Research Note

Persistent Unequal Sex Ratio in a Population of Grayling (Salmonidae) and Possible Role of Temperature Increase

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Abstract: *In some fishes, water chemistry or temperature affects sex determination or creates sex-specific selection pressures. The resulting population sex ratios are hard to predict from laboratory studies if the environmental triggers interact with other factors, whereas in field studies, singular observations of unusual sex ratios may be particularly prone to selective reporting. Long-term monitoring largely avoids these problems. We studied a population of grayling* (Thymallus thymallus) *in Lake Thun, Switzerland, that has been monitored since 1948. Samples of spawning fish have been caught about 3 times/week around spawning season, and water temperature at the spawning site has been continuously recorded since 1970. We used scale samples collected in different years to determine the average age of spawners (for life-stage specific analyses) and to identify the cohort born in 2003 (an extraordinarily warm year). Recent tissue samples were genotyped on microsatellite markers to test for genetic bottlenecks in the past and to estimate the genetically effective population size* (N_e). *Operational sex ratios changed from approximately 65% males before 1993 to approximately 85% males from 1993 to 2011. Sex ratios correlated with the water temperatures the fish experienced in their first year of life. Sex ratios were best explained by the average temperature juvenile fish experienced during their first summer. Grayling abundance is declining, but we found no evidence of a strong genetic bottleneck that would explain the apparent lack of evolutionary response to the unequal sex ratio. Results of other studies show no evidence of endocrine disruptors in the study area. Our findings suggest temperature affects population sex ratio and thereby contributes to population decline.*

Persistencia de Proporción de Sexos Desigual en una Población de Tímalos (Salmonidae) y el Posible Papel del Incremento de la Temperatura

Resumen: *En algunos peces, la química del agua o la temperatura afectan la determinación del sexo o crean presiones de selección específicas por sexo. Las proporciones de sexo resultantes son difíciles de predecir a partir de estudios de laboratorio si los detonadores ambientales interactúan con otros factores, mientras que en estudios de campo, observaciones singulares de proporciones de sexo inusuales pueden estar particularmente propensas a reportes selectivos. El monitoreo a largo plazo evita estos problemas. Estudiamos una población*

de tímalos (Thymallus thymallus) *en el Lago Thun, Suiza, que ha sido monitoreada desde 1948. Desde 1970, se han capturado muestras de peces reproductores 3 veces por semana durante la época de reproducción, y se ha registrado la temperatura del agua en los sitios de desove. Utilizamos muestras recolectadas en años diferentes para determinar la edad promedio de los reproductores (para análisis de etapas de vida específicas) y para identificar la cohorte nacida en 2003 (un año extraordinariamente caluroso). Se determinó el genotipo de muestras recientes de tejido con marcadores de microsatélite para buscar cuellos de botella genéticos en el pasado y para estimar el tamaño poblacional genéticamente efectivo* (N$_e$). *Las proporciones de sexo operativas cambiaron de aproximadamente 65% de machos antes de 1993 a aproximadamente 85% de machos de 1993 a 2011. Las proporciones de sexo se correlacionaron con las temperaturas del agua que los peces experimentaron durante su primer año de vida. Las proporciones de sexo fueron mejor explicadas por la temperatura promedio que experimentaron peces juveniles durante su primer verano. La abundancia de tímalos está declinando, pero no encontramos evidencia de un cuello de botella drástico que pudiera explicar la falta de respuesta evolutiva a la proporción de sexos desigual. Los resultados de otros estudios no muestran evidencia de disruptores endócrinos en el área de estudio. Nuestros resultados sugieren que la temperatura afecta la proporción de sexos en la población y por lo tanto contribuye a su declinación.*

Palabras Clave: mortalidad sesgada por sexo, proporción de sexos operativa, reversión sexual ambiental, tamaño poblacional efectivo

Introduction

River-dwelling fishes often have little possibility of moving to avoid less than optimal environmental conditions and hence can be affected by changes in water temperature or chemistry. If tolerance to stressors is sex specific (e.g., Perry et al. 2005) or if genetic sex determination is reversed by environmental conditions (Baroiller et al. 2009), environmental changes can have demographic and evolutionary effects that may be counterintuitive (Cotton & Wedekind 2009). Such effects may not be detected easily in natural fish populations if, for example, nonrandom harvest obscures demographic effects of environmental changes. However, laboratory-based studies on the possible demographic effects of, for example, temperature can be misleading (Ospina-Alvarez & Piferrer 2008).

We analyzed 63 years of sex-ratio data (1948–2011) on a population of grayling (*Thymallus thymallus*; Salmonidae) whose spawning area has been protected since 1952. The temperature in the region encompassing the spawning area increased abruptly from 1987 to 1988 (Hari et al. 2006). We observed a shift in the operational sex ratios (OSR) (i.e., the proportion of males among all breeders during the time when egg-bearing females with mature eggs could be caught at a spawning site) that may have contributed to an otherwise unexplained decrease in abundance (Wedekind & Küng 2010), and we tested for possible links between OSR and water temperature.

If a population cannot change its geographic distribution, phenotypic plasticity or evolutionary adaptation may be the only way a population threatened with extinction can persist (Hoffmann & Sgrò 2011). Magerhans et al. (2009) found paternal and maternal effects on temperature-dependent sex determination in aquaculture stocks of another salmonid species, and analogous genetic effects on sex-specific tolerance to environmental stressors seem possible (Perry et al. 2005; Hutchings 2006). A genetic bottleneck could, therefore, reduce the adaptive capacity of the population and contribute to changes in demography, for instance in OSR. We, therefore, also tested whether genetic bottlenecks could have occurred in recent years.

Methods

The study population of graylings has its main feeding territory in Lake Thun (Switzerland) and spawns in the lake outlet, the Aare River (between the lake and a weir 1.3 km downstream). Since 1948, samples of spawning fish have been caught with nets about 3 times per week around the spawning season in March and April. Yearly catches during spawning season range from 51 to 2085 grayling (median = 630), were low before sampling efforts were standardized in 1962, and declined from the 1970s to 2009 (Wedekind & Küng 2010). The large majority of these fish were released back to the spawning site after their gametes were collected (by stripping). However, in some years, especially the first years of the program, some males (0–95%) and some females (0–85%) died due to handling or were released downstream of the weir and hence were separated from their original spawning population (Fig. 1a). In 1992, 2006, and 2008, fish were marked by clipping the adipose fin or removing scales to determine whether individuals were recaptured. On average 15.0% of males (3.8–22.3%) and 0.4% of females (0–1.9%) were caught twice during the same spawning period. We used these averages to correct the OSR estimates for all years. Use of the lowest or the highest observed number of multiple captures would not have changed our conclusions and would have shifted OSR estimates only slightly (e.g., in 2011 from 0.861 to 0.877 or 0.851, respectively). We used Spearman rank-order
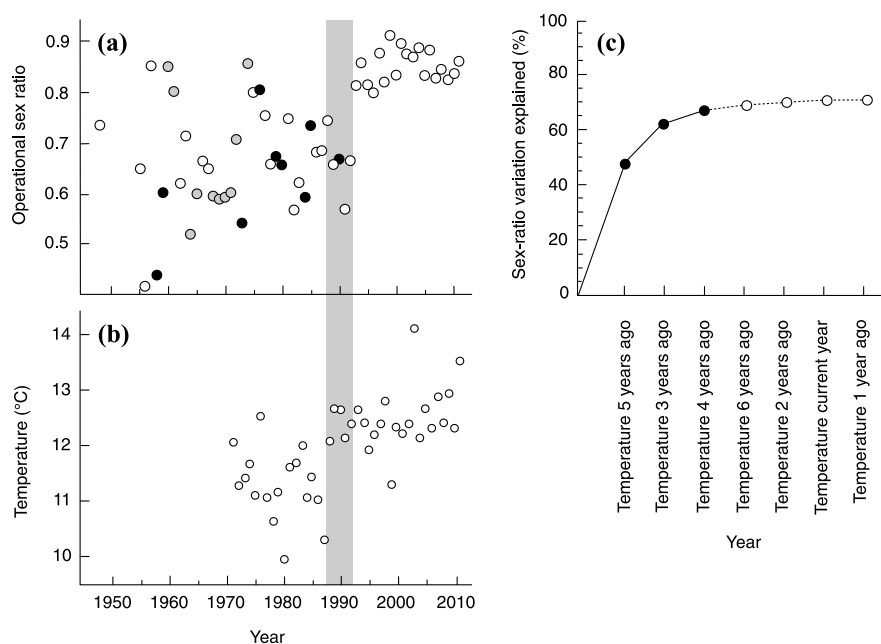
Figure 1. Operational sex ratio (OSR) and water temperature at the spawning site of grayling in Lake Thun, Switzerland: (a) OSR since 1948 (gray and black points, years in which at least 30% and 50%, respectively, more males than females died or were released downstream of a weir after stripping; shaded area in parts (a) and (b), first 5 years after an abrupt increase in temperatures from 1987 to 1988 [Hari et al. 2006]), (b) average water temperature at the spawning ground between 1 March and 31 August, and (c) amount of variation in observed OSR explained by adding the average temperature of previous (1–6 years previous) and current years to a forward stepwise regression (black circles, models significantly improved [F always >4.8, p always <0.05] by adding the latest covariate; white circles and dashed line, no statistically significant model improvement).

correlation coefficients (r_s) to test whether OSR or water temperature changed over time and to compare yearly OSR with the number of sampled females from 1948 to 2009 (as reported in Wedekind & Küng 2010) and from 2010 and 2011.

The water temperature at the spawning site of our study population has been continuously recorded at 1-min intervals since April 1970, and the timing of spawning is known for each year. We could, therefore, determine the average temperature that embryos and juveniles were exposed to at different developmental stages, here defined in intervals of 500 ATU (accumulated temperature units = degree days).

Wedekind and Küng (2010) found 2 major changes in the temperature environment of embryos and juveniles from 1971 to 2009. First, because of a temperature-linked shift in the timing of spawning and the fact that water temperatures rose more slowly in early spring than in late spring, embryos and hatchlings have been exposed to increasingly colder temperatures during their first 500 ATU. Second, summer temperatures (i.e., temperatures juveniles are exposed to during 1500–2000 ATU) have been increasing. Most grayling in our samples are 4, 5, or 6 years old (mean [SD] = 5.0 [1.2]) (Wedekind & Küng 2010). Therefore, we conducted a multiple regression

analysis (in JMP version 8.0.1; SAS Institute, Cary, NC) relating the OSR of each year to the average temperature embryos and juveniles experienced 5 years earlier during each of the 4 intervals of their first 2000 ATU of development (starting every year with the day when half the spawners of that year had been sampled). We also performed a forward stepwise regression predicting OSR on the basis of the average water temperatures of the current or the 6 previous years to evaluate the significance of the temperature during the first year of life compared with later years.

Because extraordinarily high temperatures were recorded in 2003 (Fig. 1b), we estimated the OSR of the 2003 cohort on 712 spawners that were sampled in 2008 and 2009 and aged on the basis of growth rings on scales. Age was estimated independently by 2 observers and analyzed separately per observer.

We determined the genotypes of 169 spawners collected in 2007 (Table 1) with polymerase chain reactions (PCR) to test whether genetic bottlenecks occurred in the past and to estimate the effective population size (N_e) of the population in 2007. Each PCR reaction (10 μL) was composed of 2.5 μL of DNA template extracted from fin samples with DNeasy Tissue Kit (Qiagen, Venio, NL), 1 × PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.5 μM

Table 1. Properties[a] of 9 microsatellite loci[b] used to estimate the genetic diversity in the study population of grayling in Lake Thun, Switzerland.

Locus	A	H_S	F_{IS}
SSOSL311	2	0.09	−0.05
BFRO005	2	0.31	−0.002
BFRO006	5	0.47	−0.07
BFRO0011	7	0.73	−0.02
Ogo2	8	0.43	−0.01
BFRO004	2	0.18	−0.05
BFRO0010	6	0.42	0.11
One9	4	0.37	0.07
Str73	7	0.79	0.26[c]

[a]*Abbreviations: A, number of alleles per locus; H_S, gene diversity; F_{IS}, inbreeding coefficient.*
[b]*See Gum et al. (2003) and references therein for details on the primers.*
[c]*Probability <0.05 as tested in FSTAT 2.9.3 (Goudet 1995) with 1000 permutations.*

Table 2. Results of multiple-regression analysis of the effects of average temperatures to which naturally spawned embryo and fry of graying were exposed on operational sex ratio recorded 5 years later.[a]

	Parameter estimate (SE)	t	p
Intercept	−0.07(0.32)	−0.21	0.84
0–500 ATU[b]	0.001(0.03)	0.05	0.96
500–1000 ATU	−0.01(0.02)	−0.66	0.52
1000–1500 ATU	−0.002(0.01)	−0.13	0.90
1500–2000 ATU	0.06(0.01)	4.26	<0.001

[a]*Full model: $F_{4,31} = 5.6$, p = 0.002.*
[b]*Developmental stages separated into consecutive periods of 500 accumulated thermal units (ATUs).*

of each primer, and 0.25 units of Taq DNA polymerase (Qiagen). We used the following PCR profile: initial denaturation at 94 °C for 3 min, 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature described in Gum et al. (2003), 30 s at 72 °C, and a final extension at 72 °C for 5 min. We ran PCR templates on an ABI 3100 automated DNA sequencer, used GENEMAPPER (Applied Biosystems, Carlsbad, CA) to score alleles, analyzed number of alleles and gene diversity (H_S) in FSTAT version 2.9.3 (Goudet 1995), and used BOTTLENECK (Piry et al. 1999) to test whether recent bottlenecks had occurred. We assumed a 2-phase mutation model with settings recommended by Piry et al. (1999) for microsatellite data (95% single-step mutations and 5% multiple-step mutations with variance among multiple steps = 12).

To estimate N_e we used a method that is based on linkage disequilibrium implemented in the LDNE (Waples & Do 2008). Because we did not sample all the cohorts from the population, we used only the individuals from the cohort with the greatest number of individuals (i.e., 5-year-olds, $n = 60$). This analysis provided an estimate of the effective number of male and female breeders (N_b) that we multiplied by the generation time (estimated as the average age of sampled fish [i.e., 4.97 years]) to calculate N_e (Fraser et al. 2007). We applied the rule proposed by Waples and Do (2010) when sample size is > 25 and did not consider alleles at frequency <0.02. We did not include the locus Str73 in these analyses because it significantly deviated from Hardy–Weinberg equilibrium (Table 1).

Results

Both OSR ($r_s = 0.62$, $p < 0.001$) and average spring and summer temperatures ($r_s = 0.65$, $p < 0.0001$) increased over the study period (Fig. 1a and b). The increase in OSR was correlated with the decline in the number of females ($r_s = -0.63$, $n = 58$, $p < 0.001$). The OSR was best explained by the average temperatures during the early developmental stages of these spawning fish (model including only mean temperature 5 years ago: $F_{1,34} = 32.7$, $p < 0.0001$; model including all factors: $F_{7,27} = 8.6$, $p < 0.0001$ [Fig. 1c]). Our OSR estimates of the 2003 cohort were 84.9% and 88.5% and corresponded to the OSR observed after 1992 (Fig. 1a). The average temperature juveniles experienced during 1500 and 2000 ATU (in summer) was the covariate most strongly associated with the OSR 5 years later (Table 2).

We identified genotypes of 168 individuals. Only one locus deviated significantly from Hardy–Weinberg equilibrium (Str73) (Table 1), probably due to the occurrence of null alleles. Average allele number was 4.78, and average gene diversity was 0.42 (Table 1). On the basis of Piry et al.'s (1999) criteria, we found no evidence of a significant recent bottleneck when we included or excluded locus Str73 from analyses. The N_b for 2007 was approximately 158.6 and $N_e = 788.2$.

Discussion

Male grayling stay longer at the spawning site than females and hence were more likely to be sampled. Therefore, OSR estimates of >0.5 can be expected even when sex ratios are equal. However, we found a distinct shift in water temperatures followed by high OSRs that persisted over 19 years (1993 to 2011). The ability of populations to genetically adapt to such unequal OSR depends on the selection coefficient, the population size and genetic variability, and the reproductive excess (e.g., number of eggs per female) (Hoffmann & Sgrò 2011). In grayling unequal OSRs are likely to cause strong selection and reproductive excess is large (thousands of eggs per female and year). The current genetic diversity of the study population is moderate but within the range of European grayling populations (Gum et al. 2003, 2009). The apparent lack of an evolutionary response to unequal OSR supports the view

that many salmonid populations may not be able to adapt to changing temperature (Fraser et al. 2007).

Consuegra and de Leaniz (2007) studied another salmonid and found significant variation in the sex ratios of populations that were in close geographic proximity. They suggest that spatial and temporal variation in OSR within populations may lead to sex-specific dispersal. However, connectivity between populations of many river-dwelling fish is low. This is also the case for our study population, which is largely separated from a neighboring population by a weir. Both the observed low N_e and low genetic exchange between populations may contribute to the observed lack of an evolutionary response to unequal OSR.

In fish OSR can be changed by temperature or toxicants that either affect sex determination or induce sex-biased mortality. Our study population shares its main feeding area, Lake Thun, with several whitefish (*Coregonus* sp.) populations that had an extraordinarily high prevalence of gonadal deformations in the early 2000s (Bernet et al. 2009). (So far, our study population of graylings has not been inspected for gonadal deformations.) Because the lake serves as a drinking-water reservoir for the region and cities downstream, several studies have focused on the occurrence and trends of potential endocrine disruptors in the water, sediments, and tissue of affected and unaffected whitefish (e.g., Liedtke et al. 2009; Bogdal et al. 2010). Contamination levels that have been found do not explain the malformations. Moreover, Bernet et al. (2009) raised whitefish from a population with gonadal deformation in spring water and on artificial food and found gonadal abnormalities similar to those observed in the wild. These findings suggest that toxicants are not responsible for the unequal OSR we observed.

The average water temperature at the outlet of Lake Thun (i.e., temperature embryos and juveniles are exposed to) has increased by about 1.5 °C from 1970 to 2011. Wedekind and Küng (2010) observed a shift to earlier spawning in the study population from 1948 to 2009. This shift has had several effects. First, it led to a decelerated temperature increase during the first 500 ATUs. Therefore, embryos and hatchlings are being exposed to steadily decreasing temperatures. At these life stages, salmonids are typically exposed to microbial pathogens and show behavioral and immunological reactions (Jacob et al. 2010) that are often temperature-dependent (C.W., unpublished results) and could be sex specific (Perry et al. 2005). This period also seems to be the time at which gonads go through the labile period of sex determination that can, in some salmonids, be affected by temperature (Baroiller et al. 2009; Magerhans et al. 2009). However, our results suggest that OSR is not significantly affected by environmental changes during these early developmental stages, and results of recent experiments suggest temperature does not affect sex determination in grayling (M.P. and C.W., unpublished results). Instead, in-

creasing summer temperatures seem to affect OSR, probably through sex-specific survival or sex-specific growth that influences survival later (Hutchings 2006). Not much is known about sex-specific behavior or growth and their potential temperature-dependence during early developmental stages, and it is unclear what kind of evolutionary response may arise as OSR changes. At least, the temperature effect does not seem to be linear because the extraordinarily warm summer in 2003 did not further skew the sex ratio.

Increased temperatures may contribute to the observed decline of many salmonid populations (Clews et al. 2010). Our findings suggest that sex-specific tolerance of high summer temperatures directly or indirectly accelerates extinction rates. The persistence of an unequal OSR suggests that probability of extirpation may be reduced by focusing on evolutionary processes in population management, for example, improving connectivity between populations.

Acknowledgments

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