance in *Menidia menidia*. Evolution 60: 1269-1278.

Bardonnet A. and Gaudin P. 1991. Influence of daily variations of light and temperature on the emergence rhythm of grayling fry (*Thymallus thymallus*). Canadian journal of fisheries and aquatic sciences 48: 1176-1180.

Barson N.J., Haugen T.O., Vøllestad L.O. and Primmer C. R. 2009. Contemporary isolation-by-distance, but not isolation-by-time, among demes of European grayling (*Thymallus Thymallus,* Linnaeus) with recent common ancestors. Evolution 63: 549-556.

Beacham T.D. and Murray C.B. 1985. Effect of female size, egg size, and water temperature on developmental biology of chum salmon (*Oncorhyncus keta*) from the Nitinat river, British Columbia. Canadian journal of fisheries and aquatic sciences 42: 1755-1765.

Beacham T.D. and Murray C.B. 1988. Variation in developmental biology of sockeye salmon (*Oncorhyncus nerka*) and chinook salmon (*Oncorhyncus tshawytscha*) in British Columbia. Canadian journal of zoology 67: 2081-2089.

Billerbeck J.M., Lankford T.E. and Conover D.O. 2001. Evolution of intrinsic growth and energy acquisition rates. I. Trade-offs with swimming performance in *Menidia menidia*. Evolution 55: 1863-1872.

Brännäs E. 1995. First access to territorial space and exposure to strong predation pressure: a conflict in early emerging Atlantic salmon (*Salmo salar* L.) fry. Evolutionary ecology 9: 411-420.

Bærum K.M. 2008. Morphological divergence among sympatric demes of European grayling (*Thymallus thymallus*). MSc thesis, Faculty of mathematics and natural sciences, University of Oslo.

Carlson S.M., Olsen E.M. and Vøllestad L.A. 2008. Seasonal mortality and the effect of body size: a review and an empirical test using individual data on brown trout. Functional ecology 22: 663-673.

Conover D.O. and Present T.M.C. 1990. Countergradient variation in growth rate: compensation for length of the growing season among Atlantic silversides from different latitudes. Oecologica 83: 316-324.

Conover D.O. and Schultz E.T. 1995. Phenotypic similarity and the evolutionary significance of countergradient variation. Trends in ecology and evolution 10: 248-252.

Crawley M.J. 2005. Statistics: an introduction using R. Chichester, West Sussex: John Wiley & sons ltd.

Crisp D.T. 1988. Prediction, from temperature, of eyeing, hatching and 'swim-up' times for salmonid embryos. Freshwater biology 19: 41-48.

Dobzhansky T. 1941. Genetics and the origin of species – second edition, revised. New York, New York: Columbia university press.

Elliott J.M., Humpesch U.H. and Hurley M.A. 1987. A comparative study of eight mathematical models for the relationship between water temperature and hatching time of eggs of freshwater fish. Archiv für hydrobiologie 109: 257-277.

Fox J. 1997. Applied regression analysis, linear models, and related methods. Thousand Oaks, California: SAGE publications, inc.

Fuiman L.A. and Werner R.G. 2002. Fishery science – The unique contributions of early life stages. Osney Mead, Oxford: Blackwell science ltd.

Futuyma D.J. 2005. Evolution. Sunderland, Massachusetts: Sinauer associates, Inc.

Gagliano M., McCormick M.I. and Meekan M. G. 2007. Temperature-induced shifts in selective pressure at a critical developmental transition. Oecologica 152: 219-225.

Gammelsrud S. 1982. Fordeling og ernæring hos fisken i Lesjaskogsvatnet – Konkurranse (in norwegian). MSc thesis, Department of natural resource management, the Norwegian agricultural college.

Gilbert S.F. 2001. Ecological developmental biology: developmental biology meets the real world. Developmental biology 233: 1-12.

Gíslason D., Ferguson M.M., Skúlason S. and Sigurður S.S. 1999. Rapid and coupled phenotypic and genetic divergence in Icelandic Arctic char (*Salvelinus alpinus*). Canadian journal of fisheries and aquatic sciences 56: 2229-2234.

Granath K.L., Smoker W.W., Gharrett A.J. and Hard J.J. 2004. Effects on embryo development time and survival of intercrossing three geographically separate populations of southeast Alaska coho salmon *Oncorhyncus kisutch*. Environmental biology of fishes 69: 299-306.

Gregersen F., Haugen T.O. and Vøllestad L.A. 2008. Contemporary egg size divergence among sympatric grayling demes with common ancestors. Ecology of freshwater fish 17: 110-118.

Groman J.D. and Pellmyr O. 2000. Rapid evolution and specialization following host colonization in a yucca moth. Journal of evolutionary biology 13: 223-236.

Hairston Jr N.G., Elner S.P., Geber M.E., Yoshida T. and Fox J.A. 2005. Rapid evolution and the convergence of ecological and evolutionary time. Ecology letters 8: 1114-1127.

Hare J.A. and Cowen R.K. 1997. Size, growth, development and survival of the planktonic larvae of *Pomatomus saltatrix* (Pisces: Pomatomidae). Ecology 78: 2415-2431.

Haugen T.O. and Vøllestad L.A. 2000. Population differences in early life-history traits in grayling. Journal of evolutionary biology 13: 897-905.

Haugen T.O. and Vøllestad L.A. 2001. A century of life history evolution in grayling. Genetica 112-113: 475-491.

Heath D.D. and Blouw D.W. 1998. Are maternal effects in fish adaptive or merely physiological side effects? In: Mosseau T.E. and Fox C.W. (eds) Maternal effects as adaptations. New York, New York: Oxford university press inc.

Hendry A.P., Wenburg J.K., Bentzen P., Volk E.C. and Quinn T.P. 2000. Rapid evolution of reproductive isolation in the wild: evidence from introduced salmon. Science 290: 516-518.

Humpesch U.H. 1985. Inter- and intra-specific variation in hatching success and embryonic development of five species of salmonids and *Thymallus thymallus*. Archiv für hydrobiologie 104: 129-144.

Jensen L.F., Hansen M.M., Pertoldi C., Holdensgaard G., Mensberg K.D. and Loeschcke V. 2008. Local adaptation in brown trout early life-history traits: implications for climate change adaptability. Proceedings of the royal society B 275: 2859-2868.

Jungwirth M. and Winkler H. 1984. The temperature dependence of embryonic development of grayling (*Thymallus thymallus*), Danube salmon (*Hucho hucho*), Arctic char (*Salvelinus alpinus*) and Brown trout (*Salmo trutta fario*). Aquaculture 38: 315-327

Kamler E. 2002. Ontogeny of yolk-feeding fish: an ecological perspective. Reviews in fish biology and fisheries 12: 79-103.

Kamler E. 2008. Resource allocation in yolk-feeding fish. Reviews in fish biology and fisheries 18: 143-200.

Konecki J.T., Woody C.A. and Quinn T.P. 1995. Influence of temperature on incubation rates of coho salmon (*Oncorhyncus kisutch*) from ten Washington populations. Northwest science 69: 126-132.

Koskinen M.T., Ranta E., Piironen J., Veselov A., Titov S., Haugen T.O., Nilsson J., Carlstein M. and Primmer C.R. 2000. Genetic lineages and postglacial colonization of grayling (*Thymallus thymallus*) in Europe, as revealed by mitochondrial DNA analyses. Molecular ecology 9: 1609-1624.

Kristiansen H. and Døving K.B. 1996. The migration of spawning stocks of grayling, *Thymallus thymallus*, in Lake Mjøsa, Norway. Environmental biology of fishes 47: 43-50.

Krogstad E. 2008. Early development of grayling in Lake Lesjaskogvatn: a reciprocal transplant field experiment. MSc thesis, Faculty of mathematics and natural sciences, University of Oslo.

Kruse G.H. and Dalley E.L. 1990. Length changes in capelin, *Mallotus villosus* (Müller), larvae due to preservation in formalin and anhydrous alcohol. Journal of fish biology 36: 619-621.

Lande R. 1976. Natural selection and random genetic drift in phenotypic evolution. Evolution 30: 314-334.

Lande R. 1988. Genetics and demography in biological conservation. Science 241: 1455-1460.

Laugen A.T., Laurila A., Räsänen K. and Merilä J. 2003. Latitudinal countergradient variation in the common frog (*Rana temporaria*) developmental rates – Evidence for local adaptation. Journal of evolutionary biology 16: 996-1005.

Litvak M.K. and Leggett W.C. 1992. Age and size-selective predation on larval fishes: the bigger-is-better hypothesis revisited. Marine ecology progress series 81: 13-24.

Lund E., Olsen E.M. and Vøllestad L.A. 2003. First year survival of brown trout in three Norwegian streams. Journal of fish biology 62: 323-340.

Macqueen D.J., Robb D. and Johnston I.A. 2007. Temperature influences the coordinated expression of myogenic regulatory factors during embryonic myogenesis in Atlantic salmon (*Salmo salar* L.). The journal of experimental biology 210: 2781-2794.

Miller T.J., Crower L.B., Rice J.A. and Marshall E.A. 1988. Larval size and recruitment mechanisms in fishes: toward a conceptual framework. Canadian journal of fisheries and aquatic sciences 45: 1657-1670.

Nicieza A.G., Reiriz L. and Braña F. 1994. Variation in digestive performance between geographically disjunct populations of Atlantic salmon: countergradient in passage time and digestion rate. Oecologica 99: 243-251.

Niewiarowski P.H. and Angiletta Jr M.J. 2008. Countergradient variation in embryonic growth and development: Do embryonic and juvenile performances trade off? Functional ecology 22: 895-901.

Northcote T.G. 1995. Comparative biology and management of Arctic and European grayling (Salmonidae, *Thymallus*). Reviews in fish biology and fisheries 5: 141-194.

Obedzinski M. and Letcher B.H. 2004. Variation in freshwater growth and development among five New England atlantic salmon (*Salmo salar*) populations reared in a common environment. Canadian journal of fisheries and aquatic sciences 61: 2314-2328.

Oufiero C.E. and Angilletta Jr. M.J. 2006. Convergent evolution of embryonic growth and development in the Eastern fence lizard (*Sceloporus undulatus*). Evolution 60: 1066-1075.

Pepin P., Orr D.C. and Aderson J.T. 1997. Time to hatch and larval size in relation to temperature and egg size in Atlantic cod (*Gadus morhua*). Canadian journal of fisheries and aquatic sciences 54 (Suppl. 1): 2-10.

Pinheiro J.C. and Bates D.M. 2000. Mixed-effects models in S and S-PLUS. New York, New York: Springer-Verlag New York, Inc.

Post J.R. and Parkinson E.A. 2001. Energy allocation strategy in young fish: allometry and survival. Ecology 82: 1040-1051.

Reznick D.N. and Ghalambor C.K. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. Genetica 112-113: 183-198.

Schoots A.F.M., Opstelten R.J.G. and Denucé J.M. 1982. Hatching in the pike *Essox lucius* L: evidence for a single hatching enzyme and its immunocytochemical localization in specialized hatching gland cells. Developmental biology 89: 48-55.

Schultz E.T., Conover D.O. and Ehtisham A. 1998. The dead of winter: size-dependent variation and genetic differences in seasonal mortality among Atlantic silverside (Atherinidae: *Menidia menidia*) from different latitudes. Canadian journal of fisheries and aquatic sciences 55: 1149-1157.

Simpson G.G. 1953. The major features of evolution. New York, New York: Columbia university press.

Sogard S.M. 1997. Size-selective mortality in the juvenile stage of teleost fishes: a review. Bulletin of marine science 60: 1129-1157

Stearns S.C. 1992. The evolution of life histories. Oxford: Oxford university press.

Sundström F.L., Lömhus M. and Devlin R.H. 2005. Selection on increased intrinsic growth rates in coho salmon, *Oncorhyncus kisutch*. Evolution 59:2005.

Sultan S.E. 2007. Development in context: the timely emergence of eco-devo. Trends in ecology and evolution 22: 575-582.

Teletchea F., Gardeur J.-N., Kamler E. and Fontaine P. 2009. The relationship of oocyte diameter and incubation temperature to incubation time in temperate freshwater fish species. Journal of fish biology 74: 652-668.

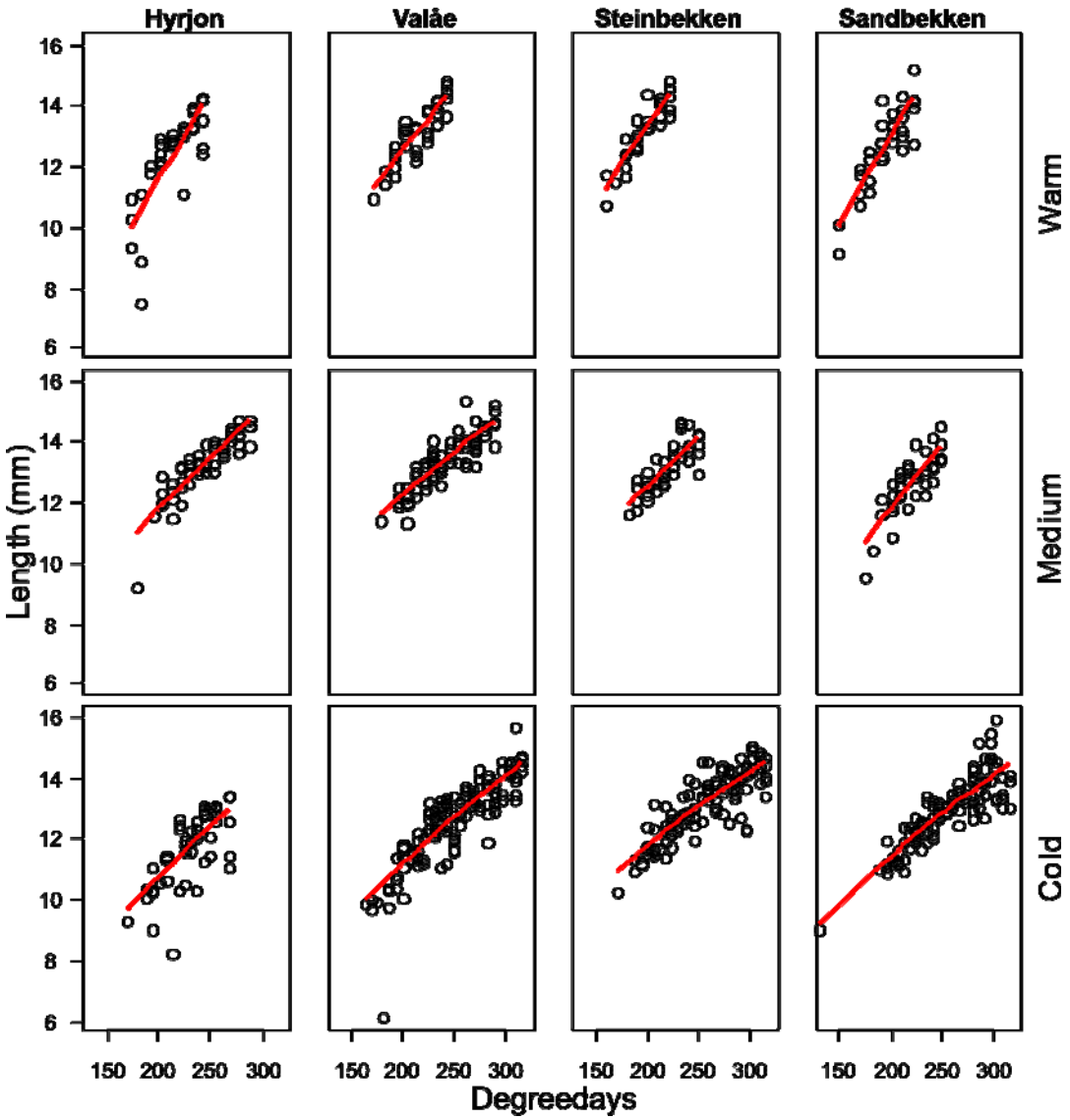
Ware D.M. 1975. Relation between egg size, growth, and natural mortality of larval fish. Journal of the fisheries research board of Canada 32: 2503-2512

Willi Y., Buskirk J.V. and Hoffmann A.A. 2006. Limits to the adaptive potential of small populations. Annual review of ecology, evolution and systematics 37: 433-458.

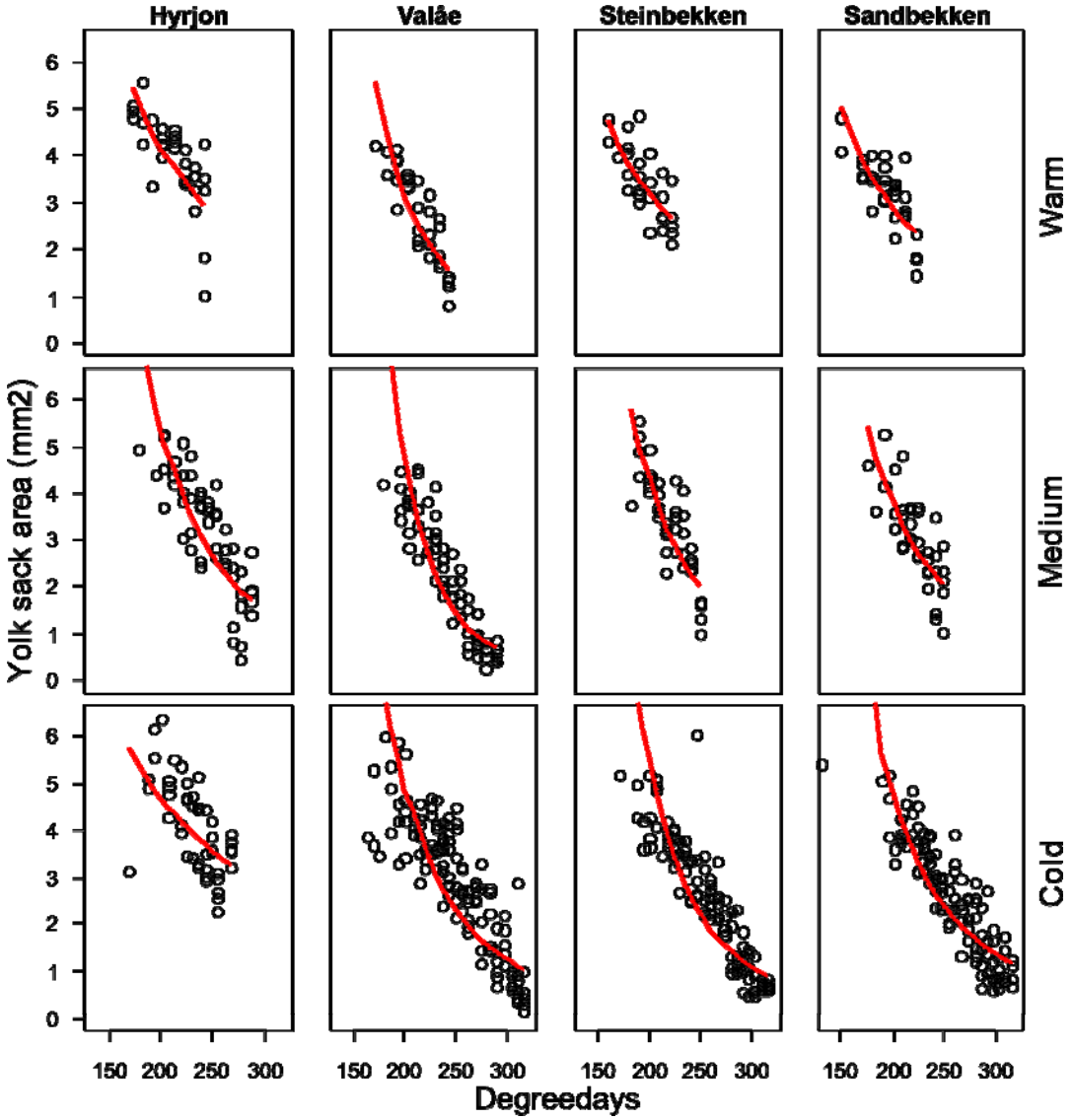
Wright S. 1931. Evolution in mendelian populations. Genetics 16: 97-159.

Yamahira K. and Conover D.O. 2002. Intra- vs. interspecific latitudinal variation in growth: adaptation to temperature or seasonality? Ecology 83: 1252-1262.

Appendices



Appendix figure 1: Scatterplots showing the length of each individual sampled from every deme at every treatment temperature. The red line represents the model fit to the data (replicate effect not accounted for).



Appendix figure 2: Scatterplots showing the yolk sac area of each individual sampled from every deme at every treatment temperature. The red line represents the model fit to the data (replicate effect not accounted for).

Appendix table 1: Parameter estimates for the factors included in the models fitted to the data. Linear mixed models (LME) for length and yolk sac area, logistic regressions for eyeing probability and hatching probability.

Trait	Factor	Estimate	SE
Eyeing probability	Intercept	8.765	0.757
	ln°d	-0.082	0.007
	Deme (Hyrjon)	-1.450	0.954
	Deme (Valåe)	0.874	0.407
	Deme (Sandbekken)	0.381	0.444
	Treatment (Cold)	-0.387	0.416
	Treatment (Medium)	1.590	0.367
Hatching probability	Intercept	30.238	3.526
	ln°d	-0.162	0.019
	Deme (Hyrjon)	0.179	1.323
	Deme (Valåe)	-0.436	1.265
	Deme (Sandbekken)	1.325	1.885
	Treatment (Cold)	-2.008	0.875
	Treatment (Medium)	3.812	1.504
Length	Intercept	-1.034	0.103
	ln°d	0.664	0.019
	Deme (Hyrjon)	-0.037	0.005
	Deme (Valåe)	-0.013	0.005
	Deme (Sandbekken)	0.034	0.006
	Treatment (Cold)	-0.078	0.004
	Treatment (Medium)	-0.002	0.004
Yolk sac area	Intercept	17.065	0.706
	ln°d	-2.950	0.132
	Deme (Hyrjon)	0.265	0.036
	Deme (Valåe)	-0.192	0.035
	Deme (Sandbekken)	-0.012	0.044
	Treatment (Cold)	0.168	0.027
	Treatment (Medium)	-0.042	0.029

Appendix table 2: Summary table for deme wise linear regressions of ln length on degreedays in the three treatment temperatures. SE indicates standard error; N indicates number of individuals on which the regression is based.

Treatment	Deme	Slope	SE	r^2 adjusted	N
Warm	Hyrjon	1.015	0.152	0.577	33
	Valåe	0.697	0.065	0.780	33
	Steinbekken	0.765	0.066	0.833	28
	Sandbekken	0.897	0.083	0.773	35
Medium	Hyrjon	0.608	0.044	0.775	56
	Valåe	0.490	0.033	0.761	70
	Steinbekken	0.553	0.062	0.657	42
	Sandbekken	0.731	0.083	0.669	39
Warm	Hyrjon	0.643	0.086	0.560	47
	Valåe	0.577	0.027	0.798	122
	Steinbekken	0.468	0.029	0.707	105
	Sandbekken	0.520	0.026	0.789	107

Appendix table 3: Summary table for deme wise linear regressions of ln yolk sac area on degreedays in the three treatment temperatures. For further details see appendix table 2.

Treatment	Deme	Slope	SE	r^2 adjusted	N
Warm	Hyrjon	-1.787	0.41	0.360	33
	Valåe	-3.747	0.41	0.721	33
	Steinbekken	-1.770	0.31	0.533	28
	Sandbekken	-1.912	0.29	0.547	35
Medium	Hyrjon	-3.148	0.41	0.517	56
	Valåe	-5.287	0.34	0.776	70
	Steinbekken	-3.423	0.40	0.643	42
	Sandbekken	-2.731	0.41	0.529	39
Warm	Hyrjon	-1.230	0.28	0.312	47
	Valåe	-3.449	0.25	0.611	122
	Steinbekken	-4.015	0.22	0.763	105
	Sandbekken	-3.065	0.21	0.673	107