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# The effect of a common micropollutant and hatchery stocking on fitness-related traits of brown trout

Palejowski Hugo

Palejowski Hugo, 2022, The effect of a common micropollutant and hatchery stocking on fitness-related traits of brown trout

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_B9121EB637C81

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Faculté de biologie
et de médecine

**Département d'écologie et évolution**

# The effect of a common micropollutant and hatchery stocking on fitness-related traits of brown trout

**Thèse de doctorat ès sciences de la vie (PhD)**

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

**Hugo Palejowski**

Master of Science in Biology, Uppsala Universitet (Sweden)

**Jury**

Prof. Richard Benton, Président
Prof. Claus Wedekind, Directeur de thèse
Prof. Michael Møller Hansen, Expert
Prof. Tadeusz Kawecki, Expert

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UNIL | Université de Lausanne

Faculté de biologie
et de médecine

**Ecole Doctorale**

**Doctorat ès sciences de la vie**

# Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

| | | | | |
|---|---|---|---|---|
| **Président·e** | Monsieur | Prof. | Richard | **Benton** |
| **Directeur·trice de thèse** | Monsieur | Prof. | Claus | **Wedekind** |
| **Expert·e·s** | Monsieur | Prof. | Michael | **Møller Hansen** |
| | Monsieur | Prof. | Tadeusz | **Kawecki** |

le Conseil de Faculté autorise l'impression de la thèse de

**Hugo Palejowski**

Master of Science in Biology, Uppsala Universitet, Suède

intitulée

**The effect of a common micropollutant and hatchery stocking on fitness of Swiss brown trout**

Lausanne, le 3 février 2023

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Richard Benton

# Acknowledgements

Firstly, I would like to thank Claus. Thank you for giving me the opportunity to do this PhD and for pushing me when I needed pushing. Despite some ups and downs I have thoroughly enjoyed the PhD and I'm extremely glad that I applied for this position and extremely thankful that you saw potential in me. Thanks also to all members of group Wedekind, for the continued experimental, statistical, and psychological support. Specifically, thanks to Christian de Guttry for your patience with me in the lab, when libraries weren't working for two years, thanks to Giulia Perroud for always being a fun office mate, thanks to Jonas Bylemans for patiently answering every single statistics- and R-related question I could think of, and thanks to David Nusbaumer for your continued support in all topics long after he had to. Field days involving 5am wakeups and working as it snowed around us that could have been horrendous under other situations were always fun thanks to the group dynamic. Thanks also to Eliane Longange, who suffered through my terrible French for a year and who really helped me learn it, and to all technicians in the Department who endured my constant questioning.

Thank you to all the people I have met during my PhD, both those in the department and outside of it. Your friendship and a steady stream of snowboarding, sauna, forest, and beach trips got me through the worst times of the PhD. I have made amazing memories from trudging through knee-deep snow on the way to a cabin in the forest that might not be there, to lying in a gently-bubbling heated pool gazing out onto a Ticinese lake. Thank you also to the Wine & Dine society for their continued support over the years. Thank you all for keeping me going.

A special thank you goes to my family. Thank you for all you have done over the decades that made it possible for me to get to this point. Thank you for giving me the fascination with travel that pushed me to move abroad in the first place, without which I wouldn't be the same person I am today. Thank you for everything.

A final thank you goes to Dr. Colin Pearson, my biology teacher at secondary school. You once told me that I should stick with education as long as I could, and should only stop once I

really could not go any further. I have thought about those words a lot over the last decade. Every time I have they have pushed me to continue, to keep travelling and learning. Dr. Pearson, I think that after 10 years of university it's finally time to stop.

# Abstract

Salmonids are a group of charismatic, socioeconomically and ecologically important fishes that are suffering widespread, multifactorial, population declines. In Switzerland, brown trout (*Salmo trutta*) populations have fallen by 80% since the 1980s: disease and poor habitat quality have been proposed as drivers. To combat declines hatchery stocking is used globally, but awareness of negative effects on the target population is growing. In this thesis we studied the trout populations of the river Aare catchment, which have been stocked and exposed to urban pollutants for decades. In a series of experiments, we tested the effect of environmentally relevant diclofenac, a common pharmaceutical micropollutant, on larval and juvenile trout, then for interactions between it and proliferative kidney disease (PKD), a putative driver of Swiss trout declines, in juveniles. Next, we assessed the effect of stocking on sex-specific life history strategies in juveniles, which have recently been implicated in declines of a grayling (*Thymallus thymallus*) population, then investigated hatchery-induced inbreeding and inbreeding depression. To better understand larval development in salmonids, we also modelled growth until the end of the larval period in trout and grayling.

We found evidence for precocious hatching in trout, a common stress response in fish and amphibians. We concluded that rapid evolution to diclofenac-induced stresses is unlikely in our study population. We also showed that diclofenac can increase measures of PKD severity, but also alleviates some negative physiological effects of PKD, highlighting that interactions between stressors demand greater attention. Diclofenac pollution may therefore be contributing to trout declines. We demonstrated that hatchery breeding affects juvenile sex-specific life history strategies and may produce highly inbred fish that are more susceptible to disease. This suggests a second possible effect on PKD virulence; however, this should be confirmed in future experiments. Finally, we showed that both studied salmonids show logarithmic-shaped larval growth, and published a tool to predict larval development in grayling. A better understanding of the interplay between diclofenac, PKD, and hatchery stocking, especially in early life stages, in observed declines is crucial to make informed decisions in Swiss brown trout management.

# Résumé

Les salmonidés sont un groupe de poissons charismatiques, importants sur les plans socio-économique et écologique, dont les populations connaissent un déclin généralisé et multifactoriel. En Suisse, les populations de truites brunes (*Salmo trutta*) ont chuté de 80 % depuis les années 1980 ; la mauvaise qualité de l'habitat et les maladies ont été proposées comme facteurs de déclin. Pour lutter contre cet affaiblissement, un alevinage massif en écloserie est utilisé. Cependant, les effets négatifs de cette pratique sur les populations cibles sont de plus en plus visibles. Dans cette thèse, nous avons étudié les populations de truites brunes du bassin versant de la rivière Aare, provenant d'écloseries, qui ont été exposées à des polluants urbains pendant plusieurs décennies. Dans une première série d'expériences, nous avons testé l'effet du diclofénac, un micropolluant pharmaceutique commun, sur les truites larvaires et juvéniles, puis les interactions entre ce produit et la Maladie Rénale Proliférative (MRP), un facteur putatif du déclin de la truite juvénile suisses. Ensuite, nous avons évalué l'effet de l'alevinage sur les stratégies d'histoire de vie spécifiques au sexe chez les juvéniles, qui ont récemment été impliquées dans le déclin d'une population d'ombre (*Thymallus thymallus*), puis nous avons étudié la consanguinité et la dépression de consanguinité induites par les écloseries. Enfin, pour mieux comprendre le développement larvaire chez les salmonidés, nous avons également modélisé la croissance de la truite brune et l'ombre jusqu'à la fin de la période larvaire.

Nous avons trouvé des preuves d'éclosion précoce chez la truite, une réponse au stress commune chez les poissons et les amphibiens. Nous avons conclu qu'une évolution rapide aux stress induits par le diclofénac est peu probable dans notre population d'étude. Nous avons également montré que le diclofénac peut augmenter les mesures de la sévérité de la MRP, mais aussi atténuer certains effets physiologiques négatifs de la MRP, soulignant que les interactions entre les facteurs de stress exigent une plus grande attention. La pollution par le diclofénac pourrait donc contribuer au déclin des truites. Nous avons démontré que la reproduction en écloserie affecte les stratégies d'histoire de vie spécifiques au sexe des juvéniles et peut produire des poissons

hautement consanguins qui sont plus sensibles aux maladies. Ceci suggère un second effet possible sur la virulence de la MRP ; cependant, ceci devrait être confirmé par des expériences futures. Enfin, nous avons montré que les deux salmonidés étudiés présentent une croissance larvaire de forme logarithmique, et publié un outil permettant de prédire le développement larvaire chez l'ombre. Une meilleure compréhension de l'interaction entre le diclofénac, la MRP et l'ensemencement en écloserie, en particulier dans les premiers stades de vie, dans les déclins observés est cruciale pour prendre des décisions éclairées dans la gestion de la truite brune en Suisse.

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# General Introduction

Salmonids are a well-studied group of culturally, socially, and economically important fishes that are native to the northern hemisphere (Fraser, 2008; Bottom et al., 2009; Fraser et al., 2011). Estimates vary but there are currently thought to be 220 species in 3 main subfamilies: Salmoninae, Coregoninae, and Thymallinae. They display extreme within-species diversity. Many species show diverse reproductive strategies, involving dominant males that compete for access to females and subdominant sneaker males that parasitize reproductive opportunities (Taborsky, 1994; Fleming, 1996). Most spectacularly, many species show diverse migration strategies, involving life history decisions over whether to migrate, to where, and when (Cucherousset et al., 2005; Strange, 2012; Ferguson et al., 2019). Because of their socio-economic importance and their fascinating intra- and inter-species diversity salmonids are now the focus of extensive research efforts worldwide.

Like many other species in the current biodiversity crisis salmonids are declining across their range (Burkhardt-Holm et al., 2002; Bottom et al., 2009; Limburg and Waldman, 2009; Wedekind et al., 2022). This has led to the loss of many local populations, and overall reductions in genetic diversity. These declines are multifactorial, species- and population-specific, and not fully understood. However, most involve overharvesting and various site-specific forms of habitat degradation such as pollution, logging, river damming, and urbanisation (Myers et al., 2004; Limburg and Waldman, 2009; Parrish et al., 2011). In many places the declines have been so severe that recolonization of habitats via dispersal is difficult or impossible (O'Reilly and Doyle, 2007).

**Salmonids in Switzerland**

Switzerland hosts four genera of salmonids and similar population declines are seen here as internationally (Burkhardt-Holm et al., 2002; Page, 2008). Most populations of salmonids in Switzerland were affected by the eutrophication crisis of the 1950s to 70s that caused bottlenecks in European freshwater fish populations (Johnson and Sumpter, 2014). Many have not fully recovered since then. Largescale restocking efforts to support populations started during this time;

however, despite these efforts some populations are still in decline. The reduction of salmonid populations is problematic for a number of reasons. Firstly, it indicates a loss of biodiversity, and therefore a loss of genetic diversity. Preserving genetic diversity within species is a key aim of modern conservation biology (Moritz, 2002; Frankham, 2005) as it is thought to i) maximise the potential for evolution in the face of environmental change, ii) provide the raw material that natural selection acts upon to generate diversification, and iii) influence both community species richness and ecosystem recovery after disturbance (reviewed in Frankham, 2005). Conversely, studies suggest that the loss of genetic diversity reinforces demographic or environmental processes that together can drive populations or species to extinction (Spielman et al., 2004). Secondly, a reduction in salmonid populations represents a departure from the intact environment decreed by Swiss law (Federal Parliament of Switzerland, 1991). Thirdly, it reduces the capacity of salmonids to act as bio-indicators of human health risks from toxic substances. This is especially true in Switzerland where drinking water is often produced from riverbank filtrate and more commonly from groundwater. Fourthly, proceeds from the annual sale of approximately 100 thousand fishing licenses are used for the maintenance of waterways in Switzerland (OFEV, 2022). If salmonid declines reduce fishing success and thus drive a decline in the sale of licenses this would reduce funding for river management. The health of salmonid populations is therefore a question of concern in Switzerland.

**Brown trout in Switzerland**

One species that has seen notable declines in Switzerland is brown trout (*Salmo trutta*) (Figure 1). As with other salmonids brown trout are highly charismatic and support fishery, restaurant, and grocery industries (Elliott, 1989; Liu et al., 2019). They are present across Switzerland and inhabit a range of freshwater habitats from lowland streams and lakes to alpine rivers (Klemetsen et al., 2003; Page, 2008). They have great ecological importance as they are a keystone species in their habitat and often a bioindicator of ecosystem quality. Even among

salmonids brown trout show remarkable diversity in life history. The species is partially migratory and shows a wide range of migration strategies, occurring as resident (no migration), fluvial-adfluvial potamodromous (downstream-upstream migration within a river system), lacustrine-adfluvial or allacustrine potamodromous (migration to and from a lake), and anadromous (migration to and from the sea) life history strategies. Some of these strategies are morphologically distinct and visually recognisable, these are referred to as "morphs". Worldwide the species is of least concern (Freyhof, 2011) but conservation status of the life history strategies vary from potentially to strongly endangered. In Switzerland, some morphs are increasing in rarity, and the anadromous morph is now extinct due to barriers that prevent migration to the sea.

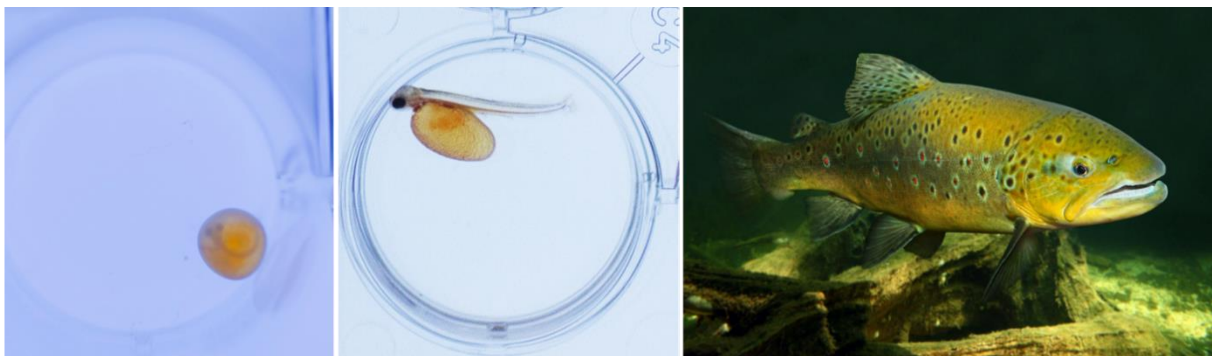

**Figure 1.** Three life stages of brown trout: embryo (left), larvae (centre), adult (right). Photos by or owned by Hugo Palejowski.

### Swiss brown trout decline

Brown trout populations are declining across alpine regions (Burkhardt-Holm et al., 2005; Lahnsteiner et al., 2009). In Switzerland research suggest that this decline has resulted in a loss of up to 80% of the trout population since the 1980s (Burkhardt-Holm et al., 2005; OFEV, 2022). A nation-wide project assessing evidence for possible causes of the observed decline identified two key factors of national or regional importance: the parasitic disease proliferative kidney disease (PKD), and habitat quality (Burkhardt-Holm et al., 2005).

#### *Proliferative kidney disease*

PKD is an emerging parasitic disease resulting from infection by the Myxozoan parasite *Tetracapsuloides bryosalmonae* (Hedrick et al., 1993). *T. bryosalmonae* spores infect the fish via the gills, then travel to the kidney and mature as sporongenic stages. Infection causes histopathological changes in the kidney structure leading to the deterioration of renal tubules and reduction in kidney function (Bailey et al., 2020). This is visible as inflammation and swelling of the kidney (Figure 2). A survey of Swiss rivers found PKD in river trout at 190 of 462 studied sites, mainly in the more densely-populated Swiss plateau (Wahli et al., 2002; Burkhardt-Holm et al., 2005). It's occurrence correlates with reported reductions in trout catch (Fischnetz, 2004). Thus PKD is now considered a potential major driver of the decline of brown trout in Switzerland (Fischnetz, 2004). Evidence for temperature-dependent virulence is now strong with increased mortality above 15°C (Okamura et al., 2011). River temperatures have risen since the 1980s and are predicted to rise further, thus the number of rivers and lakes that exceed this threshold temperature has already increased and will continue increasing (Fischnetz, 2004; Michel et al., 2022). However, stressors do not affect populations singly, and the impact of temperature on PKD virulence highlights that interactive effects between stressors are common. The extent to which other stressors interact with PKD in the wild is currently unknown.

**Figure 2.** Kidney in 0+ brown trout (A) uninfected and (B) infected with Tetracapsuloides bryosalmonae 85 days post-infection after other internal organs have been removed. Inflammation of renal tissue is visible in (B) as the kidney is swollen and turgid. Photos by Hugo Palejowski.

*Habitat quality*

Trout need natural or near-natural conditions to thrive, a far cry from the highly urbanised and canalised lowland rivers in much of Europe. Urbanisation and loss of natural river banks and overhanging vegetation reduces the availability of trout food and shelter. This also removes the buffer zone protecting the river from runoff, fine sediment (which clog streambeds and prevent trout from laying their eggs in the gravel), and leaching agricultural chemicals. Highly connected rivers would allow dispersal from population sources to sinks and thereby the recolonization of extirpated populations, but in Switzerland up to 2.5 barriers to fish dispersal exist per kilometre (Zeh-Weissmann et al., 2009). This has population-level consequences as river connectivity positively correlates with trout biomass (Burkhardt-Holm et al., 2005).

Water quality is an important component of habitat quality in aquatic ecosystems. Anthropogenic pollution into the freshwater environment has been substantially reduced since the

highs of the eutrophication crisis of the 1970s. However, waste water treatment plants frequently eliminate urban pollutants such as nutrients and synthetic chemicals incompletely (Johnson et al., 2013), and agricultural (Nusbaumer et al., 2021) and industrial (Tarazona and Munoz, 2008) pollutants are still prevalent. Therefore, pollution still enters surface waters. Both synthetic and organic chemical pollutants place important pressures on natural environments (Malaj et al., 2014; Bernhardt et al., 2017), and diluted wastewater inputs are a contributing factor to trout declines in Swiss rivers (Borsuk et al., 2006). As an example, endocrine disrupting chemicals are major pollutants of conservation concern in freshwater environments (Corcoran et al., 2010; Johnson and Sumpter, 2014). These have been shown to impact fitness Swiss salmonids (Marques da Cunha et al., 2019a, 2019b). There is extensive literature assessing the effects of various common micropollutants on freshwater species, however the majority tests the effect of acute (or at most subchronic) exposures on adults. Such studies therefore reveal little about the effects of chronic exposure on early life stages. Such exposures are highly ecologically relevant and may give distinct results to studies on adults due to embryos developing in a different chemical environment. Early life stages may also be more sensitive to stressors (Mohammed, 2013). Furthermore, traditionally stressors have been tested singly, ignoring interactive effects between them which are known to occur (e.g. Arkoosh et al., 1998; Tracy et al., 2020; Wu et al., 2022). A much greater focus of study on early life stages, chronic exposures, and interacting stressors is therefore needed to reliably inform management decisions.

**Hatchery breeding as a conservation tool**

Hatchery breeding and stocking is used internationally to prevent fish declines and support existing populations, usually with the aim of increasing the size of the naturally-reproducing wild population. In Europe, Cooke and Cowx (2006) report that some 40 billion fish are stocked into the freshwater environment annually. This practice has a long history. The possibility of artificial breeding in salmonids was first reported by Jacobi in 1762 (Birkhead and Montgomerie, 2009), and

large-scale hatchery breeding of salmonids is now over a century old (Hard et al., 1992; Lichatowich, 1999). In the present day salmonids are one of the most intensely bred species in hatchery programs (Lackey et al., 2006; Williams, 2006), including in Switzerland where over 160 million salmonids were stocked into lakes and rivers in 2019. Of these over 10 million were brown trout (OFEV, 2022). In tandem with habitat restoration and harvest quotas, hatchery breeding is therefore a key conservation tool used to conserve brown trout populations in Switzerland.

Despite having been intensely stocked for over a century brown trout populations in Switzerland are still declining (Burkhardt-Holm et al., 2005; OFEV, 2022). This raises the question of whether hatchery breeding has helped Swiss brown trout or not. Traditionally the objective of hatchery breeding has been to maximise census size. However, in recent years there has been growing concern over the potential genetic impacts stocking may be having on stocked populations (Fisch et al., 2015). If populations are shrinking and being extirpated despite widespread hatchery breeding, it is possible that management practices implemented as a conservation measure may actually be accelerating these declines. Theoretical studies suggest that captive-reared organisms can be genetically inferior to wild-reared counterparts, or may negatively affect wild populations in other ways (Ryman and Laikre, 1991; Lynch and O'Hely, 2001; Ford, 2002). Releasing these hatchery-reared individuals into the wild could therefore harm the recovery of wild populations. Three possible mechanisms through which this may function are discussed below.

*Introgression of non-local alleles*

Stocking a population with allochthonous (i.e. non-local) fish can drive the introgression of non-local alleles. This can introduce alleles maladaptive for the local environment into the gene pool, which spread into the wider population as stocked fish breed, and can cause the loss of biodiversity through the homogenisation of genetic differentiation between the populations (Araki and Schmid, 2010; Lamaze et al., 2012). The stocking with allochthonous fish is prohibited in most countries, and has been banned in Switzerland since 1991 by Federal law. Nonetheless, methods

vary between the Cantons (states) of Switzerland and trout stocking between populations and sometimes between river catchments still occurs. This may affect patterns of genetic diversity in modern Swiss salmonid populations.

*Domestication selection*

Hatchery-reared individuals are commonly found to have lower fitness than wild conspecifics (Araki et al., 2008). This phenomenon is referred to as domestication selection. Domestication selection could occur due to relaxed selection pressures in captivity allowing low-fitness phenotypes to avoid purging from the gene pool (McDermid et al., 2011). Alternatively, selection in an artificial environment (i.e. the hatchery) may produce phenotypes with low fitness in the wild, especially if a population is held in the hatchery for multiple generations (Frankham, 2008). Hatchery-reared offspring are then raised and released at a point where they have high survival rates in the wild. When they breed their maladaptive alleles spread amongst the wild population, lowering the average fitness. While most studies investigating the molecular basis for domestication selection have focused on finding evidence for selection at the genome level (Mäkinen et al., 2015; Christie et al., 2016), recent research suggests that there may be an additional epigenomic basis (le Luyer et al., 2017; Rodriguez Barreto et al., 2019). This may explain why changes in fitness have been observed in a single generation (Araki et al., 2007). Despite efforts taken to minimise the effects of domestication selection, including holding breeders for only one generation (Frankham, 2008), they can be reduced but never removed entirely (Fraser, 2008).

*Reduction of effective population size*

Hatchery facilities may inadvertently reduce the effective size of a wild population, defined as the size of an ideal model population that loses genetic variability at the same rate as the observed population, in several ways. Firstly, standard practice in hatchery facilities has traditionally been to use a limited number of individuals with favourable characteristics, frequently fewer than 50, to produce thousands of offspring (Wedekind, 2002; Fraser, 2008). Use of small breeding populations

may be due to restricted space to house larger populations, a limited number of breeders in the wild population, or the fact that female fecundity is size-linked therefore few breeders are required to produce many eggs (Einum and Fleming, 1999). High survival in hatchery facilities means that hatchery-bred offspring will make up a disproportionately large fraction of the parents of the next generation, reducing the effective population size (Ryman and Laikre, 1991). Secondly, the sexes can show drastic biological differences (Magurran and Garcia, 2000; Maitre et al., 2017; Grilo et al., 2018) even at very early developmental stages (Mousavi et al., 2021). Sex-specific selection pressures may thus act prior to release of hatchery offspring (Bolund et al., 2013; Forsman, 2018) creating biased sex ratios in the stocked population and a reduction in the effective population size. Thirdly, body size is known to correlate with fitness in salmonids (Hutchings, 1991; Einum and Fleming, 1999; Orpwood et al., 2003). If breeder selection is influenced by body size the high heritability of size may mean that closely-related individuals are selected as breeders which can directly induce inbreeding in the population which reduces effective population size (Crow and Kimura, 2017). This list is non-exhaustive but highlights three mechanisms through which Swiss hatchery stocking programs may be reducing effective population sizes in wild populations. Whatever the origin, reduced effective population sizes increase average inbreeding coefficients (Crow and Kimura, 2017) thereby increasing the risk of inbreeding depression (Charlesworth and Charlesworth, 1987), and intensify the rate of loss of genetic diversity due to genetic drift (Keller and Waller, 2002). Lower effective population sizes also reduce the effectiveness of natural selection (Frankham et al., 2002), and reduce population viability thus increasing extinction risk (Donald, 2007; Geffroy and Wedekind, 2020).

*Success of hatchery breeding*

After nearly a century of discussion, the debate over the effectiveness of stock enhancement programs has not been concluded (Needham and Slater, 1944; Greene, 1952; Rytwinski et al., 2021). Claussen and Philipp (2022) highlight that while census size may increase, decades of

research has failed to show that stocking gives a demographic boost to a self-supporting target population. A recent review by Rytwinski et al (2021) concludes that fundamentally the quality of evidence is not sufficient to determine whether hatchery breeding programs help or not. Fraser (2008b), however, argues that the weight of evidence demonstrates that there is at least the potential for such programs to induce negative effects in supported wild populations in as little as a single generation, and stresses that we probably cannot detect these until considerable damage has been done. Hatchery breeding and stocking may therefore be contributing to salmonid declines internationally, but more primary evidence is needed. The intensity of the effects on target populations likely depends on the management practices used in each program (Fraser, 2008). For example, supportive breeding is strongly believed to have fewer negative effects than captive breeding. In supportive breeding programs adults are collected from the target population (usually every year), artificially bred to create thousands of offspring, then released back into the population followed by the offspring when they are sufficiently developed to have high survival in the wild. By comparison captive breeding involves holding a breeding population in the hatchery for multiple generations. In reality the distinction is not as clearly defined and there is a continuum along which all hatchery breeding programs will fall, while programs within these categories may vary in methods and aims (Fraser, 2008). Site-specific evaluations of the effect of hatchery breeding programs are thus called for.

**Thesis outline**

In this thesis we address the topic of brown trout declines in Switzerland. We use the well-studied population of the catchment of the river Aare between Lake Thun and the city of Bern (Stelkens et al., 2012; Marques da Cunha et al., 2019b). Stocking activities in this population have been recorded since the 1980s but certainly occurred before that. This hatchery program uses typical supportive breeding measures. New breeders are collected via electrofishing every season during the migration, are held in the hatchery facility until ready to spawn, are artificially bred then

released back into their origin population, and the offspring are later released into the same population when developed enough to have high survival. In this population we investigate the effect of two drivers previously identified as likely involved in Switzerland-wide decline of trout populations. We then assess whether hatchery breeding, ostensibly meant to maintain the population, may be contributing to it in two different ways. A better understanding of the causes of the decline and the effects of the conservation measures that have been applied will allow fisheries managers to make more informed decisions regarding the management of this population and others.

Despite salmonids being the focus of intensive research internationally some basic elements of their biology are still poorly understood. Larval growth patterns are one such topic, despite the fact that larval growth rates are comparatively well-studied. In chapter I we modelled growth patterns of grayling *Thymallus thymallus*, a close relative of brown trout, until the end of their yolk sac period when, in the wild, they would emerge from the sediment salmonid eggs had been laid into. We developed a model that allows us to predict timing of and size at emergence, two highly fitness-linked traits. We then applied the model to test how a common bacterial infection affects grayling growth patterns in very early life stages. This chapter develops a modelling tool that can be used by other researchers to study growth patterns in other grayling, or salmonid, populations.

In chapter II we study how one aspect of habitat degradation, pollution by a common micropollutant, affects brown trout early-stage fitness. We assess effects of environmentally relevant concentrations of diclofenac on six traits in embryonic trout produced via full factorial breeding from wild breeders. We also model growth patterns until emergence using the modelling approach developed in chapter I. In order to assess whether trout in these populations may be able to rapidly adapt to diclofenac stress we test for adaptive genetic variance for tolerance to it.

Chapter III extends our investigation of the effects of diclofenac on trout by investigating whether this pollutant affects the virulence of proliferative kidney disease (PKD). PKD is a previously-identified driver of the decline in Swiss brown trout populations whose virulence is

known to be dependent on environmental conditions. We exposed hatchery-bred brown trout, the F1 offspring of wild-caught breeders, to test the effect of diclofenac and PKD on juvenile brown trout in a full-factorial experiment.

In order to support declining trout populations hatchery breeding is employed extensively in Switzerland and has been for at least 40 generations. In chapter IV we assess whether such activities may be creating a biased sex ratio in the stocked population which may have consequences for population viability. We employ both a large-scale field experiment and controlled rearing of juveniles under laboratory conditions to test for sex-specific growth and survival under a range of environmental conditions.

Demographic changes are only one way hatchery breeding could be disrupting wild populations. In Chapter V we continue our investigation of the effects of hatcheries by assessing evidence of inbreeding and inbreeding depression in a population of hatchery-bred brown trout. These are the F1 offspring of wild breeders collected from a population that has been stocked for many generations.

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# Contribution to chapters

I contributed to the experimental design of chapters II-III-IV-V. I contributed to the breeding of fish, and generation of the dataset, in chapters I-II-V, in the 2018 experiment in chapter III, and in the laboratory experiment in chapter IV. I raised the fish in chapters II-V, in the 2018 experiment in chapter III, and in the laboratory experiment in chapter IV. I performed the molecular laboratory work in chapters III-V and in the laboratory experiment in chapter IV. I performed statistical analyses for all chapters, and contributed to the interpretation of all results. I contributed to the development and publishing of the modelling tool in chapter I. I wrote the first draft, and contributed to the final draft, of all chapters.

# Chapter I - Predicting the end of the larval period in European grayling (*Thymallus thymallus*)

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Chapter status: unpublished

**Author contributions**

LG and CW designed and organised the fieldwork for fertilisations and breeding, with assistance from HP. LG reared and photographed the fish. HP, SEO, and MM developed and implemented the modelling approach. HP and MM prepared and published the open source code. HP and CW wrote the manuscript with input from all authors, who approved it before publishing.

**Abstract**

Due to the socioeconomic, ecological, and scientific importance of salmonids their development is well-studied. However, development patterns at very early life stages, and the effect of family and disease on these, remain unexplored, despite the critical importance of these stages for survival. Here we report an experiment where we raised European grayling *Thymallus thymallus* to the end of the yolk sac stage and modelled their patterns of yolk consumption and growth. We show that yolk consumption is approximately linear, while growth is logarithmic, and that infection by the bacterium *Pseudomonas fluorescens* significantly delays yolk consumption patterns thus reducing length throughout the larval period. Families show different yolk consumption and growth patterns, and different growth responses to *P. fluorescens* infection. We develop models to predict the end of the larval stage and length at this point in grayling, which are made available for use and development by future researchers. Our results highlight that care should be taken when assuming linear growth patterns in juvenile salmonids, and we stress that more research on salmonid and other freshwater fishes' early-stage development patterns is needed.

**Introduction**

In recent years Salmonid fishes have become model organisms for questions on a wide range of research topics (Nusbaumer et al., 2019; Pompini et al., 2013; von Siebenthal et al., 2009), but despite their socioeconomic, ecological, and scientific importance some basic elements of their biology remain unknown. One example is that the shape of the curves (hereafter referred to as the pattern) of salmonid growth and yolk sac consumption at juvenile stages are still unexplored, despite the transition from larval (here defined as from hatching to the end of the yolk sac period) to exogenously-feeding juvenile stages being considered one of the most crucial phases in the life of a fish (Rønnestad et al., 1999). This prevents the development of models predicting when an individual will end the larval stage or its size at that point. It also means that we have little knowledge of how parental identity, disease, experimental treatments, or other manipulations affect these patterns. This hole in our knowledge of basic salmonid biology should be filled.

Growth in fish has been studied for years (Hopkins, 1992) but the precise shape of curves that different species take has received less attention. Highly-cited papers maintain that growth (here defined as increase in length over time) follows logarithmic trends (Hopkins, 1992), and this is believed to be the case in salmonids as well. However, logarithmic growth may not be universally true (Vasbinder & Ainsworth, 2020) and is a generalisation that may overlook other patterns that might occur at very early developmental stages. Studies frequently simplify growth to a linear relationship for ease of comparison (Imsland et al., 1996; McCormick & Hoey, 2004; Nusbaumer et al., 2019; Thia et al., 2018) but doing so ignores all intermediate growth patterns which may be non-linear. If growth is not assumed to be linear then it is frequently modelled for inter-population comparisons of growth patterns with models such as the von Bertalanffy and Gompertz models (Hopkins, 1992; Kamler, 2008; Lugert et al., 2016). However, such methods focus on mean population growth patterns and ignore sources of variation within that population. The study of patterns of growth, and sources of intra-population variation in those patterns, is therefore underdeveloped.

Patterns of growth and yolk sac consumption (hereafter collectively referred to as patterns of development) in larval stages of salmonid fishes are especially significant due to the critical importance of this time for survival. Salmonid eggs are laid in the hyporheic zone, the sediment layer at the bottom of water bodies, where they develop before emerging shortly before the end of the larval period to become free-swimming and exogenously-feeding. Their ability to swim is hindered by their external yolk sac (Scott, 1985). Thus how quickly they can end the yolk sac stage is highly fitness-linked in many species, as is their body size at the end of the larval stage, resulting in faster development and larger size increasing survival immediately following emergence (Beacham et al., 1988; Brannas, 1995; Einum & Fleming, 2000; Mason & Chapman, 2011; Pine & Allen, 2001). Development speed's effect on fitness could be due to territoriality (Grant & Kramer, 1990), with fast development allowing the earlier establishment of feeding territories (Einum & Fleming, 2000). Body size's effect could be due to larger size giving greater swimming ability especially against otherwise-fatal high stream flow (R. A. Clark, 1992; Harvey, 1987). Alternatively, larger size could reduce predation which is frequently size-selective (Anderson, 1988; Bailey & Houde, 1989; Cushing, 1990; Miller et al., 1988; Raventos & Macpherson, 2005); however see (Litvak & Leggett 1992; Pepin et al. 1992; Meekan & Fortier 1996; Dibattista et al. 2007; Slotte et al. 2019). The crucial importance for survival of this stage makes a better understanding of larval fish development, and the factors influencing it, imperative.

In this study we thus investigate developmental patterns at early stages in a salmonid, and explore how disease and parental identity affect these patterns in order to better understand what influences passage from larval to the juvenile stage. To do this we use the European grayling *Thymallus thymallus* as a model species. Grayling are highly charismatic and socioeconomically important in Europe, and have recently become scientifically important (Maitre et al., 2017; Selmoni et al., 2019; Wedekind et al., 2013). The species is a good study model being a close relative to other highly important salmonids such as trouts and salmons, and is an external fertiliser that is easy to handle, breed, and rear. Globally the species is not at risk of extinction risk (categorised as

Least Concern by the Red List Index) but suffers population-level threats due to anthropogenic pressures (Augustyn & Nowak, 2014; Fjeld et al., 1998) causing population decline in Europe (Gum et al., 2009; Mueller et al., 2018; Weiss et al., 2013) including Switzerland (Wedekind & Küng, 2010). As a result, it is on the appendix III of the Bern Convention.

To investigate the effect of disease infection on Grayling development patterns we use *Pseudomonas fluorescens*, an opportunistic fish pathogen (Austin & Austin, 2016) which is one of the most frequently-diagnosed epizootic risk factors affecting fish populations around the world (Hoole et al., 2003; Schuwerack et al., 2001; Zhou et al., 2009). Fish larvae are very susceptible to bacterial infection (Ellis, 2005) and *P. fluorescens* has previously been found to have significant effects on salmonids in early life history stages (Pompini et al., 2013; von Siebenthal et al., 2009; Wedekind, 2002; Wedekind et al., 2004). Its widespread prevalence, and it's known effects on salmonids make it a good candidate to test how bacterial infection affects development.

We therefore report the results of a study where we modelled patterns of development in the larval stage European grayling, assessed the shape of these curves, then used the models to build tools predicting when an individual will end the larval period and its length at that point. With these we then test the effect of *Pseudomonas fluorescens* infection and parental identity on developmental patterns, and assess whether responses to infection in developmental curves are parent-specific.

**Methods**

*Parents' origin, fertilisations, and bacterial infection*

European grayling (*Thymallus thymallus*) were sampled from a captive breeding stock in Kandersteg, Switzerland that consists of the F1 of the population studied in Wedekind et al. (2013). On 17$^{th}$ April 2019, 8 males and 8 females were stripped for their gametes then returned to the breeding stock. Female gametes were collected in plastic trays while male gametes were collected by drops in 145mm petri dishes (Greiner Bio-One, Kremsmünster, Austria). These gametes were

then used for *in vitro* fertilisations, avoiding sperm drops contaminated with faeces or blood and pale-coloured eggs, in a 8 x 8 full factorial breeding design, to produce 64 sibgroups where all eggs from a given sibgroup were held together in a 145mm petri dish (Greiner Bio-One, Kremsmünster, Austria). Water used for fertilisation was chemically standardised (OECD Guidelines for Testing of Chemicals 203 (Fish, Acute Toxicity Test), 1992) and aerated prior to use. All standardised water used in this experiment followed this protocol. Fertilised eggs were left for 2 hours to harden then transported to a climate-controlled room at (5.9ºC, SD=±0.45ºC, range=3.6-6.9ºC) on a 12:12 light:dark cycle. They remained here until the end of the experiment. On the same day as fertilisation eggs were washed by sibgroup under tap water running at a flow rate of 4L/minute and 48 embryos per sibgroup were distributed singly to 24-well plates (Greiner Bio-One, Kremsmünster, Austria) filled with 1.8 mL standardised water in a block-wise design.

After 24 days post-fertilisation (142 degree days) embryos were treated with *P. fluorescens* (high virulence strain DSM 50090) that has been linked to mortality in other salmonid species (von Siebenthal et al., 2009), or a sham treatment (hereafter "control"). See Clark et al. (2013a) for growing, washing, and counting protocols for *P. fluorescens*. 200 µl of *P. fluorescens* solution was added to the wells in order to obtain a final concentration of $10^6$ CFU/mL in each well. 200 µL of autoclaved standardized water were added for sham treated embryos. Embryos from columns 1-3 on the 24-well plate were sham treated while embryos in columns 4-6 were inoculated with *P. fluorescens*, such that all sibgroups underwent both sham and *P. fluorescens* treatments equally.

*Embryo rearing, photographs, and data extraction*

Hatching occurred from 31 to 44 days post-fertilisation (183-260 degree days) and peaked at 34 days post-fertilisation (201 degree days). At hatching embryos were transferred to 12-well plates (CytoOne, Starlab, UK) organised by hatching date such that fish hatching on the same day occupied the same plate, and filled with 1.2ml autoclaved standardised water. Pictures were taken of each hatchling under standardised lighting and distance conditions using a custom-built

photobox and a Canon EOS 80D camera body with a Canon EF 50mm f/2.5 Compact Macro lens (Canon, Japan). Water volume of each well was then adjusted to 3 ml. Pictures were taken of each fish at 3, 5, and 7 days post-hatching, then regularly until the death of the hatchling.

A subset representing approximately 1/3 of all available fish was haphazardly chosen by selecting the first plate of fish that hatched on a given day and all subsequent plates that were multiples of 3. From these pictures data on fish length, yolk sac length, and yolk sac width was extracted using the open-access software ImageJ 1.53a (https://imagej.nih.gov/ij/). Fish length was measured as the most anterior point of the head to the most posterior point of the visible spine following the curve of the body. Yolk sac length was measured anterior to posterior, and yolk sac width was measured at the midpoint along that line. Yolk sac volume was estimated following Jensen et al. (2008) as $V=(\pi/6) \cdot L \cdot W^2$ where $L$ is yolk sac length and $W$ is yolk sac width. In total data was collected from 213 fish from 42 sibgroups, from both *P. fluorescens* and control treatments (n=114 and 99, respectively). 1962 length measurements were taken from all fish (range 1-14 measurements per individual, mean=10.7, median=11). 2004 yolk sac volume values were calculated from these individuals (range 3-16 per individual, mean=12.7, median=7). Hereafter this is referred to as the "whole dataset". The number of length and yolk sac volume measurements per individual varies due to the frequency with which an individual was photographed.

The whole dataset was subsetted to include only control individuals with more than 8 length measurements. This threshold was chosen as a trade-off between having many datapoints per individual in order to more accurately model growth of an individual, and having a sufficient number of individuals to representatively model growth in the population. This resulted in 80 individuals from 33 sibgroups with 837 length values (range 9-14 per individual, mean 11.4, median 11), and 855 yolk sac volume values (range 11-16 per individual, mean=13.4, median=13). Hereafter this is referred to as the "small" dataset. Length and yolk sac volume data from all individuals with more than 8 length measurements was visually checked for deviations from the trends for each individual and deviating measurements were remeasured. Remeasured values were

accepted if they differed from the first measurement by more than 0.5mm (length) or 0.5mm$^3$ (yolk sac volume) as this represents a change of approximately 10% of the range of length or yolk sac volume values for an individual. These datasets were then used for model building.

*Model fitting*

A visual diagram of model building, selection, generalisability testing, and prediction of timing of end of larval period and the size at this point is shown in Figure 1. All models were estimated and analysed in R version 4.0.2 (R Core Team, 2020). First, we used restricted maximum likelihood estimation to fit linear mixed-effects models of the pattern of yolk sac consumption via the lmer function in the *lme4* package (Bates et al., 2015). We used the number of days post-fertilisation as the response variable. We included the following explanatory variables: the number of days taken to hatch (hereafter "incubation time"), the length at hatching, the yolk sac volume at hatching, and the proportion of yolk sac remaining on a given day. Incubation time, length at hatching, and yolk sac volume at hatching are hereafter collectively referred to as "hatching characteristics". The given hatching characteristics are all believed to influence growth rates of juvenile fish (Yamagishi 1969; Phillips et al. 1995; Pine & Allen 2001; Gilbey et al. 2009; Iguchi 2011; but see Solberg et al., 2014). In addition, first order interactions between yolk sac volume at a given day and each hatching characteristic were included. Models were fitted iteratively via forward selection, incorporating the proportion of yolk sac volume remaining first as a linear predictor variable, then with the variable transformed via natural logarithm, then including polynomials of the natural logarithm-transformed variable up to 7$^{th}$ degree polynomials. The maximum polynomial degree was 6 and 4 for models of the whole and small datasets, respectively, as model fit declined above this (see Model Selection for the statistics used to evaluate goodness of fit).

Next, we used restricted maximum likelihood estimation to fit linear mixed-effects models of the pattern of growth via the lmer function again. We used length on a given number of days

post-fertilisation as the response variable. We included the following explanatory variables: the three hatching characteristics, and the number of days post-fertilisation. In addition, first order interactions between the number of days post-fertilisation and each hatching characteristic were included. Models were fitted iteratively, incorporating the number of days post-fertilisation first as a linear predictor variable, then with it transformed via natural logarithm, then including polynomials of the natural logarithm-transformed variable up to $6^{th}$ degree polynomials. The maximum polynomial degree was 3 and 4 for models of the whole and small datasets, respectively, as models failed to converge above this.

For models of both yolk sac consumption and growth individual identity was included as a random effect to account for the non-independence of samples due to the repeated measures design. Models were fitted with random slope, random intercept, or both to test which gave best model fit (see Model Selection for the statistics used to evaluate goodness of fit), and if necessary different nonlinear optimisers were tested to allow model fitting. Multicollinearity is not as relevant in predictive modelling (Shmueli 2010); nonetheless, selected models (see below) were checked for multicollinearity by calculating variation inflation factor (VIF) via the vif function from the *car* package (Fox & Weisberg, 2019). Only variables with VIF lower than 10 were included except in cases where high VIF was driven by collinearity between main effect variables and interaction terms containing those, or between variables and powers of those, checked with summary function (R Core Team, 2020). No variables were excluded following these criteria.

*Model selection*

As a purpose of this paper is to develop a suitable prediction model, the metrics chosen reflect prediction accuracy. To compare models we generated the following model metrics: conditional coefficient of determination ($R^2$), root mean standard error (RMSE), MAE (mean absolute error), and MAPE (mean absolute percentage error) (see Table S1 for description and functions used). Too strong a focus on these metrics in a given dataset can reduce predictive

accuracy and prevent generalisation of results, known as model overfitting. Therefore, we performed nested cross-validation which partitions a dataset to train a model on one partition then test it on a separate partition (Becciu et al., 2020; Pereira et al., 2009; Stone, 1974; Varoquaux et al., 2017), reducing the risk of overfitting. To do this I divided the data randomly into 80% training set and 20% testing set using the partition function from *groupdata2* package (Olsen, 2021b). Partitioning was done based on individuals' identity so that all data from a given individual was kept in the same partition thereby avoiding data leakage, when data from outside the training dataset is used to create the model (Elangovan et al., 2021; Samala et al., 2020). Partitioning of both length and yolk sac volume data was based on the individuals in the length dataset to avoid having non-identical lists of individuals in the training/testing sets in the models of yolk sac consumption and growth which could be considered data leakage. I then performed 4-fold cross-validation on the training set using a custom function modified from Olsen (2021b) and generated model metrics. This process randomly divides the training set into four quarters (again based on fish identity), trains the model on three quarters of the data in the training set, predicts the response variable using the predict function in the *stats* package (R Core Team, 2020), then compares how closely the predicted response matches the remaining quarter that was unused for model training and generates model metrics. This is repeated four times such that all quarters are used as the comparison set and four sets of model metrics are generated, which are then averaged and reported.

Cross-validation with small sample sizes can, however, lead to more uncertain results (Varoquaux, 2018), in which case model generalisation may be best estimated with the corrected Akaike's information criterion (AICc) (Burnham & Anderson, 2002; Hurvich & Tsai, 1993), a measure of out-of-sample prediction error which corrects for the tendency of AIC to overfit at small sample sizes (Claeskens & Hjort, 2008; McQuarrie & Tsai, 1998). Therefore, both cross-validation-generated model metrics and AICc were taken into account during model selection. AICc was calculated on all data, not following the 80:20 split described above, using the AICc function in the *MuMIn* package (Barton, 2020). Models were then selected based on highest $R^2$ and

AICc, and lowest RMSE, MAE, and MAPE values. Variable significance in selected models was then assessed using the summary function again, and non-significant variables were removed through stepwise backwards selection until removal of the least significant variable failed to improve model fit. This resulted in the final selected models of yolk sac consumption patterns, and of growth patterns.

*Testing model generalisability and predicting end of larval stage*

Model generalisability can be defined as how well the findings from a sample are applicable to other samples in a population (Song et al., 2021). Selected models' generalisability was assessed by training them on the full training set, predicting response variables, comparing these to the ground truth response variables in the testing set (containing randomly-selected 20% of the data which was not used for model training) and generating model metrics. However, because the sample size used for model testing is relatively small (20% of 80 individuals), its composition may influence the generated model metrics and give non-representative results. Therefore, we iterated this process by randomly selecting 100 training and testing sets, fitting the model to the training set and generating model metrics from the test set, and averaging the model metrics generated. Cross-validation and testing set model metrics were then compared to check for overfitting, which would be revealed by large differences.

Once models of yolk sac consumption and growth were selected, they were applied to predict the timing of the end of the larval period and length at this point for the 16 individuals in the testing set (Figure 1). Models of yolk sac consumption were used to predict when each individual would end the larval stage (defined as when each had consumed 80% of their yolk sac volume, discussed below), using the predict function in the *stats* package (R Core Team, 2020). Models of growth were then used to predict the length of each at this point.

*Effect of bacterial infection and parentage on development*

We then statistically tested the effect of *Pseudomonas fluorescens* infection on development patterns (i.e. both patterns of yolk sac consumption and growth) as a proof of concept for testing effect of variables. The whole dataset was filtered for all fish from both treatment groups with over 8 length measurements, resulting in 162 individuals from 40 sibgroups with 1692 length values (range 9-14 per individual, mean=11.4, median=11), and 1734 yolk sac volume values (range 11-16 per individual, mean=13.4, median=13). We then used likelihood ratio tests to test for effects of *P. fluorescens* infection. For each selected model the model's formula was fit to this dataset twice: first unaltered as a reference model, then again as a full model including *P. fluorescens* infection status in interaction with the three hatching characteristics and proportion of yolk sac volume remaining (in models of yolk sac consumption) or the number of days post-fertilisation (in models of growth). In this and all following likelihood ratio tests, we determined statistical significance differences between the models via the anova function in the *stats* package (R Core Team, 2020).

Next, we tested whether any differences in development patterns caused by *P. fluorescens* infection were driven by changes in slopes of development post-hatching. This was assessed by checking for an interaction between infection status and proportion of yolk sac volume remaining (in models of yolk sac consumption) or the number of days post-fertilisation (in models of growth) in the full models used in the likelihood ratio tests.

We tested whether infection status had caused differences in hatching characteristics. We fit linear models (lm function from *stats* package, (R Core Team 2020) with each of the three hatching characteristics as the response variable, with *P. fluorescens* infection status as the predictor variable. These models were fit to a dataset created by filtering the whole dataset for all individuals from both treatment groups with more than 8 length measurements. Significant effects of infection were assessed with the analysis of variance tables given by anova function.

We then tested the effect of parental identity on development using likelihood ratio tests. The dataset was filtered identically as in our tests of the effect of *P. fluorescens* infection on development patterns. For each selected model we then fitted the model formula to the dataset

twice, first unaltered as a reference model. The model was then fitted again as a full model including dam or sire identity (to test the effect of dam and sire, respectively, on development) in interaction with the three hatching characteristics and proportion of yolk sac volume remaining (in models of yolk sac consumption) or the number of days post-fertilisation (in models of growth). We then tested for statistically significant differences between the models. Inter-parentage differences in slope of development patterns were assessed by checking for significant interactions between dam or sire identity and proportion of yolk sac volume remaining (in models of yolk consumption) or the number of days post-fertilisation (in models of growth) in the full models. Whether parents showed differences in hatching characteristics was tested using linear models with each hatching characteristic as the response variable and dam or sire identity as the explanatory variable.

Finally, we tested whether parents show different developmental responses to infection by rebuilding the full models we used to assess effects of parental identity on development patterns. To these a three-way interaction term between dam identity, infection status, and proportion of yolk sac volume remaining (in models of yolk consumption) or number of days post-fertilisation (in models of growth) was added. Significance was assessed with analysis of variance tables via the anova function. In all analyses of effects of parentage offspring of two sires were removed from analyses as a low number of hatched offspring meant the experimental design was no longer factorial.

## Results

In all models the small dataset performed better than the whole dataset according to our model metrics. Thus all following models refer to this unless stated otherwise. Alpha value of 0.05 is used in all statistical analyses.

*Modelling yolk sac consumption*

Two models of yolk sac consumption patterns were selected based on model metrics, AICc and formula complexity. Model 1 is simpler but has slightly worse performance, while model 2 is more complex with slightly better performance (Table 1).

Model 1 gives the predictive function (1)

$$d(\rho, T, v) = \mu_1 + \beta_{T1} \cdot T + \beta_{v1} \cdot v + \beta_{\rho 1} \cdot \rho + \beta_{T \cdot \rho 1} \cdot T \cdot \rho + \beta_{v \cdot p1} \cdot v \cdot \rho \quad (1)$$

Where d is the number of days post-fertilisation at which a new fish will have proportion of yolk sac remaining $\rho$, $\mu_1$ is the intercept term in model 1, $\beta_{T1}$ is the regression coefficient of the number of days between fertilisation and incubation time in model 1, $\beta_{v1}$ is the regression coefficient of yolk sac volume at hatching of a new fish in model 1, $\beta_{T \cdot p1}$ is the regression coefficient of the interaction between incubation time and proportion of yolk sac volume remaining in model 1, $\beta_{v \cdot p1}$ is the regression coefficient of the interaction between yolk sac volume at hatching and proportion of yolk sac volume remaining in model 1, $\beta_{\rho 1}$ is the regression coefficient of the proportion of yolk sac volume remaining in model 1, and $T$ and $v$ are values measured from a new fish: respectively the incubation time and yolk sac volume at hatching (mm3).

Model 2 gives predictive function (2)

$$d(\rho, T, h, v) = \mu_2 + \beta_{T2} \cdot T + \beta_{h2} \cdot h + \beta_{v2} \cdot v + \beta_{T \cdot \log(\rho)2} \cdot T \cdot \log(\rho) + \beta_{v \cdot \log(p)2} \cdot v \cdot \log(\rho) + \beta_{\rho 2\alpha}(\log(\rho)) + \beta_{\rho 2\beta}(\log(\rho))^2 + \beta_{\rho 2\gamma}(\log(\rho))^3 + \beta_{\rho 2\delta}(\log(\rho))^4 \quad (2)$$

Where $d$ and $\rho$ are as in predictive function (1), $\mu_2$ is the intercept term, $\beta_{T2}$ is the regression coefficient of incubation time, $\beta_{h2}$ is the regression coefficient of length at hatching of a new fish, $\beta_{v2}$ is the regression coefficient of yolk sac volume at hatching of a new fish, $\beta_{T \cdot \log(p)2}$ is the regression coefficient of the interaction between incubation time and proportion of yolk sac volume remaining, $\beta_{v \cdot \log(p)2}$ is the regression coefficient of the interaction between yolk sac volume at hatching and proportion of yolk sac volume remaining, $\beta_{\rho 2\alpha}$ is the regression coefficient of the proportion of yolk sac volume remaining, $\beta_{\rho 2\beta}$ is the regression coefficient of the squared logarithm

of the proportion of yolk sac volume remaining, $\beta_{\rho2\gamma}$ is the regression coefficient of the cubed logarithm of the proportion of yolk sac volume remaining, $\beta_{\rho2\delta}$ is the regression coefficient of the fourth power logarithm of the proportion of yolk sac volume remaining, and $T$, $h$, and $v$ are values measured from a new fish: respectively the incubation time, length at hatching (mm), and yolk sac volume at hatching (mm3). See Figure 2A for visualisation of predictive functions (1) and (2).

*Modelling growth*

One model of growth patterns was selected based on model metrics and AICc (Table 1). Model 3 gives predictive function (3)

$$l(d, T, h, v) = \mu_3 + \beta_{T3} \cdot T + \beta_{h3} \cdot h + \beta_{v3} \cdot v + \beta_{h\cdot\log(d)3} \cdot h \cdot \log(d) + \beta_{d3\alpha} \log(d) + \beta_{d3\beta}(\log(d))^2 + \beta_{d3\gamma}(\log(d))^3 \qquad (3)$$

Where $l$ is the length of the fish at the number of days post-fertilisation $d$, $\mu_3$ is the intercept term, $\beta_{T3}$ is the regression coefficient of incubation time, $\beta_{h3}$ is the regression coefficient of length at hatching, $\beta_{v3}$ is the regression coefficient of yolk sac volume at hatching, $\beta_{h\cdot\log(d)3}$ is the regression coefficient of the interaction between length at hatching and the logarithm of the number of days post-fertilisation, $\beta_{d3\alpha}$ is the regression coefficient of the logarithm of number of days post-fertilisation, $\beta_{d3\beta}$ is the regression coefficient of the squared logarithm of the number of days post-fertilisation $\beta_{d3\gamma}$ is the regression coefficient of the cubed logarithm of the number of days post-fertilisation, and $T$, $h$, and $v$ are values measured from a new fish: respectively the incubation time, length at hatching (mm), and yolk sac volume at hatching (mm3). See Figure 2B for visualisation of predictive function (3). Coefficients and significance for variables for models 1, 2, and 3 are given in Table S2.

*Model generalisability and the predictive tool*

Selected models' generalisability was assessed by averaging model metrics from 100 randomly-partitioned training and testing sets. Models performed well on out-of-sample data with

high $R^2$ and low RMSE, MAE, and MAPE values (Table 1). Testing set model metrics were slightly higher than cross-validation metrics (ranging from 6-16% higher), suggesting some slight model overfitting. However, increases are small and testing set metrics are still low, thus this is considered to be acceptable. After 100 iterations predicted results of timing of end of larval stage from models 1 and 2 showed a mean average percentage error of 6.100% ±0.186 and 5.991% ±0.171 respectively from the ground truth number of days post-fertilisation; model 3 showed a mean average percentage error of 2.916% ±0.767 from the ground truth length at end of larval stage (Table 1).

We then combined these models into an R script that functions as a predictive tool. This script predicts timing of end of larval stage and length at this point for previously-unseen fish based on their hatching characteristics. We make this script freely-available to all; details of the location of the script and its dependencies are in the Supporting Information.

Applying this tool (selecting model 1 as the model of yolk sac consumption) to the 16 individuals in the first testing set showed that these were predicted to end the larval stage 60.62 to 61.23 days post-fertilisation (corresponding to 358-361 degree days), with lengths ranging from 12.56 to 14.02 mm (see Figure S1).

*Effect of bacterial infection and parentage on development*

Infection status caused significant differences in all selected models (Table 3, Table 2A), indicating that infection with *P. fluorescens* significantly alters development patterns. We found no evidence for significant differences in slope of development patterns. Testing the effect of *P. fluorescens* infection on the three hatching characteristics showed infection significantly reduced the incubation time, the length at hatch, and increased the yolk sac volume at hatch (Figure 4, Table 2C).

We then assessed the effect of parentage on overall development, slope of developmental curves, hatching characteristics, and response to *P. fluorescens* infection. Likelihood ratio tests showed yolk sac consumption was significantly affected by dam while growth was affected by both

dam and sire identity (Table 3A). No evidence was found for different slopes of yolk sac consumption between dams or sire, but dams showed different slopes of growth (Table 3B). Dam identity did not significantly affect length at hatch, but did significantly affect yolk sac volume at hatch and the incubation time, while sire identity significantly affected incubation time (Table 3C). Parent-specific responses to *P. fluorescens* in terms of yolk sac consumption were not found, but variation in both dam and sire responses to infection were found in growth (Table 3D).

**Discussion**

In this study we modelled patterns of yolk sac consumption and growth in yolk-sac stage European grayling *Thymallus thymallus*. We examined whether the assumption of linear growth is true and produced functions that can be used to predict when an individual will end the larval period and its length at this point. We make these freely available to other researchers. We then used these to test the effect of bacterial infection and parental identity on development patterns. We thus developed two models of yolk sac consumption (one slightly more accurate, one more simple) and one of growth. These show patterns of yolk sac consumption over time to be either linear or have a nearly-linear sigmoidal shape, while patterns of growth are logarithmic. Patterns of yolk sac consumption and growth varied due to parental identity and bacterial infection. *P. fluorescens* infection significantly affects the patterns of yolk sac consumption (by reducing the consumed volume at all days) and growth (by reducing length across all days). The effect of infection was mediated through effects on hatching characteristics (length at hatch, yolk sac volume at hatch, and time taken to hatch) but no differences in slope post-hatching were found. Similarly, effects of parentage on yolk sac consumption were driven by differences in hatching characteristics alone, while effects on growth were the result of differences in both hatching characteristics and in model slope. *P. fluorescens* infection affected growth, but not yolk sac consumption, in a dam- and sire-specific manner.

All models show good generalisability when tested on out-of-sample data (Table 1), with slightly better generalisability for the model of growth than those of yolk consumption. The slight increase in error values between cross-validation and testing set-generated error metrics may reveal some signs of overfitting, but this is deemed to be acceptable as RMSE, MAE, and MAPE are still low indicating little error. We chose to build models with a polynomial mixed effects model framework over the more commonly-used von Bertalanffy, Gompertz, or other published functions (Chen et al., 1992; Lugert et al., 2016) as with mixed effects models we can easily include variables believed to influence development rates (Yamagishi 1969; Phillips et al. 1995; Pine & Allen 2001; Gilbey et al. 2009; Iguchi 2011), but see (Solberg et al. 2014). Rather than just describing the shape of the population's mean development pattern, we thus statistically tested how variables of interest affect development patterns. This study shows the validity of this approach which could now be extended to include embryo characteristics that are believed to influence developmental patterns such as egg size (Leblanc et al., 2011).

The selected models of yolk sac consumption and growth were then used to develop a tool to predict timing of the end of the larval period and length at this point. Both these variables are believed to be highly fitness-linked traits (Beacham et al., 1988; Brannas, 1995; Einum & Fleming, 2000; Mason & Chapman, 2011; Pine & Allen, 2001) as described above. Thus the application of the predictive tool (available on GitHub, see Supporting Information), and others developed following these principles, will allow future researchers to predict the relative fitness of an individual raised in a similar system based solely on hatching characteristics. The predictive tool is based on polynomial regression modelling, therefore if input variables are outside of the range of those used to train the model unexpected results may occur (see Figure S2). Growth patterns are species- and environment-specific (Arul, 1991; Bagarinao, 1986; Parra & Yúfera, 2001), therefore conditions described here should be imitated as closely as possible to generate accurate predictions when applying the modelling tool.

In order to predict the timing of the end of the larval period and length at this point the larval period is defined as ending when 20% of the yolk sac remains unconsumed. Salmonids, including grayling, emerge from the hyporheic zone and begin free-swimming and exogenously-feeding before complete absorption of the yolk sac (Scott, 1985). However, there is within-population variation in the size of the yolk sacs when they begin these behaviours (Andersson et al., 2013; Scott, 1985; Sternecker & Geist, 2010). Thus, 20% is chosen as an intermediate point to account for this variation. Variation in timing of free-swimming and exogenous feeding may be driven by environmental conditions (Olsson & Persson, 1986; Skoglund et al., 2011; Sternecker & Geist, 2010; Yanagitsuru et al., 2021), or by individual-level differences such as behavioural personality type (Andersson et al., 2013; Vaz-Serrano et al., 2011). Future studies can build on the work we present here and potentially include individual-level sources of variation into predictive tools to improve accuracy.

This study found that non-linear patterns of development better describe growth (difference in AICc between best model and best linear model was 314) and may better describe yolk sac consumption (difference in AICc between best model and best linear model was 20) in our system. One peculiarity of the models of yolk sac consumption is that the volume of yolk remaining briefly goes above 100% (Figures 3A and 4A). This is due to yolk sac volume being calculated using yolk sac length but in the first days after hatching growth in body length increases the length of the sac quicker than it is being depleted, giving this anomaly. Regardless, our findings indicate that linearity should not be assumed when studying grayling growth in experimental systems such as used here with no exogenous feeding sources. One caveat to the conclusion of logarithmic growth patterns in grayling is that result was found in a system without exogenous feeding opportunities, as is common when studying larvae (E. S. Clark et al., 2014; Nusbaumer et al., 2019). As larvae in our system were not provided with external feed they therefore may have had reduced growth especially towards the end of the larval stage when nutrient insufficiency is believed to drive the initiation of exogenous feeding behaviour (Woodhead 1957; Sternecker &

Geist 2010). Thus, it is possible that logarithmic growth patterns may not occur in the wild, or in experimental settings where larvae are provided with exogenous food sources. These possibilities require further examination.

For simplicity, linear growth patterns are frequently assumed (Imsland et al., 1996; McCormick & Hoey, 2004; Nusbaumer et al., 2019; Thia et al., 2018) despite this being widely believed to be incorrect (Chen et al., 1992; Hopkins, 1992; Mommsen, 2001; Nicieza et al., 2011). However, linearity in growth may indeed be correct for some groups. A meta-analysis by Vasbinder & Ainsworth (2020) spanning 17 marine fish families found that pelagic species broadly show linear growth trends in early life. Grayling are pelagic in the post-emergent stage before switching to a near-benthic position (Bardonnet et al., 1991; Scott, 1985), so which pattern they would be predicted to follow is unclear. No salmonid or other freshwater species was included by Vasbinder & Ainsworth so whether similar patterns would be expected is unknown. Few growth curves focus on such early developmental stages, partially due to the difficulty of study, and to the best of the authors' knowledge no salmonid larval-stage growth curves have previously been published. More work is needed to clarify whether the shape of growth patterns found here are common in Salmonidae, and whether freshwater species conform to Vasbinder & Ainsworth's conclusions.

More research has been conducted on yolk and its consumption (Rønnestad et al., 1999), and diverse curve shapes are found within teleosts including logistic (Lasker, 1962; Rønnestad et al., 1999), logarithmic (Fyhn & Govoni, 1995; Laurence, 1969; Quantz, 1985), and linear (Wood, 1932) decreases. Sigmoidal patterns similar to those presented in model 2 of this paper are not well-represented in the literature but neither are studies of grayling yolk consumption. It is possible that this model is indeed overfitted despite the model metrics which advocate otherwise, and linear consumption patterns should be assumed as in model 1. Further studies of yolk consumption patterns in grayling and closely-related species are needed before this can be concluded with any certainty.

The inclusion of higher-degree polynomial relationships 4th degree in model 2, 3rd degree in model 3) improved model, indicating that developmental rates are not constant but vary. This is most clearly seen in model 2. Temporally changing development rates could reflect shifts in gene expression patterns in an individual causing periods of faster and slower growth. Alternatively, variation in feeding success is known to cause fluctuations in fish growth rates after the larval stage (Pepin et al., 2015); thus, changing development rates could be caused by changes in the content of the yolk consumed or the consumption rate (which are known to vary (Kamler 2008)). Changing development rates could also be caused by environmental changes, such as in temperature which is known to affect developmental rate (Crisp, 1981; Ojanguren et al., 1999; Wood, 1932). The temperature conditions used throughout the experiment were held stable with minimal variation (5.9ºC, SD=±0.45ºC, range=3.6-6.9ºC). However, even this small variation could have had an effect on growth patterns if individuals happened to be in a relatively cold or warm patch at a critical point which then affected short- or medium-term developmental rates from that point forward. All three of these factors could have occurred alone or in concert. Temperature variation in natural conditions is also much stronger due to weather effects and diurnal patterns, therefore could cause even greater variation in development rates especially in the case of prolonged periods of warm or cool weather. All three factors mean that individual-level larval development rates could be expected to show small- or medium-scale temporal variation in the wild.

In this study parental identity and *Pseudomonas fluorescens* infection were both found to affect hatching characteristics and development. Parent-specific development in salmonids has been previously shown in Heath *et al.* (Heath et al. 1999) and Nusbaumer *et al.* (Nusbaumer et al. 2019). We found no evidence for dam- or sire-specific yolk consumption patterns in response to *P. fluorescens* infection, but both dams and sires showed distinct growth patterns when infected. This matches previous findings of parent-specific patterns of response to bacterial infection in salmonids as shown by (Clark et al. 2013a, 2014), and the effect on sire reveals additive genetic variation for tolerance to *P. fluorescens* infection in growth in the population (Lynch & Walsh, 1998).

Our results therefore provide further support for parental identity playing an important role in development patterns at both hatching and throughout the larval period in salmonids.

*Pseudomonas fluorescens* infection was found to cause earlier hatching, smaller size at hatching, and increased yolk sac volume at hatching. As bacterial exposure occurred after spawning females were not able to alter investment into eggs; therefore, the increased yolk sac volume in infected hatchlings may reflect either delayed onset or reduced rate of embryonic yolk sac consumption (Kamler, 1992). Previous studies of *P. fluorescens* infection in salmonids have found it to induce (Pompini et al., 2013; Wedekind, 2002) and delay (E. S. Clark et al., 2014; E. S. Clark, Stelkens, et al., 2013; E. S. Clark, Wilkins, et al., 2013) hatching, and result in larger (E. S. Clark et al., 2014) and smaller (E. S. Clark, Stelkens, et al., 2013) yolk sacs at hatching. The variation in these results may be explained by the effects of infection depending on the timing of infection (E. S. Clark et al., 2014) due to differences in host developmental stage. *P. fluorescens* infection consistently results in smaller larvae at hatching (E. S. Clark, Stelkens, et al., 2013) as found in this study, a pattern that was found to continue throughout the larval stage as a result of differences in hatching characteristics. Reduced length at the end of the larval stage would have strong effects on fitness (Anderson, 1988; Bailey & Houde, 1989; Brannas, 1995; R. A. Clark, 1992; Cushing, 1990; Einum & Fleming, 2000; Harvey, 1987; Miller et al., 1988; Raventos & Macpherson, 2005) as discussed above. Thus *P. fluorescens* infection may be of conservation and pisciculture concern due to its effect on reduced cohort survival rates at the end of the larval period. Disease outbreaks primarily occur when fish undergo environmental stresses (Snieszko, 1974), therefore whether observed length differences would be lost through compensatory growth (Nislow, 2000) when the environmental stress abated is unknown. Effects of infection on fitness-related traits at the end of the larval stage are relatively understudied, despite the critical importance of this time for survival, and should be explored further.

In conclusion, in this study we present complete models of yolk sac consumption and growth patterns in European grayling *Thymallus thymallus* from hatching until end of the larval stage.

These models can be used in future research to model development of their own grayling and estimate relative fitness by predicting when an individual will end the larval stage and its size at that point. The models can be built upon to increase prediction accuracy, and expand predictive power by predicting under a wider range of environmental conditions. We show that grayling do not follow linear growth in larval stages when raised in experimental conditions such as ours, and we stress that further research into developmental patterns of freshwater fishes is needed. We confirm the results of previous studies that have found that *P. fluorescens* infection reduces length at hatching and that this continues throughout the larval period, and show that this infection causes significant differences in sibgroups' growth patterns.

## Data availability

Data will be deposited on the dryad depository upon acceptance of the manuscript

## Ethics statement

The project was approved by the Fishery Inspectorate of the Bern Canton. Additional authorisations from the cantonal veterinary office were not required as all experimental manipulations were performed prior to yolk sac absorption.


## Acknowledgements

We thank G. Perroud and D. Nusbaumer for their help in the field, D. Nusbaumer, R. Mani and staff at *fischereistützpunkt* Kandersteg for assistance breeding of the study system, and D. Rodriguez and V. Ammann for help with data collection.


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**Tables and figures**

**Table 1.** Performance of selected models of yolk sac consumption and growth in uninfected fish. Performance metrics displayed are corrected Akaike's information criterion (AICc), conditional coefficient of determination ($R^2$), root mean standard error (RMSE), mean absolute error (MAE), mean absolute percentage error (MAPE), with 95% confidence intervals. "CV" metrics are generated from averaged 4-fold cross-validation and are used for model selection; "Test" metrics are generated from averaged results of 100 train/testing set splits and are used for assessment of model generalisability. AICc is not comparable between models with different response variables so AICc of models 1 or 2 are not comparable to that of 3.

| **Model** | **Relationship type** | **AICc** | **CV $R^2$** | **CV RMSE** | **CV MAE** | **Test $R^2$** | **Test RMSE** | **Test MAE** | **Test MAPE** |
|---|---|---|---|---|---|---|---|---|---|
| *Models of yolk sac consumption* | | | | | | | | | |
| 1 | Linear | 4487 | 0.906 ±0.005 | 3.403 ±0.470 | 2.618 ±0.238 | 0.903 ±0.001 | 3.765 ±0.126 | 2.809 ±0.062 | 6.100% ±0.186 |
| 2 | 4th order polynomial | 4467 | 0.912 ±0.005 | 3.404 ±0.391 | 2.632 ±0.240 | 0.907 ±0.001 | 3.713 ±0.122 | 2.782 ±0.060 | 5.991% ±0.171 |
| *Models of growth* | | | | | | | | | |
| 3 | 3rd order polynomial | 737 | 0.950 ±0.003 | 0.406 ±0.029 | 0.312 ±0.028 | 0.947 ±0.000 | 0.469 ±0.017 | 0.351 ±0.009 | 2.916% ±0.767 |

**Table 2.** Results of tests for differences between infected and control fish in (A) growth or yolk consumption patterns, (B) slope of growth or yolk consumption patterns, (C) hatching characteristics. DF is degrees of freedom, *p* values are in bold text and given to three decimal places when significant. Models 1 and 2 are of yolk sac consumption; model 3 is of growth. Method and test statistic are given in (A), (B), and (C).

| Hypothesis tested | DF | Effect size | *p* |
|---|---|---|---|
| *(A) Development patterns are different between infected and control fish. Likelihood ratio tests ($\chi^2$)* | | | |
| - **Model 1** | **4** | **13.4** | **0.010** |
| - **Model 2** | **5** | **13.3** | **0.021** |
| - **Model 3** | **5** | **20.1** | **0.001** |
| *(B) Development patterns are different in slope between infected and control fish. Linear mixed effects models (F)* | | | |
| - Model 1 | 1 | 1.5 | 0.22 |
| - Model 2 | 1 | 0.8 | 0.38 |
| - Model 3 | 1 | 2.6 | 0.11 |
| *(C) There are differences in hatching characteristics between infected vs control fish. Linear models (F)* | | | |
| - **Incubation time** | **1** | **13.4** | **<0.001** |
| - **Length at hatch** | **1** | **9.5** | **0.002** |
| - **Yolk sac volume at hatch** | **1** | **5.0** | **0.028** |

**Table 3.** Results of tests for differences between dams and sires in (A) growth or yolk consumption patterns, (B) slope of growth or yolk consumption patterns, (C) hatching characteristics, (D) response to *P. fluorescens* infection. DF is degrees of freedom, *p* values are in bold text and given to three decimal places when significant. Models 1 and 2 are of yolk sac consumption; model 3 is of growth. Method and test statistic used is given in (A), (B), (C), and (D).

| **Hypothesis tested** | | **DF** | **Effect size** | ***p*** |
|---|---|---|---|---|
| *(A) There are parent-specific development patterns. Likelihood ratio tests ($\chi^2$)* | | | | |
| Dam | | | | |
| - | **Model 1** | **16** | **63** | **<0.001** |
| - | **Model 2** | **20** | **68** | **<0.001** |
| - | **Model 3** | **20** | **61** | **<0.001** |
| Sire | | | | |
| - | Model 1 | 28 | 34 | 0.21 |
| - | Model 2 | 28 | 40 | 0.24 |
| - | **Model 3** | **28** | **47** | **0.014** |
| *(B) There are parent-specific differences in slope of patterns. Linear mixed effects models (F)* | | | | |
| Dam | | | | |
| - | Model 1 | 4 | 1.8 | 0.14 |
| - | Model 2 | 4 | 1.3 | 0.29 |
| - | **Model 3** | **4** | **6.9** | **<0.001** |
| Sire | | | | |
| - | Model 1 | 7 | 1.0 | 0.40 |
| - | Model 2 | 7 | 1.3 | 0.34 |
| - | Model 3 | 7 | 0.77 | 0.61 |
| *(C) There are parent-specific differences in hatching characteristics. Linear models (F)* | | | | |
| Dam | | | | |
| - | **Incubation time** | **4** | **3.2** | **0.015** |
| - | Length at hatch | 4 | 1.5 | 0.21 |
| - | **Yolk sac volume at hatch** | **4** | **25** | **<0.001** |
| Sire | | | | |
| - | **Incubation time** | **7** | **8.9** | **<0.001** |
| - | Length at hatch | 7 | 1.8 | 0.10 |
| - | Yolk sac volume at hatch | 7 | 1.0 | 0.44 |
| *(D) There are parent-specific responses to P. fluorescens infection. Linear mixed effects models (F)* | | | | |
| Dam | | | | |
| - | Model 1 | 4 | 0.5 | 0.71 |
| - | Model 2 | 4 | 0.5 | 0.70 |
| - | **Model 3** | **4** | **3.2** | **0.012** |
| Sire | | | | |
| - | Model 1 | 7 | 1.1 | 0.38 |
| - | Model 2 | 7 | 1.1 | 0.39 |
| - | **Model 3** | **7** | **2.7** | **0.001** |

**Figure 1.** Diagram of steps taken in this study to build, select, and fit models of yolk sac consumption and growth, then generate predicted values for timing of end of larval period and length at this point.

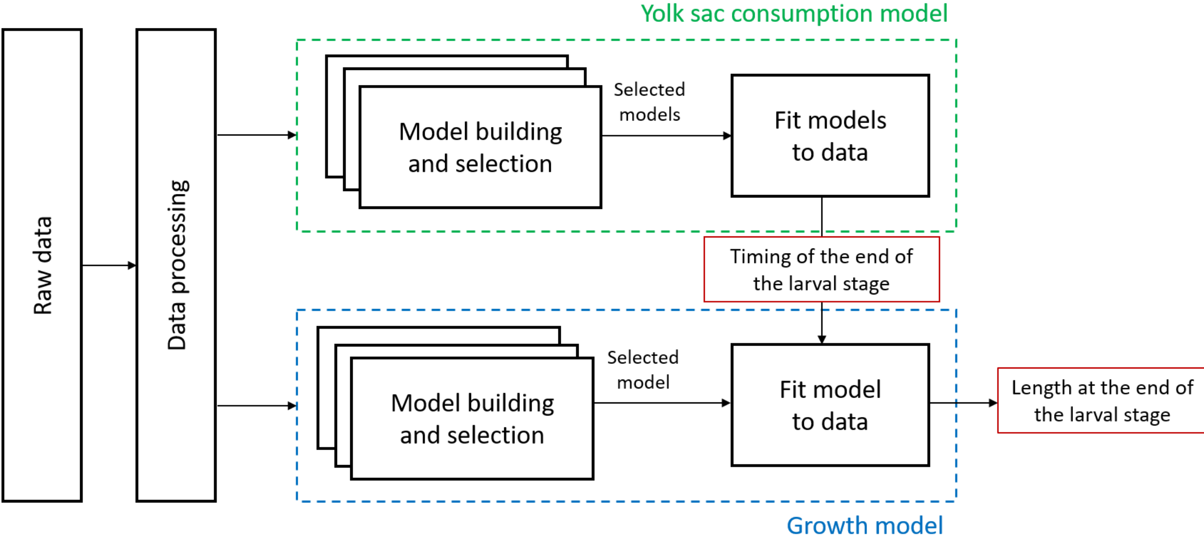


**Figure 2.** Predictive models of (A) yolk sac consumption (1, 2) and (B) growth over time (3), showing the predicted results of the selected models, plotted against all data in model training sets. Results were generated using the population average incubation time, length at hatching, and yolk sac volume at hatching (calculated from small dataset), and predicting results of models 1, 2, and 3.

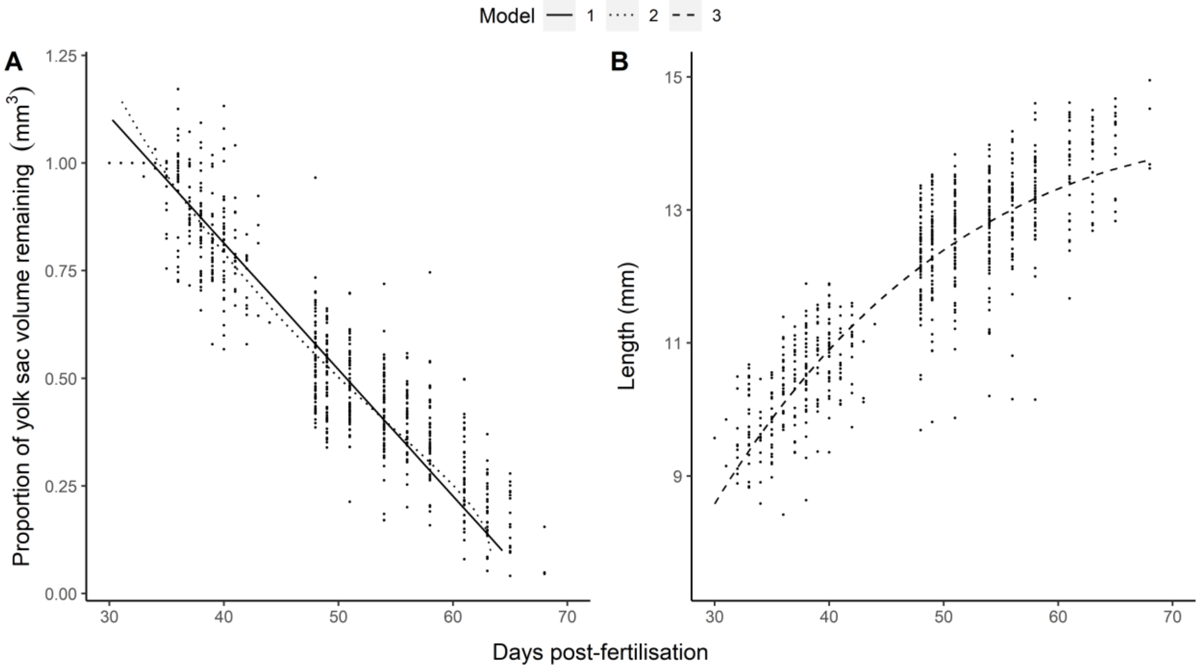


**Figure 3.** Models of yolk sac volume consumption (A, B) and growth (C) over time in control and *P. fluorescens*-infected fish. Models 1 (A) and 2 (B) describe yolk sac consumption, while model 3 (C) describes growth. Models are plotted against data from all individuals with more than 8 length measurements. Results for both control and *P. fluorescens*-infected fish were predicted from the population average incubation time, length at hatching, and yolk sac volume at hatching (calculated from all individuals with more than 8 length measurements). Thus, only infection status varies between the curves, all other variables are held the same.

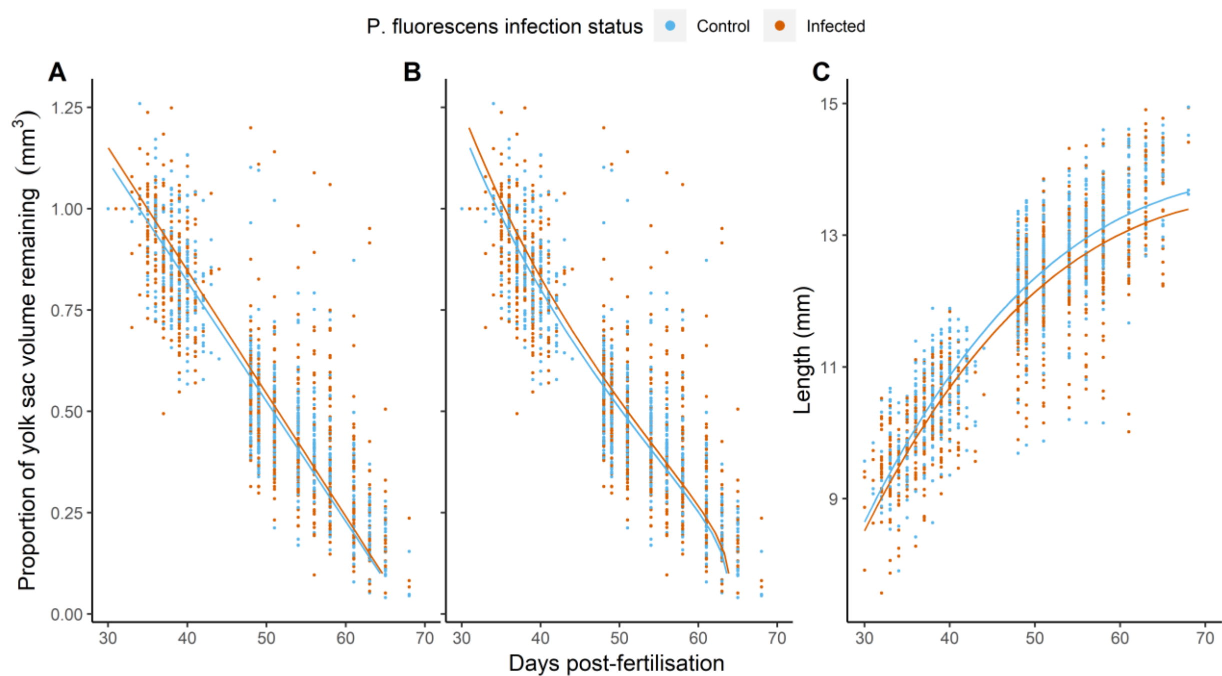
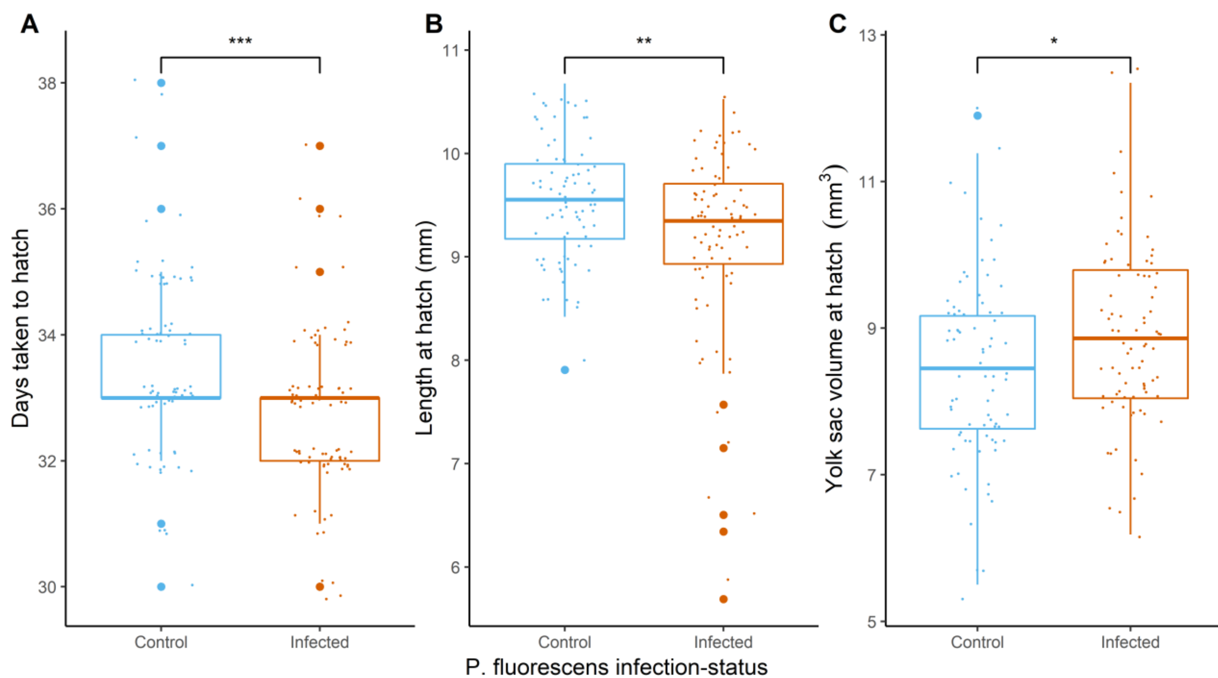


**Figure 4.** The effect of *Pseudomonas fluorescens* infection on the incubation time (A), the length at hatching (B) and the yolk sac volume at hatching (C) in grayling, after filtering the dataset for all individuals with more than 8 length measurements. Datapoints are shown jittered horizontally and vertically in (A), horizontally in (B) and (C) for clarity. Statistical significance of comparisons is given in the brackets at the top of each panel (*ggsignif* package, (Ahlmann-Eltze 2019); 0.05≥p>0.01: *, 0.01≥p>0.001: **, p≤0.001: ***.



# Supplementary information for: Predicting the end of the larval period in European grayling (*Thymallus thymallus*)

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**Content**

**Table S1**. Model metrics used to quantify errors and evaluate models built as part of the predictive tool.

**Table S2.** Coefficients of model variables in selected models predicting timing of emergence and length at that point in *Pseudomonas fluorescens*-uninfected fish

**Figure S1.** Yolk sac consumption and growth of all individuals in test set 1 plotted against models.

**Figure S 2.** Predictive models of (A) yolk sac consumption and (B) growth over time, showing the predicted results of the selected models when input variables are outside of the range of those used to train the models

**Predictive tool**

**Table S1**. Model metrics used to quantify errors and evaluate models.

| **Metric** | **Description** (Hyndman & Koehler 2006) | **Functions used - R package** |
|---|---|---|
| Conditional coefficient of determination ($R^2$) | The proportion of variation in response variable predictable from the predictor variables (fixed and random) | r.squaredGLMM - MuMIn (Barton, 2020); performance - performance (Lüdecke et al., 2021) |
| Root Mean Standard Error (RMSE) | the average error performed by the model in predicting the outcome for an observation | rmse - hydroGOF (Zambro-Bigiarini, 2020); performance_rmse - performance (Lüdecke et al., 2021) |
| Mean Absolute Error (MAE) | as RMSE but less sensitive to outliers | mae - hydroGOF (Zambro-Bigiarini, 2020); performance_mae - performance (Lüdecke et al., 2021) |
| Mean Absolute Percentage Error (MAPE) | average prediction error calculated as a percentage after pairs of results containing NAs are removed, thus slightly underestimates error | MAPE - MLmetrics (Yan, 2016) |

**Table S2.** Coefficients of model variables in selected models predicting timing of emergence and length at that point in *Pseudomonas fluorescens*-uninfected fish. Coefficients can be used to rebuild models for predictive use by others. P values are shown to 3 decimal places when significant ($\alpha$=0.05).

| Notation | Variable | Coefficient | Standard error | *p* |
|---|---|---|---|---|
| *Model 1* | | | | |
| $\mu_1$ | Intercept | 70.1 | 8.65 | <0.001 |
| $\beta_{i1}$ | Regression coefficient of incubation time | -0.08 | 0.25 | 0.74 |
| $\beta_{v1}$ | Regression coefficient of yolk sac volume at hatching | 0.04 | 0.30 | 0.89 |
| $\beta_{T \cdot p1}$ | Regression coefficient of interaction between incubation time and proportion of yolk sac volume remaining | 0.70 | 0.32 | 0.029 |
| $\beta_{v \cdot p1}$ | Regression coefficient of interaction between yolk sac volume at hatching and proportion of yolk sac volume remaining | -0.82 | 0.37 | 0.028 |
| $\beta_{p1}$ | Regression coefficient of proportion of yolk sac remaining | -50.3 | 11.0 | <0.001 |
| *Model 2* | | | | |
| $\mu_2$ | Intercept | 15.8 | 7.89 | 0.048 |
| $\beta_{T2}$ | Regression coefficient of incubation time | 0.62 | 0.19 | <0.001 |
| $\beta_{h2}$ | Regression coefficient of length at hatching | 0.39 | 0.40 | 0.34 |
| $\beta_{v2}$ | Regression coefficient of yolk sac volume at hatching | -0.65 | 0.23 | 0.006 |
| $\beta_{T \cdot log(p)2}$ | Regression coefficient of interaction between incubation time and logarithm of proportion of yolk sac volume remaining | 0.36 | 0.17 | 0.03 |
| $\beta_{v \cdot log(p)2}$ | Regression coefficient of interaction between yolk sac volume at hatching and logarithm of proportion of yolk sac volume remaining | -0.40 | 0.19 | 0.039 |
| $\beta_{p2\alpha}$ | Regression coefficient of logarithm of proportion of yolk sac remaining | -32.7 | 5.72 | <0.001 |
| $\beta_{p2\beta}$ | Regression coefficient of squared logarithm of proportion of yolk sac remaining | 0.64 | 3.03 | 0.83 |
| $\beta_{p2\gamma}$ | Regression coefficient of cubed logarithm of proportion of yolk sac remaining | 4.91 | 1.81 | 0.007 |
| $\beta_{p2\delta}$ | Regression coefficient of fourth power logarithm of proportion of yolk sac remaining | 1.05 | 0.33 | 0.002 |
| *Model 3* | | | | |
| $\mu_3$ | Intercept | 36.3 | 92.4 | 0.69 |
| $\beta_{T3}$ | Regression coefficient of incubation time | -0.20 | 0.02 | <0.001 |
| $\beta_{h3}$ | Regression coefficient of length at hatching | 2.36 | 0.41 | <0.001 |
| $\beta_{v3}$ | Regression coefficient of yolk sac volume at hatching | 0.00 | 0.03 | 0.89 |
| $\beta_{h \cdot log(d)3}$ | Regression coefficient of interaction between length at hatching and logarithm of the number of days post-fertilisation | -0.47 | 0.11 | <0.001 |
| $\beta_{d3\alpha}$ | Regression coefficient of logarithm of number of days post-fertilisation | -54.29 | 72.42 | 0.45 |
| $\beta_{d3\beta}$ | Regression coefficient of logarithm of squared number of days post-fertilisation | 20.65 | 18.91 | 0.28 |

| | | | | |
|---|---|---|---|---|
| $\beta_{d3\gamma}$ | Regression coefficient of logarithm of cubed number of days post-fertilisation | -2.11 | 1.65 | 0.20 |

**Figure S4.** Yolk sac consumption and growth of all individuals in test set 1 plotted against models. Panel labels are unique identifier of fish. Y axis of first and third columns is proportion of yolk sac volume remaining.

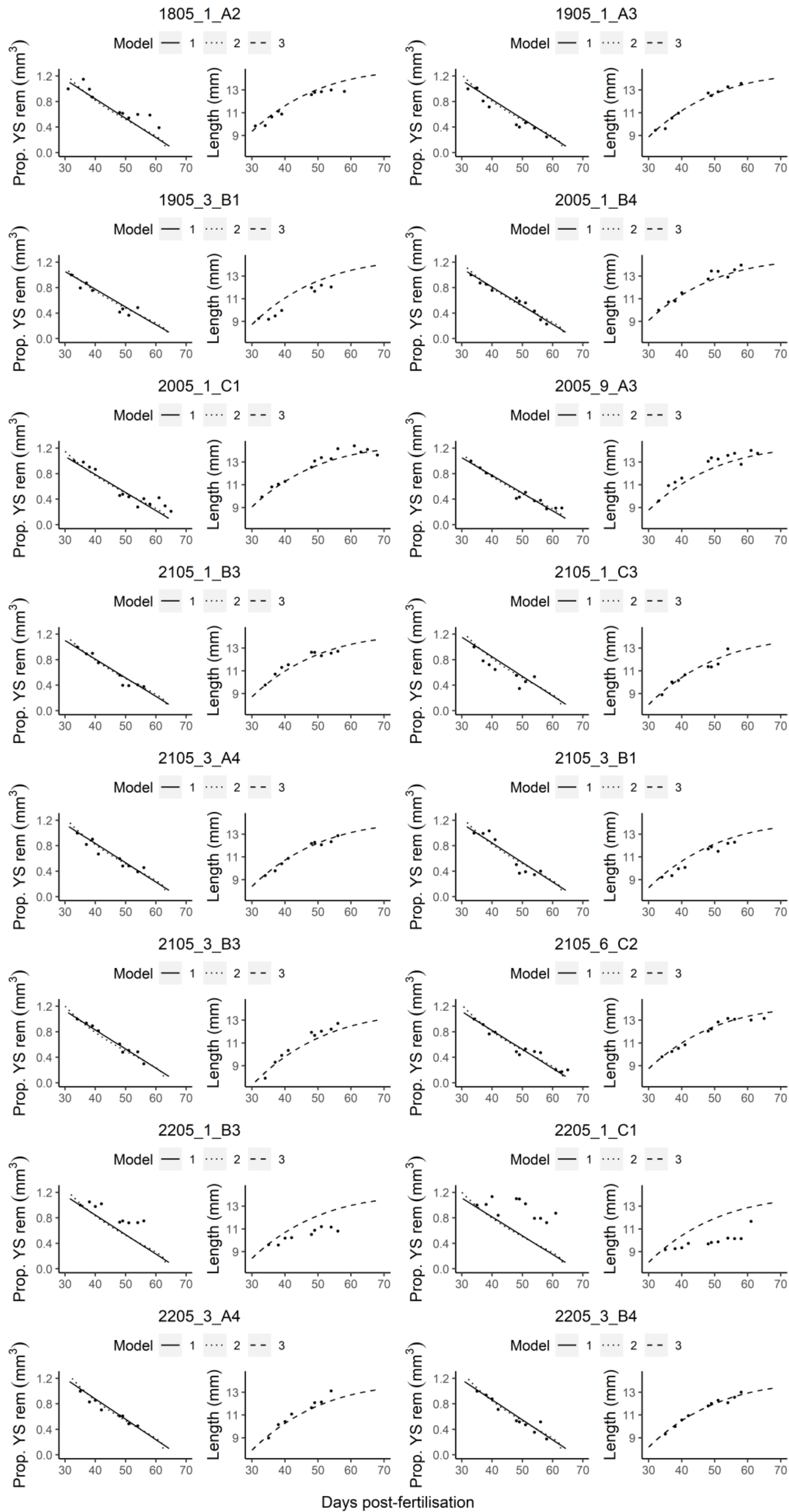
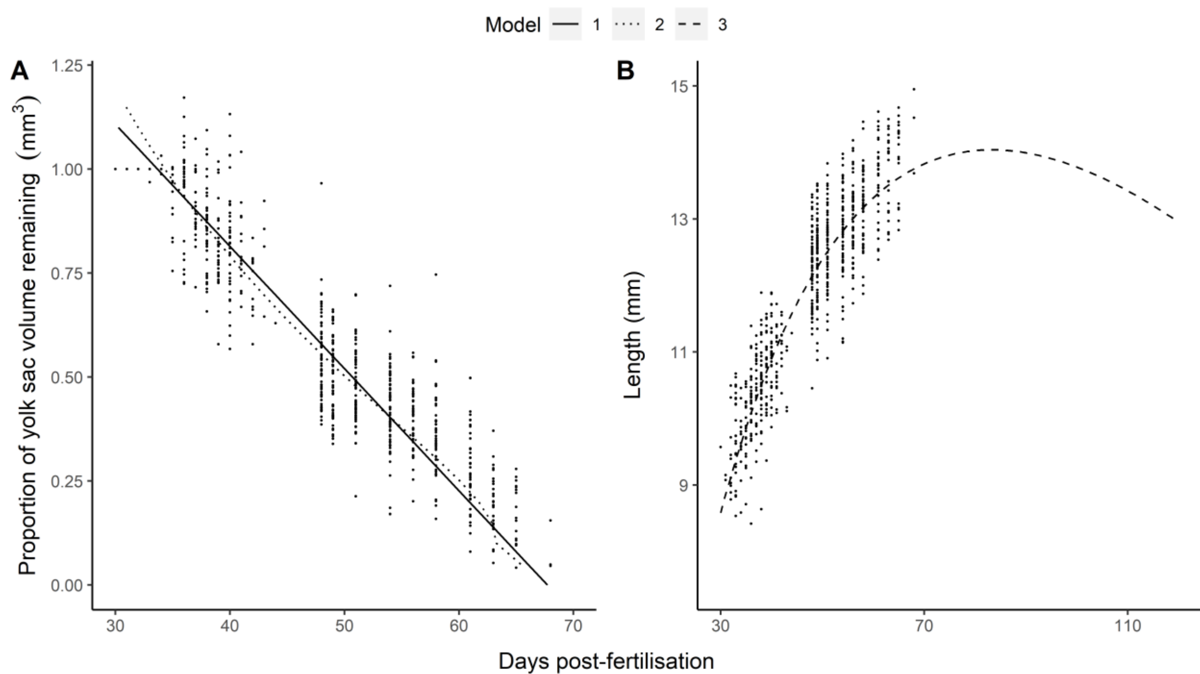

1805_1_A2
1905_1_A3
1905_3_B1
2005_1_B4
2005_1_C1
2005_9_A3
2105_1_B3
2105_1_C3
2105_3_A4
2105_3_B1
2105_3_B3
2105_6_C2
2205_1_B3
2205_1_C1
2205_3_A4
2205_3_B4
Model 1 2 3
Prop. YS rem (mm$^3$)
Length (mm)
Days post-fertilisation

**Figure S5.** Predictive models of (A) yolk sac consumption and (B) growth over time, showing the predicted results of the selected models when input variables are outside of the range of those used to train the models, plotted against all data in model training sets. Unexpected behaviour of predicted results due to inclusion of polynomials in the model can be seen in model 2 as Y values approach 0, and in model 3 as X axis values exceed 70. This is expected when predicting with polynomial models and using values of input variables beyond the range of those used to train the models.



**Predictive tool**

The R script containing the predictive tool and all its dependencies are available on GitHub at https://github.com/HugoPal/LEG.

**References for supplementary information**

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# Chapter II - Sublethal toxicity and low adaptive potential for tolerance to diclofenac pollution in a wild brown trout population (Salmo trutta)

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Chapter status: unpublished

**Author contributions**

HP and CW organized the fieldwork and designed and conducted the breeding. HP and DR reared the embryos and larvae under experimental conditions and photographed them. HP, DR, VA, MF, and JC measured larval size and growth. ELMV organized and supervised the diclofenac measurements. SEO, MM, and HP developed and implemented the polynomial regression modelling approach. HP and CW wrote the manuscript with input from all authors, who approved it before publishing.

**Abstract**

Novel stressors can elicit rapid evolutionary responses in affected populations if there is additive genetic variation in tolerance to the stressor. Diclofenac, a commonly used anti-inflammatory drug occurring as a widespread pollutant in much of Europe's surface water, is suggested to have diverse negative effects on fitness of chronically exposed fish. Populations of the socioeconomically and ecologically important brown trout (*Salmo trutta*) have thus been chronically exposed to diclofenac over many generations. This could have induced rapid evolution that would be predicted to reduce toxicity over time while using up additive genetic variance for tolerance to the stressor. To assess whether an evolutionary response to diclofenac is currently possible in a population that has been exposed to treated sewage effluent for decades, we studied differences in susceptibility to environmentally relevant levels of diclofenac pollution under experimental conditions. We sampled adult fish from the wild, used their gametes in two full-factorial *in vitro* breeding blocks (one in 2018, one in 2020) and raised embryos singly ($N_{total}$ = 2127), exposing them to 50, 250 $ng \cdot L^{-1}$ diclofenac or a sham treatment. We monitored larval characteristics at hatching and repeatedly until the end of the yolk sac period to determine growth curves. Diclofenac exposure reduced time to hatch with effect size correlating with concentration, up to 0.6% and a non-significant 0.07% in 2018 and 2020, respectively. Diclofenac did not affect length at hatching in 2018 but reduced it by 9.5% in 2020. 50 $ng \cdot L^{-1}$ was associated with increased growth but the evidence was weak. Diclofenac did not significantly affect yolk sac volume, embryo or larval survival, nor larval growth after hatching. We found no significant additive genetic variation for tolerance to diclofenac in mortality nor growth. We conclude that diclofenac can induce slight precocious hatching (a typical stress response in salmonid embryos) while no further signs of toxicity could be recorded. Rapid evolution in response to diclofenac-induced stress is currently unlikely in our study population.

**Introduction**

The resilience of natural populations is influenced by their ability to rapidly evolve to novel selection pressures (Hendry et al., 2017). For this to happen, standing genetic variation for tolerance to the new selection pressure is required, as evolution by novel mutations is a much slower process (Barrett and Schluter, 2008). Evolution of tolerance to the stressor reduces its effects on fitness but erodes the amount of genetic variation in the population: the stronger the selection pressure is the more likely that genetic variation in loci linked to the genes under selection erodes (Lynch and Walsh, 1998; Hendry et al., 2011). Anthropogenic pressures typically represent strong selection pressures, and often threaten natural populations as a result (Hendry et al., 2008). Thus, an important question in evolutionary ecotoxicology is whether there is additive genetic variation for tolerance to anthropogenic stressors (Bickham, 2011; Brady et al., 2017) such as pollutants. While studies of effects of stressors are plentiful, few assess the capacity for adaptation to that stressor (Wu et al., 2022). A widespread pollutant frequently suggested to have negative effects on the fitness of freshwater fish is the nonsteroidal anti-inflammatory drug diclofenac (Triebskorn et al., 2007; Gröner et al., 2017; Bio and Nunes, 2020). Whether standing genetic variation in tolerance to diclofenac exists, and thus whether freshwater fish populations have the ability to rapidly evolve to this stressor, is currently unknown.

Diclofenac is a popular pharmaceutical drug used worldwide in medicine as an analgesic, antiarthritic, and antirheumatic compound (Schürner et al., 2016). It is taken (ingested or used as a cream) and excreted but removal is incomplete in wastewater treatment plants (Ternes, 1998; Verlicchi et al., 2012) and it is among the most frequently detected pharmaceutical in wastewater treatment plant effluents (Verlicchi et al., 2012). As a result, it is included in the European Union watch list of chemicals under discussion as priority substances in the European Water Framework Directive (European Union, 2013). Measureable quantities are found in most European streams (Loos et al., 2009; aus der Beek et al., 2016; Simon et al., 2022), usually in the $ng \cdot L^{-1}$ to low $\mu g \cdot L^{-1}$ range. Values of 23-1080 $ng \cdot L^{-1}$ in surface water in the Czech Republic (Lacina et al., 2013; Marsik

et al., 2017), 1020 ng·L$^{-1}$ in a German stream (Ternes, 1998), and 370 ng·L$^{-1}$ at a lake's inflow in Switzerland (Buser et al., 1998) have been recorded. Concentrations are frequently highest in effluent of wastewater treatment plants, with observed concentrations of 670 ng·L$^{-1}$, 881 ng·L$^{-1}$, and 2100 ng·L$^{-1}$ in Switzerland, Sweden, and Germany, respectively (Fick et al., 2010; Morasch et al., 2010; Meyer et al., 2016). Concentrations detected in effluent probably represent the high end of exposure levels, but for effluent dominated sites the assumption that such high values could be sustained over long-term exposure is reasonable. The consumption of painkillers such as diclofenac is predicted to grow (Schröder et al., 2016). Thus, without considerable investment in retrofitting water treatment plants (reviewed in Alessandretti et al., 2021), which has been regulated by law in Switzerland (Swiss Federal Council, 1998) but may not be economically possible in all locations, occurrence in the aquatic environment will increase accordingly.

Chronic exposure studies at environmentally relevant concentrations have suggested that diclofenac has significant effects on fish. Structural changes to various organs including the kidney, liver, and gills have been noted in rainbow trout (*Oncorhynchus mykiss*) (Triebskorn et al., 2004), brown trout (*Salmo trutta*) (Hoeger et al., 2005), Nile tilapia (*Oreochromis niloticus*) (Gröner et al., 2017), and common carp (*Cyprinus carpio*) (Triebskorn et al., 2007). Impacts on the gill structure or physiology are of particular interest as adverse changes to them will likely affect oxygen supply, affecting all biochemical reactions dependent on oxygen (Hoeger et al., 2005) and causing chronic stress (Harper and Wolf, 2009) that may weaken the fish and reduce tolerance to further stressors. Indeed, evidence of an effect of diclofenac on oxidative stress has been shown in zebrafish (*Danio rerio*) (Bio and Nunes, 2020) and inanga (*Galaxias maculatus*) (McRae et al., 2018). Diclofenac has been suggested to cause alterations in enzyme activity in the liver and testes of south American catfish (*Rhamdia quelen*) (Guiloski et al., 2017), and to increase mortality rates in juvenile brown trout, reaching significance above environmentally relevant concentrations (Schwarz et al., 2017). Endocrine disrupting effects have also been detected in Japanese medaka (*Oryzias* latipes) (Hong et al., 2007), and Nile tilapia (Gröner et al., 2015, 2017). Reflecting this, a maximum concentration

of 50 ng·L$^{-1}$ is legally mandated in Swiss surface waters (Swiss Federal Council, 1998). The no observed effect concentration of diclofenac is, however, controversial (Leverett et al., 2021; Maack et al., 2022) as some studies find no effect at environmentally relevant concentrations (Stepanova et al., 2013; Praskova et al., 2014; Bickley et al., 2017). Additionally, two reviews assessing histological evidence for diclofenac's effects have suggested the no observed effect concentration to be >320 μg·L$^{-1}$(Wolf et al., 2014) or concluded that only chronic exposure to high concentrations under certain experimental conditions can cause minor changes (Wolf, 2021). However, it must be noted that both reviews were funded by a company marketing diclofenac. Despite the inconsistent findings the quantity and diversity of studies showing some element of toxicity at environmentally relevant concentrations strongly suggests that diclofenac does have toxic effects (which may be taxa or context-specific, as has been seen in other groups (Rattner et al., 2008)), but to date whether freshwater species have the capacity to rapidly adapt to these toxic effects remains unexplored.

In this study we assess the effect of, and tolerance to, diclofenac in wild populations of juvenile brown trout in two laboratory experiments over two breeding seasons. Brown trout is a socioeconomically (Elliott, 1989; Liu et al., 2019) and ecologically important salmonid that has been translocated across the globe (Halverson, 2008; Hunt and Jones, 2018; Hasegawa, 2020). However, some populations have suffered severe declines in recent decades (Burkhardt-Holm et al., 2005) making a study of tolerance to a widespread pollutant pertinent. We measured egg size, hatching success, hatching time, body length and yolk sac volume at hatching, and growth patterns over 14 and up to 102 days post-hatching. External fertilisers with no parental care such as brown trout are ideal model species with which to study genetic variation as producing many sib groups is easy, giving high statistical power to assess effects with even small effect size. In these species the components of genetic variation can be estimated using *in vitro* breeding designs to partition the variance components of phenotypes of interest (Lynch and Walsh, 1998), an approach that has been successfully employed before (Neff and Pitcher, 2005). In such designs the sire effect is a useful proxy of the additive genetic variation in a trait (Lynch and Walsh, 1998) while the effect of

the interaction between sire and the stressor tests for statistically significant additive genetic variance for tolerance to the stressor (Marques da Cunha et al., 2019a; Nusbaumer et al., 2021b). We thus test (i) whether environmentally relevant diclofenac pollution has significant effects on phenotypes in brown trout hatchlings; and (ii) whether our study population currently contains genetic variation in tolerance to diclofenac that may allow for rapid evolution.

## Methods

### *Sampling, breeding, and incubation of embryos*

During the 2018 and 2020 spawning seasons wild fish were caught via electrofishing from the Kiese river (46°49'31.8"N 7°36'04.1"E), a tributary of the River Aare, canton Bern, and transferred to the *Fischereistützpunkt Reutigen* hatchery where they were held until experimental crosses could be made. Fish were then haphazardly selected to be stripped for their gametes. Eggs of each female (dam) were about equally distributed between Petri dishes (Greiner bio-one, Austria), then milt of males (sires) was pipetted into the Petri dishes so that each male was crossed with each female within a breeding block. Using these methods, we produced one block of 6 ♀ × 10 ♂ on the 28$^{th}$ November 2018 and one block of 6 ♀ × 10 ♂ on the 25th November 2020. Standardised water (OECD, 1992) was added and the milt and eggs were mixed to induce fertilisation, then eggs were left to harden for two hours. Adult fish were released at their site of origin.

After hardening 24 and 9-12 fertilised eggs per sib group in the 2018 and 2020 experiments, respectively, were transferred to laboratory facilities. Eggs were rinsed with a tea strainer and tap water for 30 seconds at a flow rate of 2L/min before being singly distributed to 12 well plates (Cytoone, USA) previously filled with 2.7 mL autoclaved standardised water (2018 season) or 5 ml tap water (2020 season). They were held in a temperature-controlled room until diclofenac treatment exposure.

In 2018 the room was held at 5.3°C with a 12-hour day-night cycle (0800-2000). Temperature and light conditions were maintained the same throughout the experiment. In 2020 the room was held at a mean of 5.2°C with the same 12-hour day-night cycle. Due to an accident in the 2020 experiment, the room experienced a temperature spike starting 130 dpf (days post fertilisation), i.e. 626 ddpf (degree days post fertilisation), rising to 16.5°C 132 dpf (636 ddpf) so all plates were moved to another temperature-controlled room and incubated at 5.2°C until the end of the experiment.

*Treatment exposure*

In the 2018 season eggs were moved 64 dpf (336 ddpf) to individual wells in 12-well plates containing 5ml of diclofenac solution per well at one of three environmentally relevant concentrations: nominally 0, 50, or 250 $ng \cdot L^{-1}$. In the 2020 season eggs were exposed at 69 dpf (342 ddpf) to 0 or 250 $ng \cdot L^{-1}$. In both seasons solutions were produced mixing diclofenac sodium salt (CAS: 15307-79-6; Sigma-Aldrich, Switzerland) and standardised water. Eggs were assigned to wells in the plates such that fertilised eggs from each sire and dam were evenly distributed amongst all columns and plates, and that all sib groups were evenly exposed to diclofenac treatments which were arranged by columns in the plate. An identical number of control plates containing nominally 0 or 250 $ng \cdot L^{-1}$ diclofenac solution without eggs were stored in the room alongside the plates containing eggs. All plates were stored in stacks and the relative three-dimensional position of each plate was changed every weekday. The plates were then checked daily from 0900-1100 and new hatchlings were considered to have hatched on that day. Wells containing eggs that developed a pale colour and no visually discernible embryo were recorded.

*Measurement of embryo traits*

In the 2018 season photos were taken of each larva at hatching, 14 days post hatching, then every other day from 125 dpf (656 ddpf) until the end of the experiment at 189 dpf (992 ddpf) by which point 96% of all fish had died. Hatching occurred from 85 dpf (446 ddpf) until 94 dpf (494

ddpf) with the peak of hatching occurring 90 dpf (473 ddpf). In the 2020 season photos were taken of each embryo 15 dpf, then of each hatchling every day throughout the hatching period, then twice a week until the death of the last hatchling 162 dpf (836 ddpf). Hatching occurred from 90 dpf (451 ddpf) until 106 dpf (505 ddpf), with the peak of hatching occurring 99 dpf (478 ddpf). Photos were taken using a custom-made photobox with standardised lighting, using a Canon EOS 70D camera body and a Canon EF 50mm f/2.5 compact macro lens (shutter speed 1/400s, f/3.2, ISO 200, white balance 4000). Body length (most anterior point of head to most posterior visible vertebrae), yolk sac length and width, and egg diameter (measuring each egg in three different directions passing through the centre point of the egg; 2020 season only) were then measured. All measurements were done using ImageJ 1.52a. Yolk sac volume was estimated as $V = (\pi/6) \cdot L \cdot W^2$ where $L$ is the yolk sac length, and $W$ is the yolk sac width at the point midway along the yolk sac length (Blaxter and Hempel, 1963). Egg volume was calculated three times per egg, once for each measurement of egg diameter and assuming the egg to be a sphere, and the mean of the three was taken. Longevity in days was recorded for all hatched individuals based on the photos.

In both the 2018 and 2020 experiments water samples were collected every week for 7 weeks from the day of initial diclofenac exposure to determine diclofenac concentrations. Concentrations were measured using liquid chromatography – mass spectrometry. In the 2018 and 2020 experiment water samples were analysed from every timepoint except timepoint four, and from the first and last timepoint, respectively. See Supplementary Information for details.

*Statistical analysis*

We tested the effect of diclofenac and parental identity on hatching success, hatching time, length at hatching, yolk sac volume at hatching, longevity, and growth in the first 14 days. We then tested whether response to diclofenac varied with dam and sire identity. We tested the effect of parental identity on egg size 15 dpf as diclofenac treatment was applied after size measurements were collected. Linear mixed effects models of all response variables except hatching success were

built via the lmer function in the *lme4* package (Bates et al., 2015), with diclofenac treatment as a fixed predictor variable. Hatching success was analysed using generalised mixed effect models with a binomial distribution (mixed effects logistic regression) with the glmer function in the same package, including the same predictor variable in the model. One sire and one dam had low hatching success rates (1% and 6%, respectively) so were removed from analyses except that of hatching success. In species where males only fertilise eggs and provide no parental care, such as salmonids, the male effect on a given offspring phenotype is a useful proxy of additive genetic variation (Lynch and Walsh, 1998; Neff and Pitcher, 2005). The dam effect captures maternal effects (genetic and non-genetic). We therefore included terms for dam and sire as random effects modelled with random intercepts. Dam × sire effects could not be estimated due to sample size limitations caused by lower-than-expected hatching success.

The significance of each term in the model (diclofenac treatment, dam identity, sire identity) was assessed using likelihood ratio tests, comparing models including or omitting a term of interest to a reference model with the anova function from the *stats* package (R Core Team, 2020). We tested whether the response to diclofenac treatment varied by dam or sire identity by including diclofenac treatment in interaction with either identity variable within the random structure in further models. We then tested significance of the interaction with likelihood ratio tests between the reference model without the interaction and the full model including the interaction term, again with the anova function.

Due to differences in environment and developmental patterns data from the two experiments are analysed separately (Figure S2). Analysis of hatching time, length at hatching, yolk sac volume at hatching, and longevity were conducted on both experiments. Egg size measurements were not collected in the 2018 experiment, so analysis was only conducted on embryos in the 2020 experiment. Not all embryos in 2020 were exposed to diclofenac throughout the experiment, as wells' solution was replaced with standardised water when diclofenac concentration was sampled. Thus, effects of diclofenac alone or in interaction with dam or sire

identity on hatching time, length at hatching, and yolk sac volume at hatching were only tested in embryos exposed to diclofenac until hatching. Diclofenac's effects on growth over 14 days were estimated on embryos that were exposed to diclofenac until 14 days post hatching. All embryos from the 2020 experiment were, thus, omitted as 42% of experimental cells in 2020 were empty in this analysis. As we do not know when an embryo that failed to hatch would have hatched, we do not know whether it would have been exposed to diclofenac until hatching. As a result we can not test whether hatching success was affected by diclofenac exposure in the 2020 experiment, thus this was only analysed in the 2018 experiment. Similarly, larvae in the 2020 experiment were only exposed to diclofenac for a fraction of their lives before it was removed to test diclofenac levels. Therefore, analysis of larval longevity was conducted using larvae from the 2018 experiment. Longevity was recorded from photos; thus, fish that died between the photos taken 14 days post hatching and the start of regular longevity records 126dpf were marked as having longevity of 14 days. Longevity was analysed including and omitting fish that died before the start of regular longevity records.

Model assumptions were checked via visual inspection of residuals and quantile-quantile plots and transformations were applied if needed to improve data normality. Thus, egg size was transformed to the power of 2, length at hatch was transformed to the power of 5 (2018 experiment only), yolk sac volume at hatch underwent square root transformation, and longevity was transformed to the power of 2. Models were fitted with maximum likelihood estimation. Model coefficients were extracted with the summary function from the *stats* package.

Final sample sizes per analysis in the 2018 and 2020 experiment, respectively, were 904 and 194 (hatching time), 887 and 194 (length at hatching), and 884 and 195 (yolk sac volume at hatching). Hatching success and growth over 14 days were only analysed in the 2018 experiment and models had sample sizes of 1140, and 864, respectively. Larval longevity was only analysed in the 2018 experiment and models had sample sizes of 899 (including larvae that died before the start of regular longevity recording) and 123 (omitting them). Egg size was only analysed in the

2020 experiment and model sample size was 195. These and all subsequent analyses were conducted in R version 4.0.2 (R Core Team, 2020).

*Modelling growth curves*

In order to test the effect of variables of interest on growth patterns over the length of the experiment, not just growth over 14 days, we modelled changes in length over time using polynomial regression. Full details of the model building and selection processes are given in the Supplementary Information, but in brief the dataset was filtered to include only individuals from the control treatment with more than 8 length measurements (N=161 individuals from 62 sibgroups). Polynomial mixed effects models were then fit using polynomial regression using the *lme4* package. Body length was used as the response variable with incubation time, length at hatching, yolk sac volume at hatching (all mean-centered and divided by variable standard deviation due to differences in scale between the variables) included as fixed predictor variables. These variables are all believed to influence growth rates of juvenile fish (Yamagishi, 1969; Phillips et al., 1995; Pine and Allen, 2001; Gilbey et al., 2009; Iguchi, 2011); but see (Solberg et al., 2014)). The number of dpf, hereafter "age", was also included as a fixed predictor variable. First-order interactions between age and the three other predictor variables were also included. Growth patterns are modelled at the level of the individual as individual identity was included as a random effect to account for repeated measures. Models were fit with up to $5^{th}$ degree polynomials of age. We then selected the degree of polynomials of age that gave the best fit to the data based on generated model performance metrics (Table S1). This is "the selected model". We then used both forwards and backwards stepwise variable selection to assess fixed effect variable inclusion via the step function in the *stats* package. The selected model was finally evaluated for overfitting to assess the generalisability of the model to novel data.

We then tested for effects of diclofenac and parental identity on experiment-long growth patterns, and for parental effects on tolerance to diclofenac. The selected model was fitted to

datasets containing all individuals (diclofenac exposed and un-exposed) with more than 8 length measurements from the 2018 experiment. Larvae from the 2020 experiment were omitted as diclofenac solution was replaced with standardised water during the course of the 2020 experiment, and therefore these larvae were not constantly exposed to diclofenac. The effects of diclofenac treatment level, dam, and sire identity were tested using likelihood ratio tests between the selected model including or omitting the term of interest via the anova function in base R (R Core Team, 2020). Parental effects on tolerance to diclofenac were tested by including a three-way interaction between age × diclofenac treatment × dam or sire, and all lower-order interactions, and performing likelihood ratio tests between models including or omitting the three-way interaction term via the anova function. This tests whether allowing the relationship between age and parent to vary by diclofenac treatment significantly improves the model fit. In models of the effect of dam or sire on experiment-long growth, dams or sires, respectively, with fewer than four offspring were removed. In models of the effect of diclofenac in interaction with dam or sire, dams and sires with fewer than four offspring per diclofenac treatment level were removed. Thus, 90 offspring of six dams and two sires, 67 offspring of six dams, 66 offspring of two sires, 39 offspring of two dams, and 66 offspring of two sires were retained to test the effects of diclofenac, dam, sire, and diclofenac in interaction with dam, and sire, respectively.

*Extraction of variance components*

Variance components of egg size, hatching success, hatching time, length at hatching, yolk sac volume at hatching, growth over 14 days, and longevity were extracted from mixed effects models and used to calculate the components of phenotypic variation (Lynch and Walsh, 1998). Mixed effects models were built for each response variable in turn. The predictor variables included were dam identity and sire identity, and the dam × sire interaction. Models were built using the lmer and glmer functions as above. Maximum likelihood was used for the binary response variable hatching success and restricted likelihood for continuous response variables; variance components

were extracted for the control and diclofenac-exposed treatments. Assuming that epistasis is negligible, additive genetic variance ($V_A$) is calculated as four times the sire component, maternal genetic variance ($V_{Dam}$) is the dam component (including genetic, maternal environmental effects, and the interaction), dominance variance ($V_D$) as four times the dam × sire component, narrow-sense heritability ($h^2$) as $V_A$ divided by the total variance, mean-scaled additive genetic variance ($I_A$) as $V_A$ divided by the squared mean trait value, and coefficients of additive genetic variation ($CV_A$) as the square-root of $V_A$ divided by the trait mean, multiplied by one hundred (Houle, 1992; Lynch and Walsh, 1998; Hansen et al., 2011). Due to lower-than-expected hatching success calculation of $V_D$ is uncertain, therefore calculated variance components should be considered preliminary.

**Results**

*Measured diclofenac concentrations*

Diclofenac concentrations were found to be close to nominal values both in the presence and absence of eggs (Figure S1). In the 50 and 250 ng·L$^{-1}$ treatment values fell slightly over time. Control treatments at 0 ng·L$^{-1}$ were below the limit of quantitation. Diclofenac exposure is therefore deemed successful. Henceforth diclofenac treatments are referred to by their nominal values.

*Hatching success*

Hatching occurred from 85-94 and 90-106 dpf (446-494 and 451-505 ddpf) in the 2018 and 2020 experiments respectively. Final hatching success was 64% of the 1080 embryos in the 2018 season (64%, 64%, 62% hatchlings in the 0, 50, 250 ng·L$^{-1}$ diclofenac treatments, respectively) and 57% of the 687 embryos in the 2020 season (57%, 56% in the 0, 250 ng·L$^{-1}$ diclofenac treatments, respectively).

*Selected growth model*

The final selected growth model included the fixed effect variables hatching time and hatching length (both mean-centered and divided by standard deviation), in addition to first to third degree polynomials of age (Figure S3). Stepwise variable selection based on model AIC dropped the term for yolk sac volume at hatching as it did not significantly change the model fit. Model metrics generated during test set iterations were identical to those generated during 4-fold cross validation to two decimal places suggesting no model overfitting (Supplementary Information, Table S2).

*Effect of diclofenac on embryo performance*

Diclofenac had significant sub-lethal effects. We found an experiment- and concentration-specific effect on hatching time. Exposed embryos hatched 0.3% and 0.6% (1.5 and 3 degree days) faster when exposed to 50 and 250 $ng{\cdot}L^{-1}$ diclofenac, respectively, than control embryos (Table 1B) in 2018, which hatched on average 475 ddpf. In 2020 embryos hatched 0.07% faster (0.3 degree days) than controls which hatched 489 ddpf but this difference was non-significant (Table 1). We found distinct effects of diclofenac on length at hatching, as diclofenac exposed fish did not differ from control fish in 2018 but were 9.5% shorter in 2020 (Table 1, Figure 1). Diclofenac exposure altered long-term growth patterns as treatment with 50 $ng{\cdot}L^{-1}$ increased length at all points while individuals treated with 250 $ng{\cdot}L^{-1}$ did not differ in growth pattern from controls. Diclofenac had no significant effect on hatching success, yolk sac volume at hatching, short -term growth patterns, or longevity (both including or omitting individuals that died before regular longevity recording, Table S4, Figure S4).

*Parental effects and tolerance to diclofenac*

Dam identity significantly affected all measured traits in Table 1 except longevity (Figure S5). Dam effects strongly influenced experiment-long growth patterns (Figure S6), driving the bimodal growth patterns visible from 150 dpf in Figure 1F. We found no evidence for significant dam effects on tolerance for any other measured trait.

Sire identity significantly affected hatching success, hatching time, growth over 14 days (Table 1, Figure S7), and length at hatching in 2018 but not 2020 (Table 1D). We found no significant sire effects on tolerance to diclofenac in any measured variable.

Table 2 lists the variance components of the phenotypic variation in the control and 250 ng·L$^{-1}$ diclofenac treatments, the narrow-sense heritabilities ($h^2$), mean-scaled additive genetic variance ($I_A$), and the coefficients of variation ($CV_A$) in the two experiments.

*Experimental differences in embryo performance*

In addition to the distinct responses to diclofenac exposure in length at hatching and hatching time between the experiments, differences between experiments were found in hatching time (Table 1B). Embryos in the 2018 experiment hatched on average 12 degree days earlier than those in 2020 (Figure S8).

## Discussion

We conducted two breeding experiments with brown trout and exposed the resulting embryos to ecologically relevant diclofenac concentrations. We tested whether exposure to such diclofenac concentrations significantly affected several key traits at hatching, embryo or hatchling mortality, or immediate or longer-term growth patterns. We then tested for significant additive genetic variation in, and maternal effects (environmental and genetic) on, tolerance to diclofenac stress within the studied population. Finally, we calculated preliminary results for the variance components. We found that diclofenac exposure reduced time to hatch in both experiments with effect size correlating with concentration, but the effect was only significant in one. Diclofenac exposure was associated with hatching at smaller sizes in one experiment and had no effect on length at hatching in the other. Exposure to 50 ng·L$^{-1}$ diclofenac was associated with increased length, especially at later timepoints, but larvae treated with 250 ng·L$^{-1}$ did not differ in length from controls. Diclofenac exposure did not affect yolk sac volume, embryo or hatchling mortality, or

short-term growth patterns. We did not find significant additive genetic variation in tolerance to diclofenac in any measured trait.

Chronic exposure to environmentally relevant diclofenac concentrations was not found to affect either mortality rates or growth patterns; however, sublethal effects were found. The absence of significant effects on mortality matches results found in other fishes (Lee et al., 2011; Schwarz et al., 2017; Horie et al., 2019). We found some evidence for increased long-term growth patterns in response to exposure to 50 but not 250 ng·L$^{-1}$ diclofenac. While pollutants reducing fish growth is well-evidenced in the literature (Clark et al., 2013a; Affandi and Ishak, 2019; Naidoo and Glassom, 2019; Wu et al., 2022), examples of them increasing growth are rare and to the best of the authors' knowledge diclofenac has never previously been shown to increase larval growth. We therefore would have a clear *a priori* expectation regarding the effect of diclofenac on growth patterns: as a micropollutant, we expect it to reduce growth. Directional testing can only be applied in cases where *a priori* expectations of a directional effect exist (Rice and Gaines, 1994; see Herdegen-Radwan (2019) and Bray et al. (2020) for recent examples of directional testing being applied). Application of directional testing in this case concludes that the effect of diclofenac on long-term growth patterns is non-significant. Secondly, following the terminology of Muff et al. (2021) we find only moderate evidence, with small effect size, for a diclofenac-associated increase in growth rate. Based on these two points we therefore find the evidence in favour of diclofenac-induced increased growth rate unconvincing.

Size and timing of hatching are frequently highly fitness-linked in fishes, as smaller fish have lower survivorship than larger conspecifics of the same age (Meekan and Fortier, 1996; Einum and Fleming, 2000; Raventos and Macpherson, 2005), but early emergence as larvae from the sediment layer into which salmonid eggs are laid increases survival (Brannas, 1995; Einum and Fleming, 2000; Mason and Chapman, 2011). Significant changes to hatching time can either reveal variation in developmental rate (if hatching is directly linked to a developmental stage) or a behavioural response to acute stress. Diclofenac-exposed fish hatching earlier but with no

difference in lengths to unexposed fish would suggest increased developmental rate in exposed fish; conversely exposed fish hatching both earlier and at smaller sizes suggests a behavioural response to stress. In this study we find some evidence for increased developmental rate in the 2018 experiment. Potent endocrine disrupting chemicals have been shown to increase growth in teleost fish under certain conditions (Wu et al., 2022). However, salmonid larvae are not exogenously-feeding until the end of the yolk-sac stage (Woodhead, 1957; Sternecker and Geist, 2010); thus, induced increases in pre-hatch development must come from consumption of yolk stores. We found no significant effect of diclofenac on yolk sac volume at hatching, making an effect of diclofenac to significantly increase development rate unlikely. Instead, our findings that diclofenac can cause earlier hatching and at reduced lengths may suggest an action to induce precocious hatching as a behavioural response to stress; however, this requires further investigation.

Stress-induced precocious hatching is common in amphibians (Warkentin, 2011) and has been documented in salmonids (du Gas et al., 2017) but has not previously been shown in response to environmentally relevant diclofenac pollution (Memmert et al., 2013; Stepanova et al., 2013; Praskova et al., 2014; Yokota et al., 2018). Its finding in brown trout suggests taxa-specific action. Schwarz et al. (2017) found no effect of diclofenac on hatching time in brown trout even at 100 $\mu g \cdot L^{-1}$ concentrations. However, the sample size per treatment level used in that study was a quarter of the size used in this one, and embryos were not held singly, a method known to reduce measurement error as discussed below, but communally. Schwarz et al. (2017) may have missed this effect due to a type II error arising from lower statistical power.

Diclofenac did not induce embryonic or hatchling mortality in this study so precocious hatching at a reduced size may be unexpected as this strategy would gain limited fitness benefits from the early niche switch. Diclofenac pollution within ranges found in European surface water (Buser et al., 1998; Ternes, 1998; Lacina et al., 2013; Marsik et al., 2017), however, has been previously shown to have diverse sublethal effects including structural changes to organs

(Triebskorn et al., 2004; Hoeger et al., 2005; Gröner et al., 2017), endocrine-disrupting effects (Hong et al., 2007; Gröner et al., 2015, 2017), alteration of enzyme activity (Guiloski et al., 2017), and induction of oxidative stress (McRae et al., 2018; Bio and Nunes, 2020). Embryos in our study may, therefore, have been responding to these stresses with precocious hatching. Further studies are needed to assess whether early hatching allows trout embryos to reduce the sublethal negative effects of diclofenac. Our results suggest that chronic diclofenac pollution affects trout embryos in ways not measured in this study, to which they behaviourally respond.

We found no significant additive genetic variation for tolerance in any measured traits and in neither of the traits significantly affected by diclofenac. The lack of genetic variation for tolerance to diclofenac pollution means that it has either been depleted over time due to selection, or never existed in the first place. At the time of writing, we know of no other studies assessing genetic variation in tolerance to diclofenac stress so we cannot compare these hypotheses. The importance of heritable components of phenotypic variation shift throughout ontogeny (Wilson and Réale, 2006) thus it is possible that significant sire effects on tolerance would only become prominent at later life stages (Evans et al., 2010). This is speculative and beyond the scope of this paper. This possibility does not invalidate the study of effects of stressors on early life stages as these are often more sensitive than later stages (Mohammed, 2013), and may respond differently due to developing in a different chemical environment. Instead it highlights that exposure studies should be conducted on a range of life stages to best predict how a population will respond. In summary, the lack of genetic variation for tolerance to diclofenac pollution, and especially its absence in traits found to be significantly affected by diclofenac, suggests that a rapid evolutionary response to diclofenac pollution is unlikely in this population.

In this study we find convincing evidence for significant effects of diclofenac on only two measured traits, and significant additive genetic variation for tolerance in none. The lack of further significant effects of diclofenac and overall lack of significant additive genetic variation for tolerance could be due to type II false negative errors caused by a lack of statistical power, a lack

of overall genetic diversity, or a true absence of significant diclofenac effects and additive genetic variation for these traits. A type II error is caused by large measurement error, low sample size, or low effect sizes. Large measurement errors are unlikely as salmonid embryos raised singly under controlled laboratory conditions as we used have previously been shown to be sensitive indicators of environmental stressors. Similar systems to ours have been used in comparable protocols singly-rearing embryos in fully controlled laboratory conditions to test the effects of pathogens (Clark et al., 2014), organic pollution (Jacob et al., 2010; Wedekind et al., 2010), micropollutants (Brazzola et al., 2014; Marques da Cunha et al., 2019b), and waterborne cues (Pompini et al., 2013). These have successfully found significant additive genetic variation previously (von Siebenthal et al., 2009; Jacob et al., 2010; Clark et al., 2013b; Brazzola et al., 2014). These methods have been shown to be sensitive to very low doses of a stressor (Brazzola et al., 2014). The data used in our presented work is unlikely to have suffered from low sample size, with the exception of analyses of experiment-long growth which are discussed below, as we used two 6 ♀ × 10 ♂ full-factorial breeding blocks to produce 2127 embryos raised to hatching. Sample sizes similar to or smaller than ours have been used to show significant effects of diverse stressors and significant genetic variation in tolerance to those in recent years. Jacob et al. (2010) found significant effects of microbial stress and genetic variation in tolerance to it with 19 sires and 2 dams producing 2098 hatched offspring; Brazzola et al. (2014) found significant genetic variation in tolerance to 17α-ethinylestradiol (EE2) stress using 11 sires and 8 dams producing 3904 hatched offspring; (von Siebenthal et al., 2009) found significant effects of *Pseudomonas fluorescens* infection and tolerance to it with 12 sires and 4 dams producing 2304 hatched offspring; Clark, Wilkins, et al. (2013) found significant effect of *P. fluorescens* infection on expression in the major histocompatibility complex and significant additive genetic variation for this with 16 sires and 16 dams producing 832 embryos. Thus the absence of significant genetic variation in tolerance to diclofenac is unlikely to be a type II error. A lack of overall genetic diversity is also unlikely as the population network the Kiese river is part of shows high genetic diversity within and between populations (Stelkens et al., 2012) and

shows no evidence of inbreeding depression (Clark et al., 2013a; Stelkens et al., 2014). Our methods are therefore sensitive to environmental changes and would have allowed detection of phenotypic variation and any associated additive genetic variation for these if diclofenac had caused it. The lack of effects of diclofenac, and of significant additive genetic variation for tolerance, likely reflects their true absence.

One analysis that may have underestimated variable significance due to low statistical power is that of variation in experiment-long growth patterns. Models testing this included sample sizes from 52 to 91 trout and offspring from as few as 2 sires; as a result, these analyses may suffer from low statistical power and resulting type II errors. If this is the case, then future studies of the effect of diclofenac on longer-term growth patterns in brown trout may find significant genetic variation in tolerance to diclofenac pollution as we found a trend towards significance in this study. This possibility is, however, speculation, and does not alter our interpretation of the findings of this study.

Our estimates of diclofenac toxicity are likely to underestimate its true toxicity. We tested the effect of diclofenac in isolation. In the wild, however, stressors frequently occur in combination with opportunistic microbes (Wilkins et al., 2015), other micropollutants (Chèvre, 2014; Moschet et al., 2014), or parasites (Palejowski et al., in prep), and exposure to one stressor can reduce resistance to others due to immunosuppressive effects (Segner et al., 2012, 2014). Embryos were also raised singly under conditions arguably close to optimal for their development (e.g. minimised pathogen growth, no mechanical stress, no predation risk). Assessments of diclofenac toxicity are often done with acute or subchronic exposures and find No Observed Effect Concentrations above those typically found in the wild (e.g. (Nassef et al., 2009; Praskova et al., 2011; Näslund et al., 2017), but chronic exposure may have different effects due to longer-term exposure. This is especially true in tests on earlier life stages which are often more sensitive than later stages (Mohammed, 2013) and may respond differently due to developing in a different chemical environment, as discussed above. Diclofenac is, therefore, likely to be more toxic in the wild than

is usually found under controlled laboratory conditions. Furthermore, the occurrence of diclofenac is likely to increase in freshwater environments unless widespread improvements to wastewater treatment plants occurs (Schröder et al., 2016). The likely underestimation of true diclofenac toxicity and increase in freshwater diclofenac pollution, combined with its already widespread occurrence (Loos et al., 2009; aus der Beek et al., 2016), demands further investigation into if and when diclofenac pollution is toxic.

For simplicity, growth patterns in juvenile fishes are frequently assumed to be linear (Imsland et al., 1996; McCormick and Hoey, 2004; Thia et al., 2018; Nusbaumer et al., 2019) despite this being widely believed to be incorrect (Chen et al., 1992; Hopkins, 1992; Mommsen, 2001; Nicieza et al., 2011). If growth patterns deviate from linear then sizes predicted by a linear relationship will have little resemblance to reality as the linear model will underestimate true size at all intermediate points (Hopkins, 1992). In this study we modelled brown trout growth until 546 degree days post-hatching (982 ddpf) and, therefore, likely modelled hatchlings until they would be emerging from the sediment layer into which eggs are laid in the wild (Elliott and Hurley, 1998; Jensen and Johnsen, 1999; Sternecker and Geist, 2010). Growth patterns until emergence show a clear logarithmic shape which is heavily influenced by maternal identity (a common finding in studies of salmonids: (Palejowski et al.; Heath et al., 1999; Brazzola et al., 2014; Falica et al., 2017; Marques da Cunha et al., 2019a), similar to those seen in European grayling *Thymallus thymallus* (Palejowski, Ojavee, Makhmutova, Garaud, et al., in prep). In marine fish early-stage growth patterns seem to be predicted by the position a species occupies in the water column (reviewed in Vasbinder & Ainsworth (2020)), and linear growth patterns may be true for some species including pelagic marine fishes. However, no freshwater or salmonid species were included in this review so it is unknown whether freshwater species follow the same patterns as marine ones. It is possible that growth patterns observed in our study may be influenced by the experimental conditions as no external food source was provided. This may limit growth especially towards the end of the larval stage when nutrient insufficiency is thought to drive emergence behaviour and initiation of

exogenous feeding (Woodhead, 1957; Sternecker and Geist, 2010); however, further experiments that provide external food sources or sample wild larvae are needed to test this hypothesis. Our findings reinforce that the assumption of linear growth patterns in juvenile fish is incorrect, and we stress that measurements intermediate to the start and end points should be collected to better model growth patterns and fill holes in our knowledge of species-specific growth patterns.

We find distinct variables driving variation in growth in the first 14 days post-hatching and in experiment-long growth. Studies of the effects of treatments on growth frequently focus on immediate post-hatching growth (Marques da Cunha et al., 2019b, 2019a; Nusbaumer et al., 2019, 2021a) rather than growth until emergence, when salmonids emerge from the sediment as free-swimming larvae. Emergence as free-swimming larvae is, however, the stage when size differences are most important as larger size gives advantages in competitive ability (Johnson et al., 1999; Einum and Fleming, 2000), predation avoidance (Vigliola and Meekan, 2002) and overwinter survival (Hurst and Conover, 2011; Schultz et al., 2011). Growth patterns until, and size at, emergence are likely to more tightly correlate with fitness than size 14 days post-hatching, when salmonids are typically still buried in the sediment. Our findings therefore indicate that care should be taken when using immediate growth as a proxy for fitness.

We present the results of two experiments in different years, and while efforts were taken to standardise experimental conditions between them as much as possible some differences did occur. Firstly, hatching periods in days and ddpf overlapped but were not identical. Secondly, in the 2020 experiment a technical error in the temperature-controlled laboratory caused a temporary exposure to elevated temperatures. Thirdly, while the breeders used to produce the embryos in the 2018 and 2020 years were taken from the same population the individuals used were different. These experimental differences manifested as qualitatively different results of analyses of hatching time, length at hatching, and yolk sac volume. Such inter-experiment differences may result from unintended methodological differences or from the different breeders used in the experiments; unfortunately, our methodology does not allow us to tease apart these confounding factors.

Nonetheless, 11 of 15 results estimated across these three response variables in Table 1 are qualitatively similar in the two experiments. These similarities highlight the replicability of our experiments despite environmental and genetic differences.

Diclofenac pollution is widespread in European surface water and while toxic effects at environmentally relevant concentrations have been shown a debate over whether diclofenac poses a significant threat to affected populations still rages. The resilience of natural populations to novel selection pressures such as diclofenac is influenced by their ability to enact rapid evolutionary responses to them. However, such responses require standing additive genetic variation in tolerance to the stressor. We present data suggesting that diclofenac pollution at levels commonly found in nature can cause slight precocious hatching likely as a behavioural response to diclofenac-induced stresses not identified in this study. However, we find no significant additive genetic variation for tolerance to diclofenac pollution. We are, unfortunately, unable to speculate whether standing genetic variation has been used up by a previous evolutionary response or never existed. Our results suggest that diclofenac has small yet significant effects on embryonic development which may affect wild populations, but that a rapid evolution in response diclofenac-induced stress is currently unlikely in our study population.

**Acknowledgements**

We thank Benjamin Bracher, Ulrich Gutmann, and Christian Rolli for catching and handling the adult fish, Laura Garaud, Christian de Guttry, Simona Mandra, Lucas Marques de Cunha, David Nusbaumer, and Giulia Perroud for their help in the field, Fiorella Lucarini, Daniel Olbrich, and David Staedler for their work measuring diclofenac levels, and Christoph Küng and Andreas Hertig for permissions and support.

**Ethics Statement**

The study was approved by the Fishery Inspectorate of the Bern canton. Handling of adult fish happened in the context of the cantonal stocking program. Further ethical review and approval

was not required because the study was on embryos and larvae before the onset of exogenous feeding.


## Funding

We thank the Swiss National Science Foundation (31003A_182265).


## Data availability

All data will be published on the Dryad Digital Repository.

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## Figures and Tables

**Table 1.** Effects of diclofenac exposure and dam and sire identity on embryo survival, hatching characteristics, and larval growth. *P* values < 0.05 are highlighted in bold.

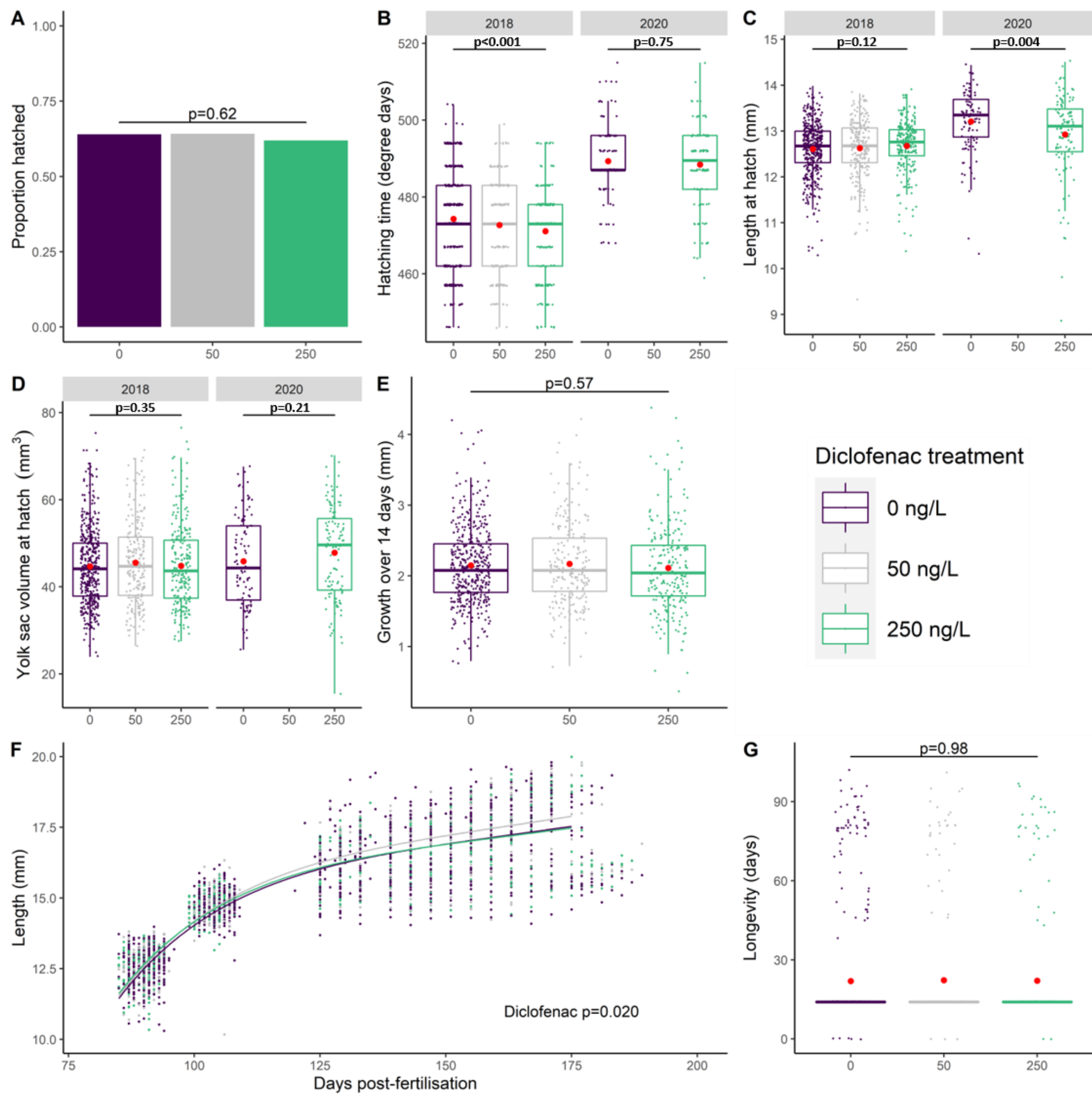
| Effect tested | $\chi^2$ | $DF_{LRT}$ | *p* | $\chi^2$ | $DF_{LRT}$ | *p* |
|---|---|---|---|---|---|---|
| | 2018 Experiment | | | 2020 Experiment | | |
| *(A) Egg size* | | | | | | |
| **Dam** | | | | **968** | **1** | **<0.001** |
| Sire | | | | 0.2 | 1 | 0.67 |
| *(B) Hatching success* | | | | | | |
| DCF | 1.0 | 2 | 0.62 | | | |
| **Dam** | **294** | **1** | **<0.001** | | | |
| **Sire** | **475** | **1** | **<0.001** | | | |
| DCF × Dam | 3.8 | 5 | 0.58 | | | |
| DCF × Sire | 1.0 | 5 | 0.96 | | | |
| *(C) Hatching time* | | | | | | |
| **DCF** | **12** | **2** | **<0.001** | 0.1 | 1 | 0.75 |
| **Dam** | **206** | **1** | **<0.001** | **86** | **1** | **<0.001** |
| **Sire** | **87** | **1** | **<0.001** | **20** | **1** | **<0.001** |
| DCF × Dam | 1.7 | 5 | 0.89 | 0.1 | 2 | 0.95 |
| DCF × Sire | 0.4 | 5 | 1.0 | 0 | 2 | 0.99 |
| *(D) Length at hatching* | | | | | | |
| **DCF** | 4.2 | 2 | 0.12 | **8.5** | **1** | **0.004** |
| **Dam** | **122** | **1** | **<0.001** | **13** | **1** | **<0.001** |
| **Sire** | **24** | **1** | **<0.001** | 1.0 | 1 | 0.31 |
| DCF × Dam | 3.4 | 5 | 0.64 | 0.0 | 2 | 1.0 |
| DCF × Sire | 2.5 | 5 | 0.78 | 0.1 | 2 | 0.94 |
| *(E) Yolk sac volume at hatching* | | | | | | |
| DCF | 2.1 | 2 | 0.35 | 1.6 | 1 | 0.21 |
| **Dam** | **460** | **1** | **<0.001** | **171** | **1** | **<0.001** |
| Sire | 3.1 | 1 | 0.08 | 2.5 | 1 | 0.12 |
| DCF × Dam | 1.7 | 5 | 0.88 | 1.2 | 2 | 0.55 |
| DCF × Sire | 2.4 | 5 | 0.79 | 0.3 | 2 | 0.86 |
| *(F) Growth over 14 days* | | | | | | |
| DCF | 1.1 | 2 | 0.57 | | | |
| **Dam** | **65** | **1** | **<0.001** | | | |
| **Sire** | **6.6** | **1** | **0.010** | | | |
| DCF × Dam | 5.3 | 5 | 0.38 | | | |
| DCF × Sire | 1.4 | 5 | 0.92 | | | |
| *(G) Experiment-long growth patterns* | | | | | | |
| **DCF** | **832** | **2** | **0.016** | | | |
| **Dam** | **618** | **5** | **<0.001** | | | |
| Sire | 0.0 | 1 | 0.85 | | | |
| DCF × Dam | 4.8 | 2 | 0.09 | | | |
| DCF × Sire | 2.7 | 2 | 0.26 | | | |
| *(H) Longevity* | | | | | | |
| DCF | 0 | 2 | 0.98 | | | |
| Dam | 0 | 1 | 1 | | | |
| **Sire** | **1526** | **1** | **<0.001** | | | |
| DCF × Dam | 0 | 5 | 1 | | | |
| DCF × Sire | 0.4 | 5 | 1 | | | |

**Table 2.** Estimates of variance components in each experiment for (A) egg size, (B) hatching success, (C) hatching time, (D) length at hatching, (E) yolk sac volume at hatching, (F) growth over 14 days, and (G) longevity. From these we calculated narrow-sense heritability, mean-scaled additive genetic variance, and coefficients of additive genetic variation. Components of egg size were not estimated for diclofenac-exposed eggs as exposure occurred after egg size measurements were collected. Components could not be estimated for length at hatching in diclofenac-unexposed embryos in 2020 as the model failed to converge.

| | $V_{Tot}$ | $V_A$ | $V_{Dam}$ | $V_D$ | $V_{Res}$ | $h^2$ | $I_a$ | $CV_A$ |
|---|---|---|---|---|---|---|---|---|
| *(A) Egg size* | | | | | | | | |
| **2018** | | | | | | | | |
| Control | 259.97 | 0.00 | 217.81 | 9.32 | 39.83 | 0.00 | 0.00 | 0.00 |
| *(B) Hatching success* | | | | | | | | |
| **2018** | | | | | | | | |
| Control | 9.37 | 19.40 | 1.90 | 6.50 | 1.00 | 2.07 | 19.40 | 440.45 |
| 50 ng·L$^{-1}$ | | | | | | | | |
| 250 ng·L$^{-1}$ | 10.29 | 19.67 | 3.17 | 4.84 | 1.00 | 1.91 | 19.67 | 443.49 |
| *(C) Hatching time* | | | | | | | | |
| **2018** | | | | | | | | |
| Control | 162.80 | 66.42 | 42.22 | 10.72 | 101.28 | 0.41 | <0.01 | 1.72 |
| 50 ng·L$^{-1}$ | 147.21 | 49.11 | 37.48 | 0.00 | 97.45 | 0.33 | <0.01 | 1.48 |
| 250 ng·L$^{-1}$ | 151.77 | 50.99 | 46.86 | 23.88 | 86.19 | 0.34 | <0.01 | 1.52 |
| **2020** | | | | | | | | |
| Control | 117.25 | 34.56 | 50.20 | 61.28 | 43.09 | 0.29 | <0.01 | 1.20 |
| 250 ng·L$^{-1}$ | 119.95 | 33.37 | 53.94 | 50.54 | 45.03 | 0.27 | <0.01 | 1.18 |
| *(D) Length at hatching* | | | | | | | | |
| **2018** | | | | | | | | |
| Control | 0.33 | 0.04 | 0.05 | 0.02 | 0.26 | 0.13 | <0.01 | <0.01 |
| 50 ng·L$^{-1}$ | 0.42 | 0.11 | 0.07 | 0.00 | 0.33 | 0.25 | <0.01 | 2.60 |
| 250 ng·L$^{-1}$ | 0.34 | 0.01 | 0.07 | 0.00 | 0.27 | 0.03 | 0.03 | 0.84 |
| **2020** | | | | | | | | |
| Control | | | | | | | | |
| 250 ng·L$^{-1}$ | 0.71 | 0.00 | 0.95 | 0.00 | 0.62 | 0.00 | 0.00 | 0.00 |
| *(E) Yolk sac volume at hatching* | | | | | | | | |
| **2018** | | | | | | | | |
| Control | 84.51 | 3.22 | 36.37 | 2.57 | 46.70 | 0.04 | 0.00 | 4.02 |
| 50 ng·L$^{-1}$ | 96.44 | 0.00 | 48.35 | 5.82 | 46.63 | 0.00 | 0.00 | 0.00 |
| 250 ng·L$^{-1}$ | 96.67 | 0.60 | 43.29 | 2.70 | 52.56 | 0.01 | 0.00 | 1.73 |
| **2020** | | | | | | | | |
| Control | 117.67 | 1.35 | 81.78 | 23.49 | 29.69 | 0.01 | 0.00 | 2.55 |
| 250 ng·L$^{-1}$ | 153.54 | 9.81 | 106.16 | <0.01 | 44.63 | 0.06 | <0.01 | 6.90 |
| *(F) Growth over 14 days* | | | | | | | | |
| **2018** | | | | | | | | |
| Control | 0.29 | <0.01 | 0.03 | 0.05 | 0.24 | 0.02 | <0.01 | 0.02 |
| 50 ng·L$^{-1}$ | 0.37 | 0.06 | 0.02 | <0.01 | 0.37 | 0.15 | <0.01 | 0.05 |
| 250 ng·L$^{-1}$ | 0.41 | <0.01 | 0.06 | 0.00 | 0.35 | 0.02 | <0.01 | 0.02 |
| *(G) Longevity* | | | | | | | | |
| **2018** | | | | | | | | |
| Control | 744.73 | 2805.7 | 0.00 | 0.00 | 43.31 | 3.77 | 5.66 | 238.00 |
| 50 ng·L$^{-1}$ | 749.98 | 2816.6 | 0.00 | 0.00 | 45.82 | 3.76 | 5.49 | 234.30 |
| 250 ng·L$^{-1}$ | 726.35 | 2638.0 | 0.00 | 0.00 | 66.85 | 3.63 | 5.33 | 230.78 |

*Abbreviations:* $V_{Tot}$ total variance; $V_A$ additive genetic; $V_{Dam}$ maternal; $V_D$ dominance; $V_{Exp}$ experiment; $V_{Res}$ residual; $h^2$ narrow-sense heritability; $I_A$ mean-scaled genetic variance; $CV_A$ coefficients of genetic variance.

**Figure 1.** Effect of diclofenac exposure on (A) hatching success, (B) time taken to hatch (degree days), (C) length at hatch (mm), (D) yolk sac volume at hatch ($mm^3$), (E) growth in the first 14 days post-hatching (mm), (F) larval length over time, and (G) longevity (days after hatching) in brown trout larvae. In (B), (C), and (D) boxplots are facetted by experiment. In (A), (E), (F), and (G) measurements are from 2018 only. In boxplots means per group are given as a red dot. To plot (F), the selected model was modified by adding a variable describing diclofenac treatment in interaction with days post-fertilisation, then trained on all individuals with more than 8 length measurements from the 2018 experiment. Predictions of the model were then generated based on the population average incubation time and length at hatching calculated from the same individuals. The results were plotted against data from all individuals excluding those treated with 50 $ng \cdot L^{-1}$ diclofenac.


A
Proportion hatched
p=0.62
B
Hatching time (degree days)
2018
2020
p<0.001
p=0.75
C
Length at hatch (mm)
p=0.12
p=0.004
D
Yolk sac volume at hatch (mm3)
p=0.35
p=0.21
E
Growth over 14 days (mm)
p=0.57
Diclofenac treatment
0 ng/L
50 ng/L
250 ng/L
F
Length (mm)
Diclofenac p=0.020
Days post-fertilisation
G
Longevity (days)
p=0.98

# Supplementary information for: Sublethal toxicity and low adaptive potential for tolerance to diclofenac pollution in a wild brown trout population (*Salmo trutta*)

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## Measuring diclofenac concentrations

### *Sample collection*

Water samples were collected to measure the uptake of diclofenac by eggs and to determine diclofenac concentrations. In 2018, 300 µl was collected every week for 7 weeks from the day of initial diclofenac exposure ending 106 dpf. Water samples were collected from each of 80 randomly-chosen wells containing eggs treated with 50 $ng \cdot L^{-1}$ or 250 $ng \cdot L^{-1}$ diclofenac, and 80 eggless wells with 0 $ng \cdot L^{-1}$ and 250 $ng \cdot L^{-1}$ diclofenac. Samples were held in 15 ml Falcon tubes (Greiner bio-one, Austria). Wells to sample were chosen without replacement such that all wells were sampled with similar frequency. In 2020, the entire solution content of a well was collected every week for 7 weeks from the day of initial diclofenac exposure ending 113 dpf. Wells were initially randomly assigned to timepoints for sampling, then assignments were haphazardly altered such that all dams had as close to 10 eggs as possible in the first and last sample. At each sampling the entire solution content of a well was removed by pipette and pooled by timepoint and treatment in 50 ml Falcon tubes (Greiner bio-one, Austria), the well was then filled with 5ml standardised water. All samples were stored at -20°C until analysis. Water samples were then analysed using liquid chromatography – mass spectrometry to assess diclofenac concentrations. In the 2018 and 2020 experiment water samples were analysed from every timepoint and from the first and last timepoint, respectively.

## Solid-phase extraction of water samples

### *2018 experiment*

Sample processing procedures were dependent on nominal concentrations. For the 250 $ng \cdot L^{-1}$ treatment, 1 mL of sample was pipetted into a glass vial, spiked with 50µL of an internal standard solution (Diclofenac (D4) 20 $ng \cdot L^{-1}$; TRC-Canada), briefly vortexed and then centrifuged (10 min). The upper layer was transferred to a new vial for measurement. For the 50 $ng \cdot L^{-1}$ treatment, 1 mL of sample was spiked with 50µL of a 1:5 diluted internal standard solution

(Diclofenac (D4) 4 ng.ml$^{-1}$) and briefly vortexed before solid-phase extraction (SPE). Ten mL of control sample in a glass vial was spiked with 50µL of the 1:5 diluted internal standard solution (Diclofenac (D4) 4 ng.mL$^{-1}$) and briefly vortexed before SPE. Controls and 50 ng.mL$^{-1}$ treatment samples were extracted using a 12-port vacuum manifold SPE system. SPE cartridges (Strata X 60 mg/3 mL; Phenomenex Helvetia GmbH, Basel, Switzerland) were conditioned using two times 1 mL of methanol and two times 3 mL of water. Then, samples were passed through the cartridges (3-4 drops per second), cartridges were washed with two times 3 mL of water, dried under vacuum for about 1 min and eluted sequentially with three times 0.5 mL of methanol into a glass vial. Extracts were reduced under a gentle nitrogen stream to dryness. Afterwards each sample vial was filled up with water to a final volume of 0.2 mL and vortexed. As a result, SPE samples were enriched five or 50 times.

*2020 experiment*

Dependent on nominal samples concentration, 2 g (250 ng·L$^{-1}$ samples) or 25 g (blanks) of sample was weighed into a PP-Greinertube and spiked with 25 µL of internal standard solution (Diclofenac (13C6) 20 ng.mL$^{-1}$; prepared from 35361-10MG; BCCD7644, SIAL/Merck). Subsequently, samples were extracted using a SPE 12-port vacuum manifold system. SPE cartridges (Strata X 60 mg/3 mL) were conditioned using two times 1 mL of methanol, and two times 3 mL of water. Then, samples were passed through the cartridges (3-4 drops per second), cartridges were washed with two times 3 mL of water, once with 1 mL 50% methanol, dried under vacuum for about 1 min and eluted sequentially with three times 0.4 mL of methanol into a glass vial. Extracts were reduced under a gentle nitrogen stream to dryness. Afterwards each sample vial was filled with water to a final volume of 0.5 mL and vortexed. As a result, samples were enriched four or 50 times.

**LC-MS/MS analysis of diclofenac**

*2018 experiment*

Chemical analysis of diclofenac was performed in positive mode with an ESI-source on an Agilent G6495B Triple Quadrupole (QQQ) Mass Spectrometer coupled to an UHPLC-System for chromatographic separation (Agilent 1290 Infinity II, Waters ACQUITY UPLC BEH Shield RP18, 130Å, 2.1 mm X 100 mm, 1.7 column (p/n 186002854) with a 5 mm precolumn (p/n 186003977)). A binary gradient method with flow rate of 0.5 mL/min was run with 0.1% formic acid as Solvent A and methanol as Solvent B. Column temperature was kept at 40°C. The gradient profile was programmed as follows: Prerun 3 min 10% B; 10% B (0-0.19 min); 10–30% B (0.19–2 min); 30-60% B (0.2–10.2 min.); 60-100% B (10.2–11 min); 100% B (11-14 min). Sample injection volume was 50 µL.

An internal standard (IS) calibration with matching D4 marked analyte and interpolation using a linear regression model with six levels between 20 and 1250 $ng \cdot L^{-1}$ was performed using Agilent MassHunter Quantitative Analyses. Identification of diclofenac was performed based on two Multiple Reaction Monitoring (MRM) transitions (quantifier 296>214, qualifier 296>215; IS quantifier 300>218; IS qualifier 300>219) between the precursor ion and the two most abundant product ions. The first transition was used for quantification purposes ("quantifier"), whereas the second ("qualifier") was used to confirm the presence of the target compound in the sample. Quantified diclofenac was verified further by comparing the retention time (11.9 min) of the corresponding standard and the ratio between two ion transitions recorded (± 30%) in the standard and water samples. Limit of detection (LOD) was determined at a signal-to-noise (S/N) ratio of 3:1 and limit of quantification (LOQ) at a S/N ratio of 10:1. LOQ for the 50 fold enriched sample was 0.5$ng \cdot L^{-1}$.

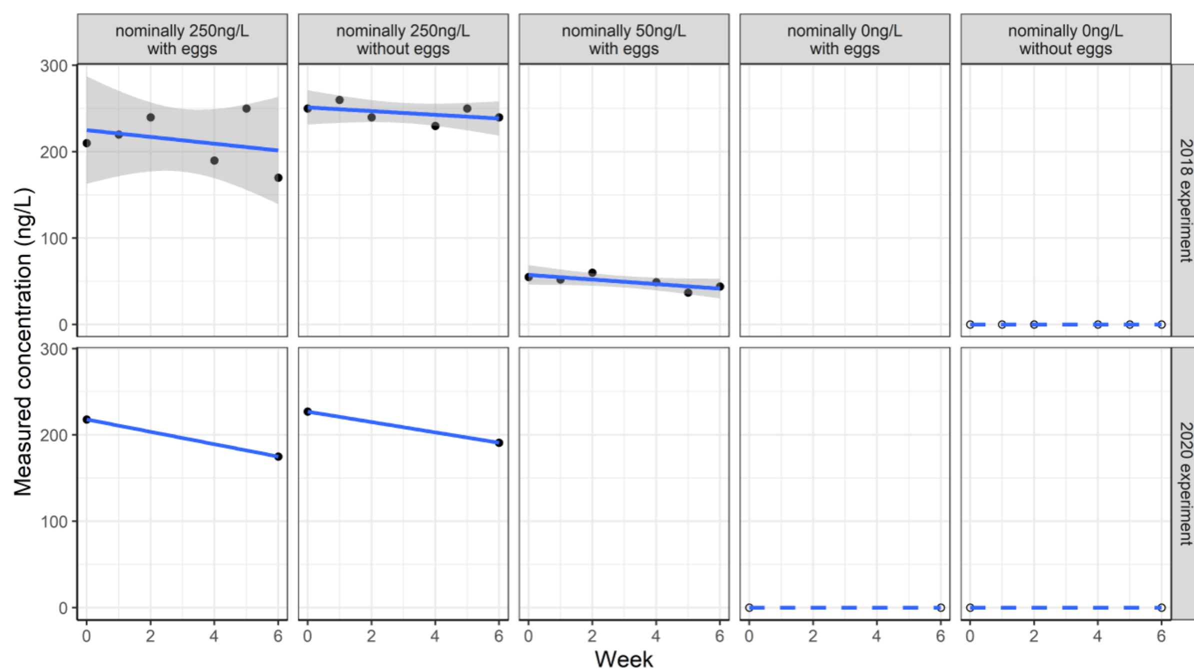
*2020 experiment*

Several methods changes occurred compared to the 2018 spawning season. Chemical analysis of diclofenac was performed in positive mode with an ESI-source on an Agilent G6495ATriple Quadrupole (QQQ) Mass Spectrometer coupled to an UHPLC-System for

chromatographic separation. Chromatography involved the same column, temperature and mobile phases as before but we used a slightly different mobile phase protocol: 10% B (0-0.19 min); 10–30% B (0.19–2 min); 30-60% B (0.2–10.2 min); 60-100% B (10.2–14.2 min.); 100% (14.2-16 min); 100-10% B (16–16.1 min); 10% B (16.1-20 min). Sample injection volume was again 50 µL.

The IS calibration used a matching 13C marked analyte and interpolation using a linear regression model with 11 levels between 10 and 2800 $ng \cdot L^{-1}$. Data analyses were performed using Agilent MassHunter Quantitative Analyses. Identification of diclofenac was based on the MRM transitions: quantifier 296>214, qualifier 298>214; IS quantifier 302>220; IS qualifier 304>222. The retention time (11.4 min) was shifted earlier compared to the 2018 analysis. LOQ for the 50 fold enriched sample was again 0.5 $ng \cdot L^{-1}$.

**Figure S1**. Measured diclofenac concentrations in treatments and controls. Values in the panels nominally 0ng·L$^{-1}$ with eggs and without eggs were below the limit of quantitation of 0.5ng·L$^{-1}$. Open dot and dashed line indicates sampling points with data below limit of quantitation.



### Modelling experiment-long growth of brown trout

*Model building and selection*

To model growth of brown trout we filtered the dataset to include only individuals from the 0 $ng \cdot L^{-1}$ diclofenac treatment with more than 8 length measurements. This resulted in a dataset of 161 individuals from 62 sibgroups with 2114 length values ranging from 9-25 per individual (mean=13.1, median=13). Restricted maximum likelihood estimation was then used to fit polynomial mixed effects-models via polynomial regression of the length of hatchlings over time with the *lme4* package (Bates et al., 2015). Body length was used as the response variable, with incubation time, length at hatching, yolk sac volume at hatching (all scaled and centered with the scale function in base R, and believed to influence growth rates of juvenile fish (Gilbey et al., 2009; Iguchi, 2011; Phillips et al., 1995; Pine & Allen, 2001; Yamagishi, 1969; but see Solberg et al., 2014)) and the number of days post-fertilisation as predictor variables. First-order interactions between the number of days post-fertilisation and the three other predictor variables were also included. Models were built incorporating the number of days post-fertilisation as a linear predictor variable, transformed via natural logarithm, then with polynomials of the natural logarithm-transformed days post-fertilisation up to 5$^{th}$ degree polynomials, which allow increasing model flexibility to better fit the data. Model fit failed above 5$^{th}$ degree polynomials, thus higher degree polynomials were not tested. For all models individual identity was included as a random effect to account for the non-independence of samples due to the repeated measures design. Random slope, random intercept, and models incorporating both were tested, and if necessary different nonlinear optimisers were tested to allow model fitting (testing optimisers offered in the *lme4* and *optimx* (Nash & Varadhan, 2011) packages. Multicollinearity is not as relevant in predictive modelling (Shmueli, 2010), but nonetheless selected models' (see below) variance inflation factor was checked and values higher than 10 were excluded except in cases where this was driven by collinearity between main effect variables and interaction terms containing those, or between variables and powers of those. No variables were excluded following these criteria.

Models then underwent nested cross-validation whereby the dataset was split into a training and testing set containing 80% and 20% of the data respectively, partitioning based on individual identity to avoid data leakage (Samala et al., 2020). We then performed 4-fold cross-validation on the training set with a custom function modified from (Olsen, 2021), generating the following model metrics: conditional coefficient of determination ($R^2$), root mean standard error (RMSE), MAE (mean absolute error), and MAPE (mean absolute percentage error). These were used to quantify model fit and error rates; see Table S1 for definitions and functions used. A model was then selected which best fit the data (henceforth: "the selected model"). Polynomials and random effect structure were chosen based on both the generated model metrics and AICc as calculated on the entire dataset using the AICc function in the *MuMIn* package (Barton, 2020). Main effect variables and their interactions were chosen via forward and backward variable selection using the step function from *lmerTest* function (Kuznetsova et al., 2017). The generalisability of the selected model was then assessed by comparing predicted response variables generated from the entire training set to those in the testing set, then iterating this process 100 times and averaging model metrics generated. The model was checked for overfitting by comparing model metrics generated by the 4-fold cross validation and test set iterations, which would be revealed by large differences in generated model metrics.

We compared growth patterns in the 2018-19 and 2020-21 seasons, and therefore test the extent to which growth patterns varied by experiment. Growth patterns show bimodal trajectories so we explored if this was due to parental identity by modelling growth per dam and sire. To assess the effect of each variable on growth patterns we performed likelihood ratio tests (hereafter "LRTs") with the anova function in base R between the selected model including and omitting the variable from the model. Finally, we tested the effect of diclofenac on growth patterns with analysis of variance via the Anova function from the *car* package (Fox & Weisberg, 2019). We report the change in AIC (hereafter "ΔAIC") as AIC of model omitting variable minus AIC of model including variable for each term of interest, calculated by the anova function from the LRT. Models

with ΔAIC <2 are considered to not be significantly different from one another. Season, dam identity, and sire identity were modelled as random effects with random intercepts, while diclofenac treatment was modelled as a fixed effect in interaction with all main effects. Filtering the dataset for individuals with more than 8 length measurements left highly unbalanced treatment groups (0 $ng \cdot L^{-1}$ diclofenac n=160, 50 $ng \cdot L^{-1}$ diclofenac n=24, 250 $ng \cdot L^{-1}$ diclofenac n=143), and the 50 $ng \cdot L^{-1}$ treatment is confounded with experiment, therefore all individuals treated with 50 $ng \cdot L^{-1}$ treatment were removed from analyses. In all cases where variables of interest were modelled as fixed effects in interaction with others type 3 sums of squares were calculated first, and these are reported if significant, then type 2 sums of squares were calculated and are reported if no evidence was found for a significant interaction with the variable of interest. For all analyses model assumptions were found to not be violated after visual inspection of residuals and quartile-quartile plots.

**Table S1.** Model metrics used to quantify errors and evaluate models.

| **Metric** | **Description** (Hyndman & Koehler 2006) | **Functions used - R package** |
|---|---|---|
| Conditional coefficient of determination ($R^2$) | The proportion of variation in response variable predictable from the predictor variables (fixed and random) | r.squaredGLMM - *MuMIn* (Barton, 2020); performance – *performance* (Lüdecke et al., 2021) |
| Root Mean Standard Error (RMSE) | the average error performed by the model in predicting the outcome for an observation | rmse – *hydroGOF* (Zambro-Bigiarini, 2020); performance_rmse - *performance* (Lüdecke et al., 2021) |
| Mean Absolute Error (MAE) | as RMSE but less sensitive to outliers | mae – *hydroGOF* (Zambro-Bigiarini, 2020); performance_mae – *performance* (Lüdecke et al., 2021) |
| Mean Absolute Percentage Error (MAPE) | average prediction error calculated as a percentage after pairs of results containing NAs are removed, thus slightly underestimates error | MAPE – *MLmetrics* (Yan, 2016) |

*Performance of selected model*

After testing 18 models of growth one was selected based on model metrics and AIC (Table S2). Model metrics generated during test set iterations are almost identical to those generated during 4-fold cross validation (Table S2), suggesting no model overfitting. After 100 iterations the predicted results of length showed a low mean average percentage error of 3.8% ±0.037 from the ground truth results. The selected model gives predictive function (1)

$$l(d, T, h) = \mu + \beta_T \cdot T + \beta_h \cdot h + \beta_{T \cdot \log(d)} \cdot T \cdot \log(d) + \beta_{h \cdot \log(d)} \cdot h \cdot \log(d) + \beta_{d\alpha} \log(d) + \beta_{d\beta} (\log(d))^2 + \beta_{d\gamma} (\log(d))^3 \quad (1)$$

where $l$ is the length of the fish at the number of days post-fertilisation $d$, $\mu$ is the intercept term, $\beta_i$ is the regression coefficient of incubation time in degree days post-fertilisation, $\beta_h$ is the regression coefficient of length at hatching, $\beta_{i \cdot \log(d)}$ is the regression coefficient of the interaction between incubation time in degree days post-fertilisation and the logarithm of the number of days post-fertilisation, $\beta_{h \cdot \log(d)}$ is the regression coefficient of the interaction between length at hatching and the logarithm of the number of days post-fertilisation, $\beta_{d\alpha}$ is the regression coefficient of the logarithm of number of days post-fertilisation, $\beta_{d\beta}$ is the regression coefficient of the squared logarithm of the number of days post-fertilisation $\beta_{d\gamma}$ is the regression coefficient of the cubed logarithm of the number of days post-fertilisation, and $T$, and $h$ are values measured from a new fish: respectively the incubation time in degree days post-fertilisation, and length at hatching in mm. Stepwise variable selection with the step function removed yolk sac volume at hatching from the final model as it did not improve model fit. The predictive function is visualised in Figure S2. Coefficients and significance of variables are given in Table S3.

**Table S2**. Performance metrics of models built and tested to best fit *Salmo trutta* growth patterns across both experiments. Models with 4$^{th}$ degree polynomials of log-transformed days post-fertilisation and above failed to converge and are not shown. T = incubation time in degree days post-fertilisation, L = length at hatching, V = yolk sac volume at hatching, D = days post-fertilisation; Int = Intercept, Sl = Slope. Relationship type gives the format D appears in the model: Linear = linear, Log(D) = logarithm-transformed D, 2$^{nd}$ poly log(D) = logarithm-transformed D and 2$^{nd}$ polynomial of logarithm-transformed D, 3$^{rd}$ poly log (D) = logarithm-transformed D and 2$^{nd}$ and 3$^{rd}$ polynomial of logarithm-transformed D. Performance metrics displayed are conditional coefficient of determination (R2), root mean standard error (RMSE), mean absolute error (MAE), with 95% confidence intervals. Cross-validation metrics are generated from averaged 4-fold cross-validation and are used for model selection; test set metrics are generated from averaged results of 100 train/testing set splits and are used for assessment of model generalisability. Selected models highlighted in bold.

| Fixed effects | Relation-ship type | Random effects | AIC | $R^2$ | RMSE | MAE | $R^2$ | RMSE | MAE |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Cross-validation | | | Test set | | |
| T + L + V + D + T:D + L:D + V:D | Linear | Int | 4225 | 0.881 | 0.925 | 0.655 | | | |
| T + L + V + D + T:D + L:D + V:D | Linear | Int + Sl | 3228 | 0.902 | 0.920 | 0.642 | | | |
| T + L + V + D + T:D + L:D + V:D | Linear | Sl | 3890 | 0.900 | 0.919 | 0.645 | | | |
| T + L + V + T:D + L:D + V:D + log(D) | Log(D) | Int | 3788 | 0.900 | 0.863 | 0.617 | | | |
| T + L + V + log(D) + T:D + L:D + V:D | Log(D) | Int + Sl | 2874 | 0.949 | 0.864 | 0.599 | | | |
| T + L + V + T:D + L:D + V:D + log(D) | Log(D) | Sl | 3706 | 0.904 | 0.861 | 0.615 | | | |
| T + L + V + T:D + L:D + V:D + log(D) + $\log(D)^2$ | 2nd poly log(D) | Int | 3353 | 0.916 | 0.800 | 0.581 | | | |
| T + L + V + T:D + L:D + V:D + log(D) + $\log(D)^2$ | 2nd poly log(D) | Int + Sl | 2572 | 0.949 | 0.798 | 0.575 | | | |
| T + L + V + T:D + L:D + V:D + log(D) + $\log(D)^2$ | 2nd poly log(D) | Sl | 3270 | 0.919 | 0.799 | 0.581 | | | |
| T + L + V + T:D + L:D + V:D + log(D) + $\log(D)^2$ + $\log(D)^3$ | 3rd poly log(D) | Int | 3348 | 0.916 | 0.797 | 0.579 | | | |
| T + L + V + T:D + L:D + V:D + log(D) + $\log(D)^2$ + $\log(D)^3$ | 3rd poly log(D) | Int + Sl | 2779 | 0.950 | 0.813 | 0.585 | | | |
| T + L + V + T:D + L:D + V:D + log(D) + $\log(D)^2$ + $\log(D)^3$ | 3rd poly log(D) | Sl | 3266 | 0.919 | 0.797 | 0.579 | | | |
| **T + L + T:D + L:D + log(D) + $\log(D)^2$ + $\log(D)^3$** | 3rd poly log(D) | **Sl** | **3257** | **0.919** | **0.788** | **0.572** | **0.923** | **0.778** | **0.572** |

**Figure S2.** Growth of *Salmo trutta* hatchlings unexposed to diclofenac per experiment. Plotted are the predicted results of the selected model formula, modified to include experiment as a fixed effect in interaction with all main effects. Results were then generated using the population average incubation time and length at hatching calculated from the same individuals. The results are plotted against data from all individuals from the 0ng·L$^{-1}$ diclofenac treatment group.

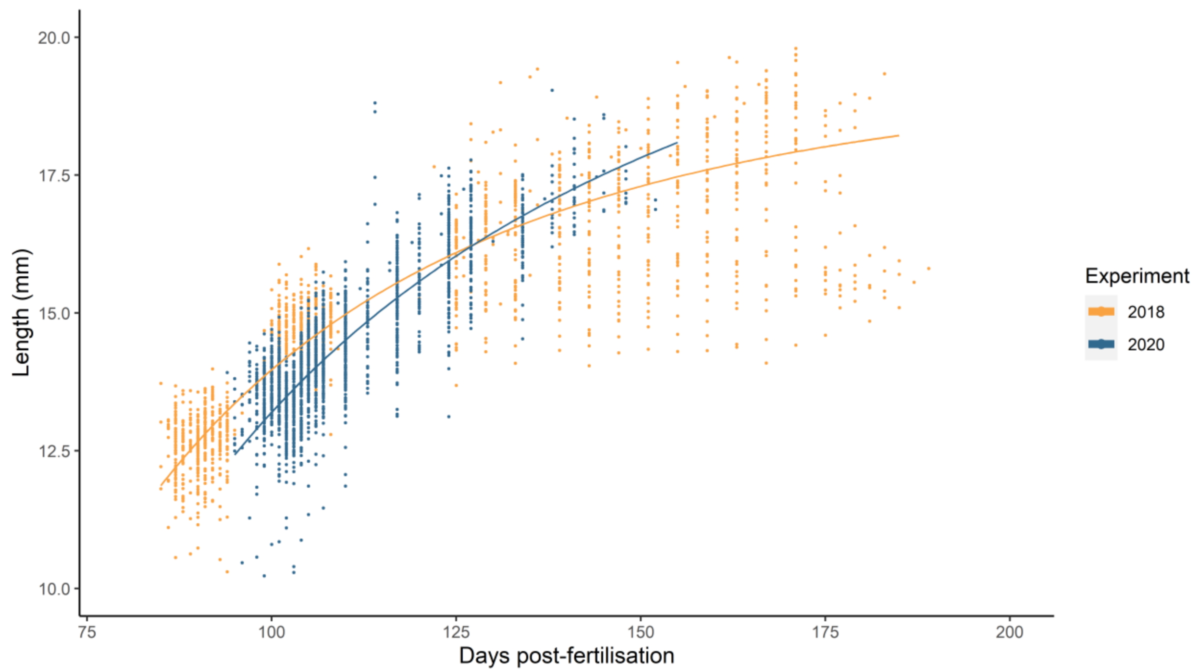


**Figure S3**. Growth of *Salmo trutta* hatchlings unexposed to diclofenac. To plot, the selected model was trained on all individuals with more than 8 length datapoints from the 0ng·L$^{-1}$ diclofenac treatment group. Results were then generated using the population average incubation time and length at hatching calculated from the same individuals. The results are plotted against data from all individuals from the 0ng·L$^{-1}$ diclofenac treatment group.

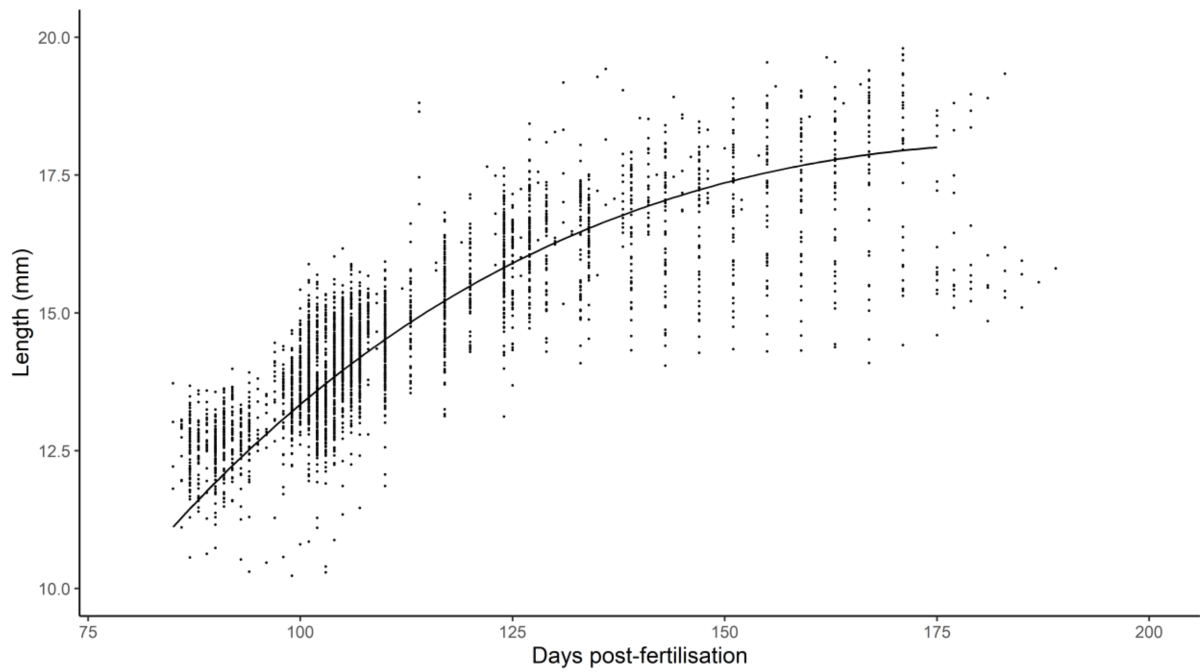


**Table S3.** Coefficients of model variables in the selected model predicting growth in *Salmo trutta* hatchlings unexposed to diclofenac. *P* values are shown to 3 decimal places when significant ($\alpha$=0.05).

| Notation | Variable | Coefficient | Standard error | *p* |
|---|---|---|---|---|
| $\mu$ | Intercept | 327 | 303 | 0.28 |
| $\beta_{i}$ | Regression coefficient of incubation time in degree days post-fertilisation | -6.69 | 0.515 | <0.001 |
| $\beta_{h}$ | Regression coefficient of length at hatching | -1.45 | 0.507 | 0.004 |
| $\beta_{i \cdot log(d)}$ | Regression coefficient of interaction between incubation time in degree days post-fertilisation and logarithm of the number of days post-fertilisation | 1.34 | 0.107 | <0.001 |
| $\beta_{h \cdot log(d)}$ | Regression coefficient of interaction between length at hatching and logarithm of the number of days post-fertilisation | 0.394 | 0.107 | <0.001 |
| $\beta_{d\alpha}$ | Regression coefficient of logarithm of number of days post-fertilisation | -254 | 188 | 0.18 |
| $\beta_{d\beta^2}$ | Regression coefficient of logarithm of squared number of days post-fertilisation | 63.2 | 38.8 | 0.10 |
| $\beta_{d\gamma^3}$ | Regression coefficient of logarithm of cubed number of days post-fertilisation | -4.97 | 2.67 | 0.06 |

**Table S4.** Statistical results of the effect of diclofenac exposure, dam and sire identity on longevity (after square transformation) of individuals that survived until the start of regular longevity recording. Offspring of two sires and six dams survived until this point. Model sample size is 123.

| Effect tested | $\chi^2$ | $DF_{LRT}$ | *p* | $\chi^2$ | $DF_{LRT}$ | *p* |
|---|---|---|---|---|---|---|
| | 2018 Experiment | | | 2020 Experiment | | |
| DCF | 0.1 | 2 | 0.97 | | | |
| Dam | <0.1 | 1 | 1.0 | | | |
| Sire | <0.1 | 1 | 0.92 | | | |
| DCF × Dam | <0.1 | 5 | 1.0 | | | |
| DCF × Sire | 0.2 | 5 | 1.0 | | | |

**Figure S4**. Boxplot of longevity of *Salmo trutta* hatchlings untreated and treated with diclofenac that survived until the start of regular longevity recording 126dph. Mean per group is shown as a red dot.

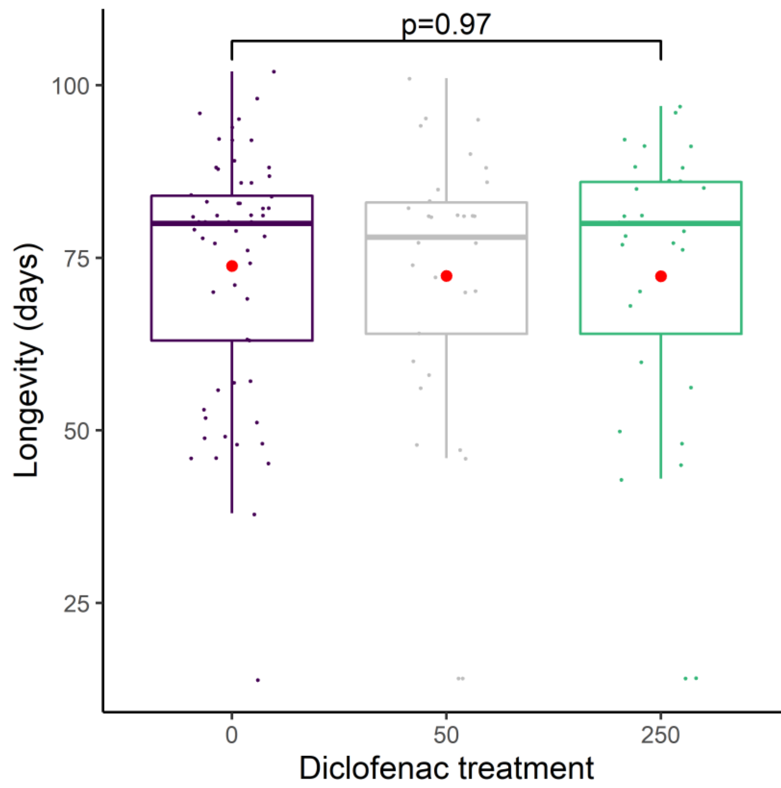


**Figure S5.** Box- and barplots of the effect of dam identity (X axis for all panels) on (A) egg size in mm3, (B) hatching success, (C) hatching time in degree days, (D) length at hatch in mm, (E) yolk sac volume at hatching in mm3, (F) longevity in days, (G) growth until 14 days post-hatching in mm. Dams coloured in orange (2018) and blue (2020). See Table 1 for statistics. Dam KLS had low hatching success, therefore was removed from analyses and is included in panel (A) and (B) only.

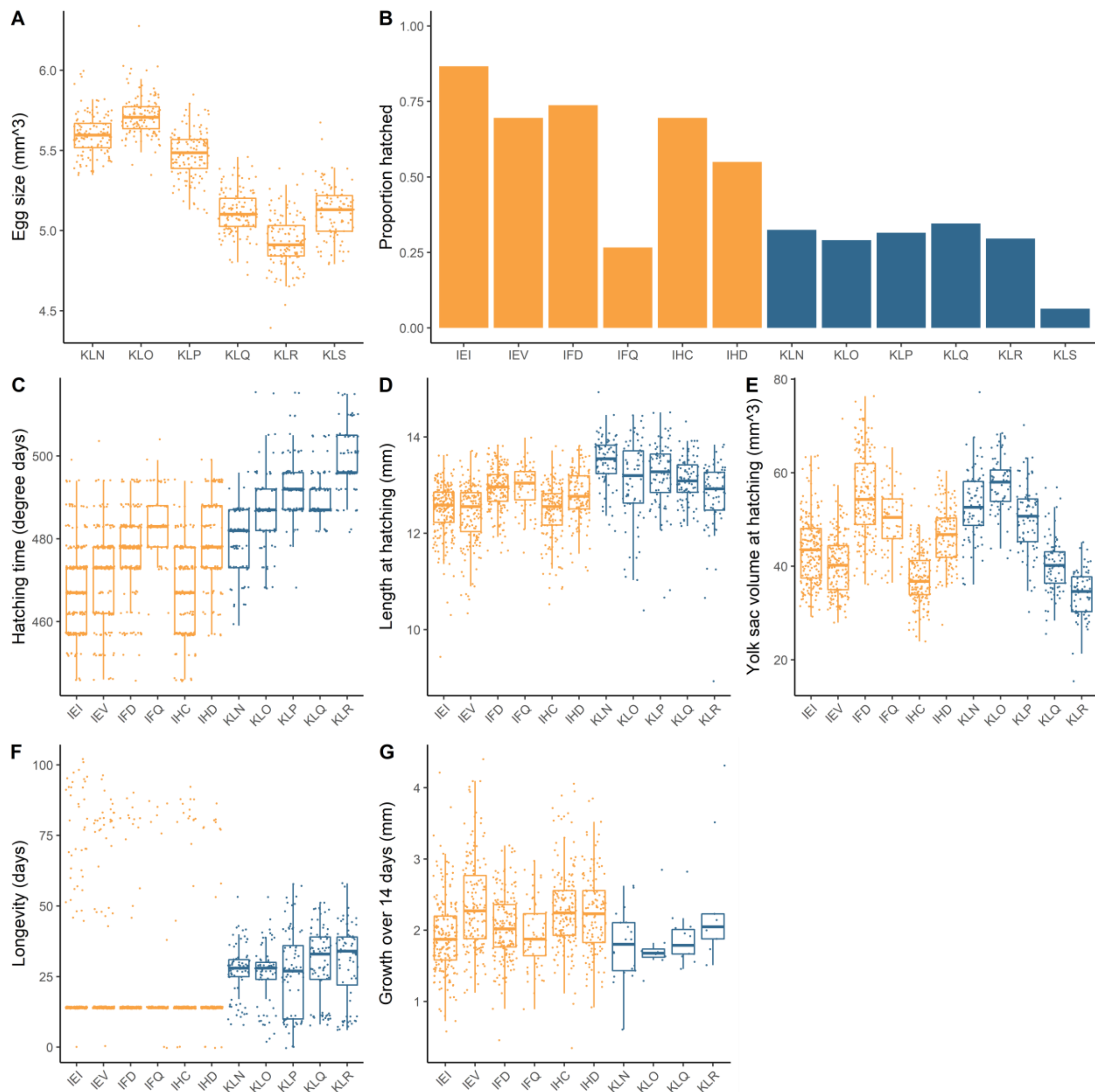


**Figure S6.** Growth in *Salmo trutta* hatchlings per dam identity in the 2018 experiment. Plotted are the predicted results of the selected model formula, modified to include dam as a fixed effect in interaction with all main effects. The model was trained on all individuals with more than 8 length measurements, removing offspring of dams with fewer than 5 offspring, then results were generated using the population average incubation time and length at hatching calculated from all individuals with more than 8 length measurements. Results are plotted against all data from the 2018 experiment.

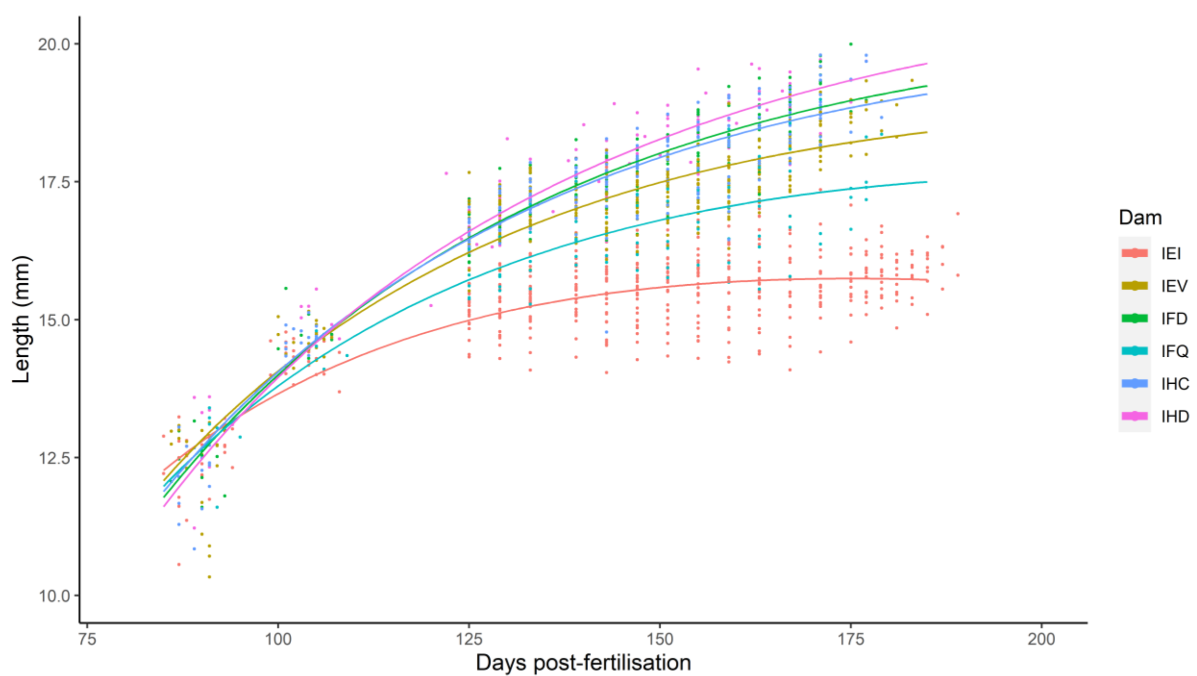


**Figure S7.** Box- and barplots of the effect of sire identity (X axis for all panels) on (A) egg size in mm3, (B) hatching success, (C) hatching time in degree days, (D) length at hatch in mm, (E) yolk sac volume at hatching in mm3, (F) longevity in days, (G) growth until 14 days post-hatching in mm. Sires coloured in orange (2018) and blue (2020). See Table 1 for statistics. Sire 2033 had low hatching success, therefore was removed from analyses and is included in panel (B) only.

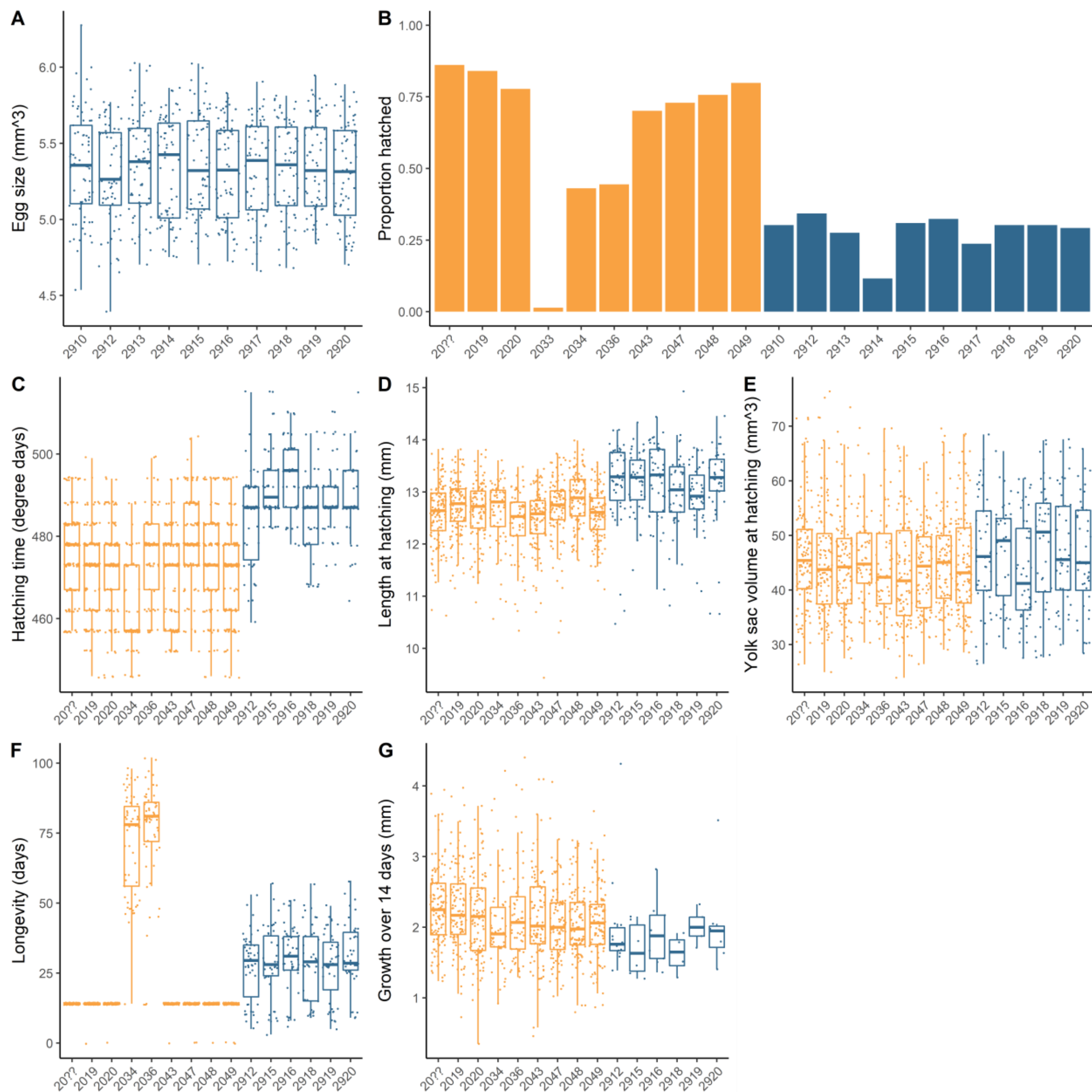
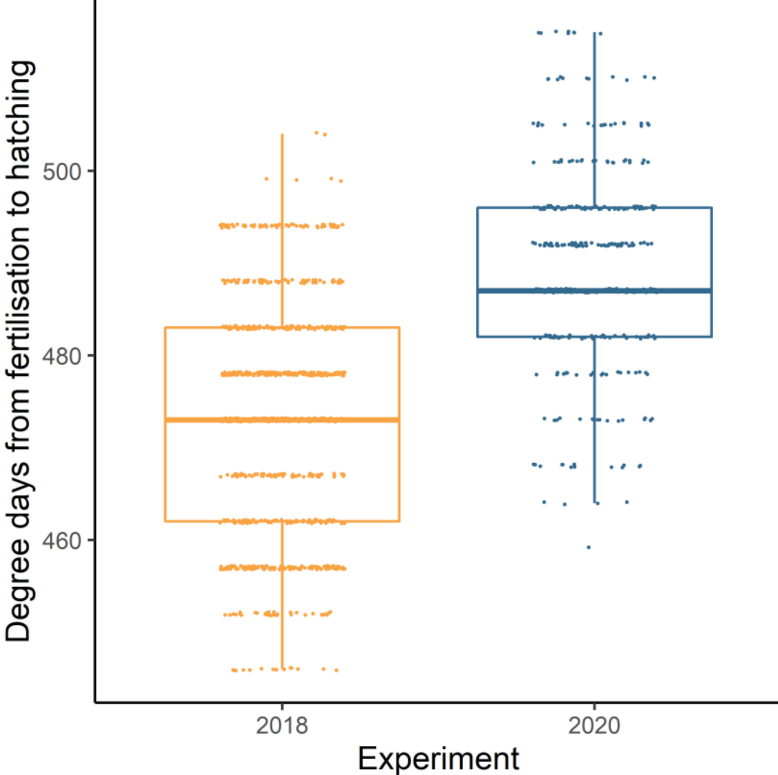


**Figure S8.** Boxplot of hatching time in the two experiments.



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# Chapter III – Effects of co-occurring micropollution by diclofenac and PKD infection

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Chapter status: unpublished

**Author Contributions**

Fish were raised and data collected by AU and LMC in the 2014-15 experiment, and HP and VA in the 2018-19 experiment. ELMV organized and supervised the diclofenac measurements. HP, AU, and CW conceptualised and planned the study, with oversight from HH who helped obtaining *Tetracapsuloides bryosalmonae*-infected bryozoan populations. Data was analysed and the manuscript written by HP and CW. All authors contributed to the article and approved the submitted version.

**Abstract**

Declines have been observed in many populations of brown trout (*Salmo trutta*), an ecologically and socioeconomically important species, most notably in Switzerland where populations are believed to have fallen by up to 80% since the 1980s. These population declines have frequently been attributed to proliferative kidney disease (PKD), an emerging disease caused by the myxozoan parasite *Tetracapsuloides bryosalmonae* resulting in kidney inflammation and frequently death. However, stressors do not occur singly and the effect of multiple stressors on severity is little explored. We study the possible role of the non-steroidal anti-inflammatory drug diclofenac on PKD symptoms. This common micropollutant has been suggested to have toxic effects, including on the kidney where the effects of PKD are concentrated. We conducted two experiments exposing 0+ brown trout to *T. bryosalmonae* infection, diclofenac pollution at environmentally relevant concentrations, both, or neither, and measured the effects on five indicators of PKD. Sex effects on stressor tolerance can be important, threatening population viability if severe enough to cause biased sex ratios. We found, however, that neither diclofenac or PKD had sex-specific effects on any measured variable. Alone diclofenac did not affect length, or haematocrit, but did raise weight-length ratio ($K_i$) by 0.6% in one experiment. *T. bryosalmonae* exposure reduced haematocrit, increased nephrosomatic index, and increased $K_i$ in one experiment. At the number of days post-infection at which sampling occurred nephrosomatic index significantly predicted $K_i$ and explained 28% of variation in it, strongly suggesting PKD was causing increased nephrosomatic index in both experiments. Diclofenac had divergent effects on *T. bryosalmonae*-exposed fish in the two experiments, increasing $K_i$ (and therefore, we argue, nephrosomatic index) in one and having no effect on nephrosomatic index in the other. Divergent effects of diclofenac on *T. bryosalmonae*-exposed fish could be explained by the concentration of diclofenac used and / or the water temperature, both of which differed in the experiments. Diclofenac-exposure also reduced the negative effect of PKD on haematocrit. We conclude that diclofenac can exacerbate some negative effects of PKD, and that higher stream water

temperatures and pollutant concentrations are likely to push diclofenac to have this effect more often, this study does not show that diclofenac consistently increases severity of PKD in brown trout.

**Introduction**

Stressors do not affect wild populations singly. Environmental conditions can determine disease virulence or severity resulting in nonlinear effects (Segner et al., 2014). Temperature is the most widespread determinant of disease virulence (Harvell et al., 2002; Altizer et al., 2013); however, chemical pollution is pervasive and toxic effects are common (Malaj et al., 2014; Bernhardt et al., 2017; EEA, 2018). Understanding how such stressors interact and their effects on wildlife can be important to protect wild populations of conservation concern.

Brown trout (*Salmo trutta*) is a widespread species native to Europe but that has been translocated across the globe (Halverson, 2008; Hunt and Jones, 2018; Hasegawa, 2020). Within its native range it is a keystone species and of great socioeconomic value (Elliott, 1989). In recent decades declines have been noted in many European populations (Burkhardt-Holm et al., 2005; Lahnsteiner et al., 2009; Waldner et al., 2020), most notably in Switzerland where the census size is believed to have fallen by up to 80% since the 1980s (Burkhardt-Holm et al., 2005; OFEV, 2022). In Switzerland proliferative kidney disease (PKD), an emerging disease caused by infection by the myxozoan parasite *Tetracapsuloides bryosalmonae* (Hedrick et al., 1993), is considered to be playing a major role (Wahli et al., 2002, 2007; Borsuk et al., 2006; Burkhardt-Holm and Scheurer, 2007; Schager et al., 2007). PKD manifests primarily as inflammation in renal tissue (Figure S1) caused by proliferation of pre-sporogenic phases of *T. bryosalmonae* in the host (Hedrick et al., 1993; Okamura et al., 2011). The effects of PKD are known to depend on environmental conditions (Hedrick et al., 1993), with strong temperature effects on mortality rates and disease progression (Bettge et al., 2009a; Okamura et al., 2011). However, as chemical pollution is known to put important pressures on the natural environment (Malaj et al., 2014; Bernhardt et al., 2017), and more than half of European rivers have poor chemical state (EEA, 2018), chemical pollutants may also be affecting the severity of PKD in *T. bryosalmonae*-infected populations.

The nonsteroidal anti-inflammatory drug diclofenac is a likely contender in potential PKD-micropollutant interactions. It has already been identified as the major cause of the Indian vulture

crisis, where the vulture population in the Indian subcontinent declined by over 90% (Prakash et al., 2003), due to renal failure as a consequence of exposure to veterinary diclofenac through carcasses (Oaks et al., 2004). In Europe freshwater diclofenac pollution is widespread (Loos et al., 2009), including in Switzerland (Buser et al., 1998), as removal via waste water treatment plants is incomplete, and usually occurs in the $ng^{-L}$ to low $\mu g^{-L}$ range (Ternes, 1998; Marsik et al., 2017). While the no observed effect concentration is still debated (Wolf, 2021), diverse toxic effects at environmentally relevant concentrations have been shown in both model and non-model freshwater organisms. These include structural changes to organs (Triebskorn et al., 2004; Hoeger et al., 2005; Gröner et al., 2017), including to the kidney as in vultures (Hoeger et al., 2005; Triebskorn et al., 2007), oxidative stress (McRae et al., 2018; Bio and Nunes, 2020), alteration of enzyme activity (Guiloski et al., 2017), endocrine disrupting effects (Hong et al., 2007; Gröner et al., 2015, 2017), and induction of slight yet significant precocious hatching (Palejowski et al., in prep). In Switzerland, a maximum concentration of 50 $ng \cdot L^{-1}$ in surface waters is legally mandated, to reflect this (Swiss Federal Council, 1998). As both diclofenac and PKD cause kidney damage an amplification of the negative effect of the micropollutant and disease is possible: chronic diclofenac pollution may be weakening fish and increasing the severity of PKD. PKD has been detected across Europe and North America, therefore the action of diclofenac pollution on PKD may be of widespread conservation concern (Hedrick et al., 1993).

It is increasingly recognised that reactions to environmental stresses are frequently sex specific (Stinson, 1985; Pérez-Cerezales et al., 2018). Sex specific life history strategies and susceptibility to environmental stressors have recently been identified at early developmental stages in brown trout and several closely related salmonids (Maitre et al., 2017; Nusbaumer et al., 2021a; Palejowski et al., 2022). These can have demographic consequences that amplify the relevance of PKD, or diclofenac, as a stressor of conservation concern (Wedekind, 2012).

Here we use wild brown trout populations to experimentally test i) whether exposure to an environmentally relevant concentration of diclofenac increases severity of PKD, and ii) whether the sexes differ in their responses to the pollutant or the pathogen.

## Methods

### 2014-15 experiment

#### *Producing study populations*

Brown trout embryos were artificially fertilised during the 2014 breeding season from breeders caught via backpack electrofishing in various tributaries of the river Aare, canton Bern. Hatched alevins were raised in the *Fischereistützpunkt Reutigen* in a large tank with circulating water current. On the 17th July 2015 704 0+ brown trout were then moved to a temperature- and light-controlled room where they were haphazardly distributed to 12 pump-filtered, aerated, 200L aquaria filled with 130L of tap water in a temperature and light controlled room, with 58 or 59 fish per aquaria. Light conditions were a 12:12 light:dark cycle. Temperature was held at 12°C for one month. Temperature was then raised to 18°C by increasing 1°C per day. Throughout the experiment fish were fed *ad libitum* with size-appropriate commercial dry fish food. One of four treatments was then applied to each aquarium, with two aquaria per treatment: *T. bryosalmonae* exposure (hereafter referred to as "PKD"), diclofenac, PKD × diclofenac, and non-treated controls. The position of each treatment in the room was randomly chosen. EE2 was included for a separate manuscript and is not considered in the context of this study.

#### *Treatment exposure*

On the 28th August 2015, bryozoan were collected from two streams known to be infected with *Tetracapsuloides bryosalmonae* (see Supporting Information). They were transferred to the University of Lausanne on ice, where they were crushed into a paste using a pestle and mortar and stored in a 2L beaker. The solution was thoroughly mixed then about evenly distributed to the

tanks designated for PKD treatment. The filters of the exposed tanks were turned off for two hours to give parasitic spores enough time to infect the fish (McGurk et al., 2006).

Two weeks prior to infection with *T. bryosalmonae* spores, diclofenac sodium salt (SIGMA-ALDRICH, USA; CAS: 15307-79-6) was dissolved in tap water to give a spike solution. This was then added to aquaria designated for diclofenac treatment such that final diclofenac concentrations were 200 $ng \cdot L^{-1}$.

*Measuring traits*

On the 12th November 2015 (76 days post spore-exposure) all surviving trout in the diclofenac, PKD, or PKD × diclofenac treatments were euthanised with an overdose of KoiMed® Sleep (KOI&BONAI, Bühlertann, Germany). They were photographed, from which length was measured (most anterior point of snout to most posterior point in longest lobe of caudal fin), and wet weight was measured. Proliferative kidney disease causes inflammation and swelling of the kidney, therefore we calculated weight-length ratio as $K_i = (\frac{W_i}{L_i^3}) \times 100$ where W is the weight (g) and L is the total length (cm) for each fish (Musseau et al., 2018). The $K_i$ is therefore used as a proxy for PKD-associated swelling.

2018-19 experiment

*Producing study populations*

Information on producing the study population has been published as part of the laboratory study in Palejowski et al. (2022), reported here in brief. Breeding adults were collected via backpack electrofishing then kept in the *Fischereistützpunkt Reutigen* until gamete stripping. Adult breeders were then narcoticised using Tricaine Methanoesulfonate (i.e. 0.075 g/L buffered with 0.15 g/L $NaHCO_3$) and stripped for gametes. Gametes were fertilised using typical hatchery breeding methods by mixing all gametes from one streamlet in a container and adding water. Palejowski et al. (2022) reported that the study population was created through full-factorial breeding with adults from the Kiese river. However, microsatellite analysis in the present study

reveals that this was not the case, i.e., that the fish stem from routine hatchery breeding of from one of the tributaries of the Aare river between Thun and Bern (likely due to a labelling error). The peak of hatching occurred on 17th February 2019. Further reports of fish age are based on this hatching date. Post-hatching fry were kept in a common environment in the hatchery in large tanks with a circulating water current, at a water temperature of 9°C, and fed *ad libitum* with size-appropriate commercial dry fish food.

In total 816 0+ brown trout were transferred to a temperature-controlled laboratory at the University of Lausanne at 190 dph (days post hatching). They were haphazardly divided into 12 groups of 68 fish with each group housed in a pump-filtered, aerated, aquaria filled with 120 litres of tap water. A small water pump was included to provide a water current, and a submerged structure and an opaque cover on the front of the aquaria were added to reduce stress in the fish. Fish were allowed to acclimatise in their tanks at a room temperature of 10.4°C for two weeks then the room temperature was raised to a final mean water temperature of 14.6°C (SD = 0.49°C). Water was initially changed daily, then every weekday from 246 dph onwards, changing 15 L until 250 dph then 40 L per change until the end of the experiment. Fish were fed *ad libitum* with size-appropriate commercial dry fish food. Water quality was monitored weekly using JBL PROSCAN® (Germany) test strips and deviations from values safe for freshwater aquaria were corrected if necessary by adding JBL Denitrol® (Germany). Because effects of fish density on length had been observed in the 2014-15 experiment (Figure S5) and are known to affect salmonids (Pickering and Pottinger, 1987; Mazur and Iwama, 1993), fish number per aquaria was equalised once a week by reducing density to the level of the least dense aquaria if mortality had occurred by randomly selecting and euthanising individuals from the higher-density aquaria (see below).

*Treatment exposure*

The 12 aquaria were assigned to one of 6 treatment groups with two replicate aquaria each: one of three environmentally relevant diclofenac concentrations (0, 50, 250 $ng \cdot L^{-1}$) (Huebner et al.,

2015) and either exposed to *T. bryosalmonae* spores or a sham water treatment. Treatments were fully factorial and were assigned to aquaria following a block-wise randomization method whereby each treatment's position in a block of 6 was randomized.

Diclofenac treatment occurred 208 dph. Diclofenac sodium salt (SIGMA-ALDRICH, USA; CAS: 15307-79-6) was dissolved in standardised water (OECD, 1992) to give a 1mg/L solution. This solution was added to the respective tanks to give a final nominal concentration of 50 $ng \cdot L^{-1}$ or 250 $ng \cdot L^{-1}$ in the diclofenac-treated groups. The other tanks were sham treated. Diclofenac solution was then re-added daily in line with the daily volume of water changed to maintain the nominal concentrations.

Treatment with *T. bryosalmonae* spores occurred from 216 to 219 dph. Bryozoans from a population known to be infected with *T. bryosalmonae* (Sieber et al., 2020) were collected and used for exposure to the fish as in the 2014/15 study (Strepparava et al., 2020)(Supporting Information). Sham treatments with standardised water occurred to treatment groups not selected for *T. bryosalmonae* exposure.

*Measuring traits*

Five juvenile trout per aquaria were then randomly selected for sampling every two weeks from 220 dph until the end of the experiment 304 dph. This gave seven sampling timepoints in total collected on the day of diclofenac exposure, then 11, 25, 39, 53, 67, 81, and 95 days post-exposure to diclofenac. Random selection occurred by dividing the aquaria into an eight by ten numbered grid, using a random number generator to select a grid cell, and sampling the fish nearest to that cell. Selected fish were then euthanised with an overdose of KoiMed® Sleep (KOI&BONAI, Bühlertann, Germany) at a concentration of 0.7 ml/L. At timepoint three and all subsequent timepoints blood samples were taken using 80 µL microcapillary tubes to determine haematocrit values, an indicator of red blood cell volume and therefore an important component of blood oxygen carrying capacity known to be fitness-linked (Munkittrick and Leatherland, 1983;

Pearson and Stevens, 1991) and affected by parasite infection (Filipsson et al., 2017). Haematocrit values were measured following Filipsson et al. (2017) as the fractional red cell volume following centrifugation of blood in the capillary tube at 10,000 rpm for five minutes. All fish euthanised at each sampling point were photographed and were measured for weight and body length as before. Two haphazardly selected fish per aquaria then underwent histological analysis for a parallel study on gonad development (Palejowski et al., 2022). The remaining three fish per aquaria had their kidney removed and weighed to calculate nephrosomatic index as kidney weight divided by total body weight. Tissue samples were taken from all fish for genetic sexing following the protocol in Palejowski et al., (2022).

In the 2018-19 experiment, diclofenac concentration in each aquarium was determined by collecting water samples from each tank the day after initial diclofenac spiking (timepoint 0), then 25, 53, and 81 days post-exposure to diclofenac (timepoints 2, 4, and 6). Diclofenac in the samples was quantified by liquid chromatography–mass spectrometry.

*Statistical Analysis*

In weeks three to six after exposure in the 2018-19 experiment, all fish in 5 of the 12 aquaria died for unknown reason. This included both replicate aquaria of the 250 $ng \cdot L^{-1}$ diclofenac treatment uninfected with *T. bryosalmonae,* i.e., the experimental design was no more factorial with regard to the 250 $ng \cdot L^{-1}$ diclofenac treatment. Because a main aim of the study was to test for diclofenac x PKD effects on severity, the remaining 250 $ng \cdot L^{-1}$ diclofenac treatment groups were therefore removed from the analyses.

Linear mixed effects models were constructed using the lme4 package (Bates et al., 2015) to analyse the effect of the variables of interest on length, $K_i$, haematocrit, and nephrosomatic index. Main effect terms for diclofenac exposure and *T. bryosalmonae* infection, both modelled as fixed main effects, were included, as well as a term for the interaction between the two. In models of data from the 2018-19 study a three-way interaction term of timepoint × diclofenac × PKD was

included, as well as all lower-order interaction terms, to investigate whether the effect of diclofenac on PKD severity varies as disease symptoms progress. Aquarium was included as a random effect with varying intercept to account, for example, for density effects, which are known to affect salmonid health and may influence development strategy (Pickering and Pottinger, 1987; Mazur and Iwama, 1993). Model assumptions were checked via visual inspection of residuals and quantile-quantile plots, a 5$^{th}$ root transformation was applied to $K_i$ and a log transformation applied to nephrosomatic index. Variable significance was then assessed using the *Anova* function from the car package, conducting Wald F tests, calculating type 3 sums of squares, and applying a heteroscedasticity-corrected coefficient covariance matrix (based on Long and Ervin (2000)) if the response variable showed heteroscedasticity. Non-significant interaction terms were stepwise removed except the term for diclofenac × PKD, the focus of this study. If after this process no significant interaction terms remained, then significance was calculated with type 2 sums of squares. Tukey post-hoc tests were conducted, and estimated marginal means were obtained, via the *emmeans* function in the emmeans package (Lenth et al., 2022). Models had final sample sizes of 173 (length 2014-15), 172 ($K_i$ 2014-15), 78 (length 2018-19), 78 ($K_i$ 2018-19), 72 (haematocrit), and 58 (nephrosomatic index). All analyses were conducted in R (R Core Team, 2020).

We expect the effects of diclofenac on PKD to be most evident when the disease has developed in the infected fish, not immediately after infection. Therefore, the effect of diclofenac on PKD severity was assessed from the onset of PKD symptoms.

In the 2014-15 experiment tank effects were assessed by building linear mixed effect models of each response variable with terms for PKD and diclofenac treatment, and their interaction, with a random intercept term for aquarium. Significance was assessed using likelihood ratio tests as before.

Sample sizes were not large enough to estimate the effect of sex on response variables in the same model as the diclofenac × PKD interaction. Therefore, linear mixed effects models were constructed to test the effect of sex as a main effect and in interaction with either stressor. Sex

specific effects can arise at early life history stages (Maitre et al., 2017; Nusbaumer et al., 2021a; Palejowski et al., 2022); therefore, models of sex effects were built using individuals from all timepoints of the 2018-19 experiment to detect potential early effects. Models were built with the *lmer* function in the lme4 package with length, $K_i$, haematocrit, and nephrosomatic index as response variables, timepoint, PKD treatment, and sex as predictor variables, modelled as main effects, and including terms for the three-way interaction and all two-way interactions. Identical models were then built replacing PKD treatment with diclofenac treatment. In all models a term for aquarium identity was included also as a random effect with varying intercept. Variable significance was then assessed and reported identically as above. Models had final sample sizes of 189 (length), 180 ($K_i$), 114 (haematocrit including PKD treatment), 134 (haematocrit including diclofenac treatment), and 134 (nephrosomatic index).

**Results**

*2014-15 experiment*

Neither diclofenac exposure at a nominal concentration of 200 $ng \cdot L^{-1}$, *T. bryosalmonae* exposure, nor the interaction between the two had a significant effect on trout length (Table 1) (Figure 2A). Conversely, $K_i$ was 0.6% and 4.9% higher in the DCF-exposed and *T. bryosalmonae*-exposed treatments, respectively, than in unexposed fish, while simultaneous diclofenac and *T. bryosalmonae* exposure resulted in a 13.8% increase in $K_i$ (Table 1A, Figure 1B). No significant tank effects were found in either length (df=1, $\chi^2$=2.0, p=0.16) nor $K_i$ (df=1, $\chi^2$=0, p=1) (Figures S4, S5).

*2018-19 experiment*

As expected, diclofenac concentrations in aquaria that were not exposed to diclofenac remained around 0 $ng \cdot L^{-1}$ (Figure S3). In the treatment groups, levels of measured diclofenac deviated from the nominal value (50 $ng \cdot L^{-1}$) and varied over the course of the experiment. Measured concentrations fell from a mean value of 30 $ng \cdot L^{-1}$ (range = 5.5 to 46.9) to 8.2 $ng \cdot L^{-1}$

(2.9 to 15) then increased over time to a final mean concentration of 106 ng·$L^{-1}$ (84.8 to 117.2) by timepoint 6.

Length, haematocrit values, and nephrosomatic index did not significantly differ between the sexes (Table 2, Figure S8). Overall $K_i$ in males was significantly higher than in females (mean difference = 0.2%). But, the effect of sex on $K_i$ varied over time in the 2018-19 experiment with females' $K_i$ higher than males' at timepoint 1 and lower at timepoint 7 (significant sex and sex × timepoint interaction effects in Table 2; Figure S8F). Due to the absence of significant interactions between sex and either stressor the factor sex was therefore ignored in all further analyses.

PKD symptoms became evident after timepoint 4, as nephrosomatic index in *T. bryosalmonae*-exposed fish showed clear differences from unexposed fish starting at timepoint 5 (Figure S2B). The effects of diclofenac on PKD severity are therefore analysed using fish sampled at timepoints 5 to 7.

Over the course of the infection, *T. bryosalmonae*-exposed fish showed increasing levels of kidney swelling, as measured by nephrosomatic index (Figure S2B). By the end of the observation period (timepoint 7), kidney swelling was common in *T. bryosalmonae*-exposed fish and nephrosomatic index correlated positively with $K_i$ ($R^2 = 0.28$; Figure S6), revealing body swelling in exposed fish. Analysis of variance of the linear regression model of $K_i$ as a function of nephrosomatic index shows that, by the end of the observation period, nephrosomatic index significantly correlated with $K_i$ ($F = 7.6$, $df = 1$, $p = 0.01$).

As expected, average lengths increased over time (Table 2), however this effect was not significant when only considering fish in the last three timepoints (Table 1A). Neither diclofenac exposure, *T. bryosalmonae* exposure, nor their interaction significantly influenced length or $K_i$ in the 2018-19 experiment (Table 1B). However, the treatments affected the fish as haematocrit levels in *T. bryosalmonae*-exposed fish were 23.0% lower than in control fish, while simultaneous exposure to diclofenac and *T. bryosalmonae* resulted in levels 17.7% lower than in control fish (Diclofenac × PKD interaction, Table 1B). *T. bryosalmonae*-exposed fish had haematocrit levels lower than

unexposed fish at timepoint 5, but the difference between them did not significantly change between timepoints 5 and 7 (no significant timepoint x PKD interaction in Table 1B; Figure S7). Mean nephrosomatic index in *T. bryosalmonae*-exposed fish were 285% higher than in unexposed fish (Figure S2A). We found no effect of the interaction between diclofenac and *T. bryosalmonae* exposure on nephrosomatic index (Table 1B). Variation in nephrosomatic index was higher in *T. bryosalmonae*-exposed than unexposed fish: from timepoint 5 onwards, when nephrosomatic indexes in the two treatments had diverged, nephrosomatic index values within the range of those seen in the *T. bryosalmonae*-unexposed treatment were found in 5/61 sampled individuals. These individuals probably did not develop PKD.

**Discussion**

In this study we tested what effect the ubiquitous micropollutant diclofenac, has on *T. bryosalmonae*-infected brown trout in two laboratory experiments. In one experiment we observed the inflamed kidney expected during PKD, while in the other we found evidence consistent with this. In the first experiment we found *T. bryosalmonae* infection significantly increased $K_i$, a measure of body swelling, compared to control fish. Importantly, *T. bryosalmonae* exposure in combination with diclofenac pollution further increased $K_i$. In the second experiment we newly measured nephrosomatic index and found that it significantly predicted $K_i$ at a timepoint equivalent to that at which $K_i$ was measured in the first experiment. We can therefore be confident that, in the first experiment, higher $K_i$ in fish exposed to both stressors compared to those only exposed to *T. bryosalmonae* means that diclofenac was increasing nephrosomatic index in those fish. In the second experiment, $K_i$ was not significantly affected by the treatments alone or in combination. Nephrosomatic index was significantly higher in fish exposed to *T. bryosalmonae* but was not significantly affected by treatment with diclofenac. Haematocrit was reduced significantly in fish showing PKD, and simultaneous diclofenac exposure reduced the effect of PKD on haematocrit.

Our two experiments differed in nominal diclofenac concentration, temperature, and possibly in other environmental characteristics which may explain the differences in results that we find. We found the effect of neither stressor to be significantly influenced by sex. Our results therefore suggest that diclofenac increases parasite-induced kidney swelling under some environmental conditions, but also alleviates parasite-induced reduction of haematocrit. Diclofenac thus has mixed effects on the health of parasite-exposed trout, and we cannot conclude that diclofenac increases PKD severity. Neither diclofenac nor PKD affected populations in a sex-specific manner.

Small differences in environmental conditions can lead to large differences in measured variables (Elliott and Hurley, 1998; Hoffmann and Merilä, 1999). In our first experiment fish were treated with 200 $ng \cdot L^{-1}$ diclofenac and held at 18°C and diclofenac significantly increased PKD-induced kidney swelling. In our second experiment fish were treated with 50 $ng \cdot L^{-1}$ diclofenac and held at 14.5°C and no effect of diclofenac was found on PKD-induced kidney swelling. Differences in the response to sympatric diclofenac pollution and *T. bryosalmonae* exposure may therefore be driven by the concentration of diclofenac and/or the temperature conditions at the time of development of PKD symptoms. PKD severity is already well recognised to be affected by variation in temperature, with 15°C acting as a threshold above which PKD symptoms, and resulting mortality rates, increase (reviewed by Okamura et al. (2011)). In laboratory settings mortality rates of up to 46% have been documented at 16°C and up to 85% at 19°C (Bettge et al., 2009b, 2009a). This could suggest an interaction between diclofenac, PKD, and temperature. Our results suggest that 15-17°C could represent a threshold, similar to that found by other authors. Similar interactions between temperature and pollutants on virulence of parasitic infection have previously been shown in the aquatic environment (Tracy et al., 2020). Both temperature and diclofenac concentration are confounded with other experimental differences, including parental identity and populations being at slightly different developmental stages due to temperature differences (Nicieza and Metcalfe, 1997; Koumoundouros et al., 2001). Our experimental design

therefore prevents us from distinguishing which factor drove the distinct effects of diclofenac on PKD severity. However, we can speculate that a temperature effect on this system may be expected as increased temperatures have widespread effects on fishes' stress responses (Alfonso et al., 2021). Freshwater temperatures are predicted to increase due to climate change. Unless widespread improvements to waste water treatment plants occur, occurrence of painkillers such as diclofenac as pollutants in freshwater environments is likely to increase due to predicted increases in their consumption (Schröder et al., 2016). Therefore, both the possible effects of temperature and diclofenac concentration on the relationship between PKD and diclofenac warrant further investigation in order to better manage affected populations worldwide.

Analysis of our second experiment was limited by low sample sizes, with between 58 and 78 individuals per model. Effects on disease may best be studied when the disease is present, i.e. when the symptoms are strongest. Comparing uninfected treatment groups to infected groups that have not yet developed the disease may dilute effect signal and therefore miss important effects. One approach would be to focus only on the timepoint where symptoms are most severe, in this case timepoint 7 in the second experiment. This was not possible due to low sample size. We therefore focused on all timepoints that showed clear PKD-related symptoms as a trade-off between sufficient sample size and focusing on relevant disease stages. Density effects are also important in salmonids (Pickering and Pottinger, 1987; Mazur and Iwama, 1993), as we saw in our first experiment. Controlling for density differences between aquaria further reduced sample sizes but was unavoidable. Finally, as these were level 3 experiments our sample size was also constrained by Swiss ethical regulations.

Despite being well-studied (Lonappan et al., 2016) and widespread in much of the world's surface water the question of whether diclofenac has toxic effects on freshwater species when at environmentally relevant concentrations is still unresolved. Diverse toxic effects have been shown (Hong et al., 2007; Gröner et al., 2015, 2017; Guiloski et al., 2017; Bio and Nunes, 2020); however, many published studies (Praskova et al., 2011; Stepanova et al., 2013; Bickley et al., 2017) have also

failed to find an effect at concentrations typical in European surface waters (Buser et al., 1998; Ternes, 1998; Lacina et al., 2013; Marsik et al., 2017). Two methodological reviews have questioned the results of several studies claiming to find effects (Wolf et al., 2014; Wolf, 2021), however the objectivity of these reviews may themselves be questioned as they were funded by industries marketing diclofenac. In this study we find that, when alone, chronic diclofenac pollution did not significantly affect length, agreeing with published findings (e.g. Memmert et al., 2013; Yokota et al., 2018), haematocrit, or nephrosomatic index. Alone, diclofenac was only found to be associated with a 0.6% increase in $K_i$ in one of our experiments. We thus find no evidence for diclofenac toxicity at the concentrations and on the traits studied here on brown trout in the absence of other stressors.

Maintaining diclofenac concentration at nominal levels was difficult (Figure S3), but measured concentrations are well within the range of values seen in streams of the Swiss plateau. An important consideration is that in this and many other studies of the effect of stressors embryos are raised under near-optimal conditions (e.g. stressors often tested individually, no predation, fed *ad libitum*, perfect water conditions, raised singly). Laboratory studies are therefore likely to underestimate true toxicity of substances in the wild. The consumption of painkillers such as diclofenac is also predicted to increase (Schröder et al., 2016), while future rapid evolution in response to diclofenac-induced stresses may be unexpected (Palejowski et al., in prep). The widespread occurrence of diclofenac pollution (Lonappan et al., 2016) therefore demands further research into diclofenac toxicity to understand if and when it has ecologically important effects.

In salmonids sex differences in embryo and juvenile stages, especially of non-migratory species, have been generally understudied. Studies of population fitness typically ignore sex, assuming that it is of little relevance for demography and population growth (Nislow and Armstrong, 2012). However, in salmonids sex-specific responses to stressors have been demonstrated in grayling (Selmoni et al., 2019) and lake char (Nusbaumer et al., 2021b) which could have consequences for conservation of these populations (Wedekind, 2012). There is a growing

appreciation for sex-specific drug action and toxicity in general (Mennecozzi et al., 2015), and sex-specific effect of diclofenac specifically have now been seen in humans (Mennecozzi et al., 2015) and both model (Romanowska et al., 2008) and non-model species (Yokota et al., 2018). However, in this study we find no sex-specific action of either diclofenac or proliferative kidney disease. Thus our results suggest that neither diclofenac pollution nor PKD are likely to result in biased sex ratios.

The experiments presented here assess the effect of diclofenac pollution and *T. bryosalmonae* exposure over a narrow time window, from *T. bryosalmonae* infection until 12 weeks post-infection and only in 0+ fish, and under laboratory conditions. Susceptibility to diclofenac pollution is suspected to vary over ontogeny (Gröner et al., 2017; Schwarz et al., 2017), and the effects of PKD have already been shown to depend on age at time of *T. bryosalmonae* infection (Bailey et al., 2021). Therefore, the effect of diclofenac pollution on PKD severity may vary depending on age at time of *T. bryosalmonae* infection or diclofenac exposure. Furthermore, in any laboratory study questions arise over to what extent results can be extrapolated to wild populations. Bylemans et al. (2022) demonstrate that trout performance in laboratory settings is a useful predictor of performance in natural settings, thus our results are likely relevant for wild populations.

Across Switzerland, populations of brown trout have shown severe unexplained declines since the 1980s (Burkhardt-Holm et al., 2005; OFEV, 2022). These have often been attributed to proliferative kidney disease (PKD), a widespread emerging parasitic disease (Borsuk et al., 2006; Burkhardt-Holm and Scheurer, 2007). However, stressors do not occur alone. Diclofenac pollution is ubiquitous in most of the world's surface water (reviewed in Lonappan et al., 2016), and has been suggested to have toxic effects at concentrations seen in European waters including on the kidney (Triebskorn et al., 2004), where PKD causes large-scale inflammation (Okamura et al., 2011). Populations with PKD are thus likely simultaneously exposed to the two stressors which may alter the effects of both. Using two laboratory experiments we found that diclofenac does not always, but can, increase PKD-induced kidney swelling, but also alleviates PKD-induced reduction of haematocrit. We speculate that the effect of diclofenac on PKD-associated kidney swelling may be

affected by environmental conditions, especially temperature, at the time of pollutant exposure or disease infection. We conclude that diclofenac has mixed effects on PKD severity and trout health, that are dependent on environmental conditions, but that neither diclofenac nor PKD has sex-specific effects on trout that may threaten population sex ratios.

**Ethics Statement**

The study was approved by the Fishery Inspectorate of the Bern canton and the Veterinary Office of the Canton of Vaud (approval number VD3464). Experimental exposure to diclofenac and infection with *Tetracapsuloides bryosalmonae* was classified as severity level 3 (on the scale from 0 to 3) while the catching of wild adults, the keeping of these animals for some days at the *Fischereistützpunkt Reutigen*, and the collection of gametes was part of the cantonal routine breeding for the annual stocking.

**Data availability**

All data will be published on the Dryad Digital Repository.

**Acknowledgements**

We thank B. Bracher, U. Gutmann, and C. Rolli for breeding and rearing the fish, D. Buffat, J. Buser, I. Castro, C. de Guttry, L. Garaud, G. Glauser, E. Lavanchly, E. Longange, D. Nusbaumer, S. Mandra, I. Mattatia, D. Olbrich, and F. Pasquier for assistance and discussion, and C. Küng and A. Hertig for permissions.

**Funding**

The project was supported by the Swiss National Science Foundation (31003A_159579 and SNF 31003A_182265).

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**Figures and tables**

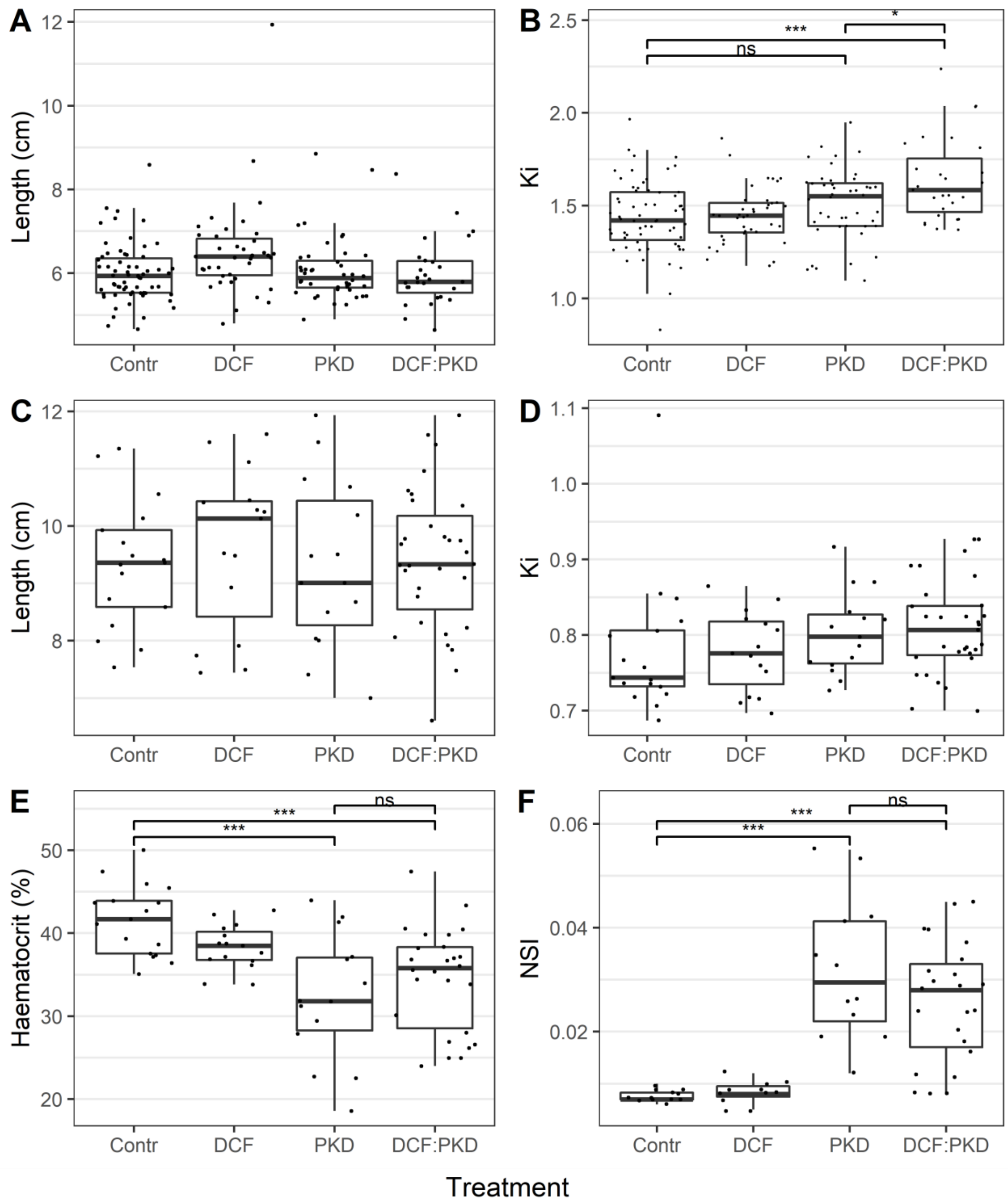
**Table 1.** Statistical results of models on the effects of diclofenac, PKD, timepoint, and their interactions on juvenile phenotypes in the A) 2014-15 and B) 2018-19 experiments. Significant results are highlighted in bold. Non-significant interaction effects were removed from the model prior to final analysis, with the exception of the Diclofenac × PKD interaction term.

| Response variable | Effect tested | DF | $\chi^2$ | P |
|---|---|---|---|---|
| *A) 2014-15 experiment* | | | | |
| Length [$^{1/3}$] | Diclofenac | 1 | 1.1 | 0.28 |
| | PKD | 1 | 0.2 | 0.65 |
| | Diclofenac × PKD | 1 | 0.7 | 0.41 |
| $K_i$ | **Diclofenac** | **1** | **5.1** | **0.024** |
| | **PKD** | **1** | **18.7** | **<0.001** |
| | **Diclofenac × PKD** | **1** | **4.0** | **0.046** |
| *B) 2018-19 experiment* | | | | |
| Length | Timepoint | 1 | 0.1 | 0.72 |
| | Diclofenac | 1 | 0.2 | 0.65 |
| | PKD | 1 | 0.1 | 0.73 |
| | Diclofenac × PKD | 1 | 0.1 | 0.80 |
| $K_i$ [$^{1/5}$] | Timepoint | 1 | 1.8 | 0.18 |
| | Diclofenac | 1 | 0.0 | 0.96 |
| | PKD | 1 | 0.9 | 0.34 |
| | Diclofenac × PKD | 1 | 0.0 | 0.94 |
| Haematocrit | **Timepoint** | **1** | **4.5** | **0.034** |
| | Diclofenac | 1 | 0.2 | 0.63 |
| | **PKD** | **1** | **25.6** | **<0.001** |
| | **Diclofenac × PKD** | **1** | **4.6** | **0.032** |
| Nephrosomatic index [$\log_{10}$] | **Timepoint** | **1** | **0.0** | **0.94** |
| | Diclofenac | 1 | 0.7 | 0.41 |
| | **PKD** | **1** | **99.2** | **<0.001** |
| | Diclofenac × PKD | 1 | 1.6 | 0.20 |

**Table 2.** Statistical results of models on the effects of the stressors, sex, timepoint, and their interactions on juvenile phenotypes in the 2018-19 experiment. Sample sizes were not large enough to estimate the effect of a sex × PKD × diclofenac interaction. Two models were therefore constructed per response variable, one including a sex × PKD interaction and the other a sex × diclofenac interaction. Models were constructed using individuals from all timepoints. Significant results are highlighted in bold.

| | | Model of sex × PKD | | | Model of sex × diclofenac | | |
|---|---|---|---|---|---|---|---|
| Response variable | Effect tested | DF | $\chi^2$ | P | DF | $\chi^2$ | P |
| Length | **Timepoint** | **1** | **71.0** | **<0.001** | **1** | **74.7** | **<0.001** |
| | Sex | 1 | 0.3 | 0.53 | 1 | 0.4 | 0.53 |
| | PKD | 1 | 0.2 | 0.60 | | | |
| | Sex × PKD | 1 | 0.4 | 0.53 | | | |
| | Diclofenac | | | | 1 | 0.3 | 0.60 |
| | Sex × diclofenac | | | | 1 | 2.9 | 0.09 |
| $K_i$ [$^{1/5}$] | Timepoint | 1 | 2.3 | 0.13 | 1 | 2.1 | 0.14 |
| | **Sex** | **1** | **6.3** | **0.01** | **1** | **6.2** | **0.01** |
| | PKD | 1 | 1.9 | 0.17 | | | |
| | Sex × PKD | 1 | 0.5 | 0.49 | | | |
| | Diclofenac | | | | 1 | 0.1 | 0.72 |
| | Sex × diclofenac | | | | 1 | 0.0 | 0.96 |
| | **Sex × timepoint** | **1** | **9.4** | **0.002** | **1** | **9.0** | **0.003** |
| Haematocrit | **Timepoint** | **1** | **22.1** | **<0.001** | **1** | **24.4** | **<0.001** |
| | Sex | 1 | 0.2 | 0.65 | 1 | 0.0 | 0.87 |
| | **PKD** | **1** | **3.8** | **0.050** | | | |
| | Sex × PKD | 1 | 0.3 | 0.56 | | | |
| | **Timepoint × PKD** | **1** | **11.9** | **<0.001** | | | |
| | Diclofenac | | | | 1 | 0.3 | 0.60 |
| | Sex × diclofenac | | | | 1 | 1.5 | 0.22 |
| Nephrosomatic index | **Timepoint** | **1** | **26.5** | **<0.001** | **1** | **23.8** | **<0.001** |
| | Sex | 1 | 0.0 | 0.92 | 1 | 0.0 | 0.94 |
| | **PKD** | **1** | **12.7** | **<0.001** | | | |
| | Sex × PKD | 1 | 0.0 | 0.84 | | | |
| | **Timepoint × PKD** | **1** | **59.1** | **<0.001** | | | |
| | Diclofenac | | | | 1 | 0.0 | 0.94 |
| | Sex × diclofenac | | | | 1 | 0.1 | 0.80 |

**Figure 1.** Boxplots of the effect of treatment (Contr = control, DCF = exposed to diclofenac, PKD = exposed to *Tetracapsuloides bryosalmonae*, DCF:PKD = exposed to diclofenac and *T. bryosalmonae*) on (A, C) length in cm, (B, D) $K_i$, (E) haematocrit, and (F) nephrosomatic index in the (A-B) 2014-15 and (C-F) 2018-19 experiments. Median is shown, as are upper and lower quartiles, and variability outside the of those. Relevant Tukey pairwise significances are given where the effect of PKD is significant ($p > 0.05$ = ns, $0.05 > p > 0.01$ = *, $0.01 > p > 0.001$ = **, $0.001 > p$ = ***). Raw measurements are shown as points with jitter.


A
B
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Length (cm)
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Haematocrit (%)
NSI
Contr
DCF
PKD
DCF:PKD
ns
***
*
Treatment

# Supplementary information for: Micropollution by diclofenac increases virulence of PKD infection in brown trout

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**Treatment Exposure**

2014-15 experiment

*PKD infection*

On the 28th August 2015 bryozoan were collected from a section of the Alte Aare (47.111333, 7.318056) and a section of the Lyssbach (47.049583, 7.354278) both of which are known to be infected with *Tetracapsuloides bryosalmonae.*

The bryozoa were collected in the river using forceps to pull them off and falcon tubes to store them (filled with the surrounding river water). After collecting about 8 tubes worth of the bryozoa, we poured them into two 1L bottles which were then filled with the river water. These bottles are then placed into a small polystyrene box with a small amount of ice for transport back to Lausanne.

Once in Lausanne, the bryozoa are crushed to a paste using a pestle and mortar, and the resulting paste is kept in a 2L beaker. Once all the bryozoa are crushed, the solution is thoroughly mixed and then evenly distributed to the infection tanks. The filters of the tanks were turned off for about 2 hours to ensure the parasites were not removed before having a chance to infect the fish. We did not have an analogous solution to sham treat the control tanks.

Following infection, all water removed from treatment tanks was placed in one of the large green containers in each room. Bleach was added in a 1:100 dilution to kill remaining spores, and then the water was left standing 48 hours before being disposed down the drain (spores generally don't survive 12-24 hours without a host).

2018-19 experiment

*PKD infection*

On the 13th September 2019 bryozoans were collected from the river Glâne (46.702488,6.932779) which is known to be infected with *T. bryosalmonae.*

Bryozoa were collected using forceps to remove them from stones, then they were stored in 50ml falcon tubes filled with surrounding river water. After collecting approximately 5 tubes worth of bryozoa they were transported to the University of Lausanne on ice.

In the laboratory collected bryozoans were placed in 1L bucket filled with aerated tap water to allow spores to spread throughout the water. The water was left for 24 hours, then 100ml was removed and added to all aquaria treated with *T. bryosalmonae* spores. This was repeated a second day. On the third day the bryozoans were removed from the bucket, broken up with foreceps, and added to the *T. bryosalmonae*-treated aquaria equally with all water remaining in the bucket.

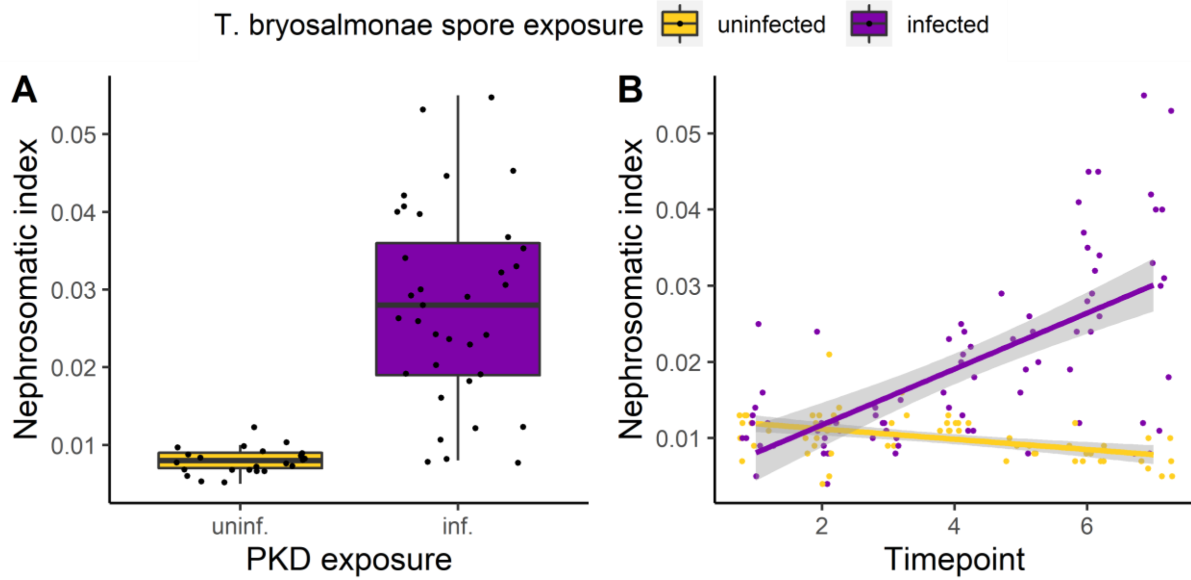
## Treatment confirmation

*PKD infection*

Visual inspection of renal tissue and comparison of nephrosomatic index in fish infected vs uninfected with T. bryosalmonae spores shows that fish in the PKD-treated group were successfully infected with PKD (Figure S1, Figure S2). No increased nephrosomatic index in the PKD uninfected group suggests that cross-contamination did not occur.

**Figure S1.** Kidney in 0+ brown trout (A) uninfected and (B) infected with *Tetracapsuloides bryosalmonae* 85 days post-infection after all other internal organs have been removed. Inflammation of renal tissue is visible in (B) as the kidney is swollen and turgid.

**Figure S2.** The effect of treatment with *Tetracapsuloides bryosalmonae* spores on nephrosomatic index in the 2018-19 experiment (A) calculated from timepoints 5-7, and (B) across all timepoints. In panel (A) values are displayed as boxplots showing median, upper and lower quartiles, and variability outside of those. In panel (B) points are jittered on the X axis for visibility and a linear regression of X ~ Y with confidence intervals is applied by treatment.



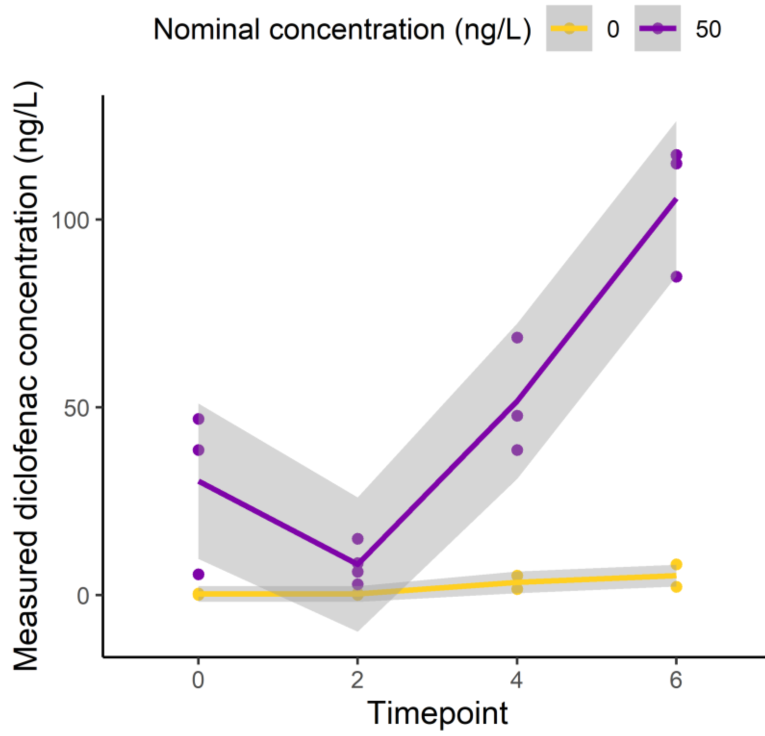
*Diclofenac exposure*

Measured diclofenac concentrations in aquaria that were not exposed to diclofenac remained close to 0 ng·L$^{-1}$ throughout the experiment. By timepoint four measured concentrations in the two aquaria in this treatment had risen to 1.6 and 5.2 ng·L$^{-1}$, and by timepoint six to 2.2 and 8.2 ng·L$^{-1}$, respectively. This might be explained by slight contamination. Such low concentrations do not affect interpretation of the results of this study. Conversely, in the nominally 50 ng·L$^{-1}$ treatment measured concentrations varied from approximately 10 ng/L (timepoint three) up to an average of 106 ng·L$^{-1}$ at timepoint seven (Figure S1). The sample at the first timepoint was taken the day after the initial diclofenac spiking, thus before much diclofenac had been bioaccumulated and metabolised by the trout in the aquaria. The one low concentrations in the 50 ng·L$^{-1}$ treatment in timepoint one is likely due to human error adding the wrong concentration. Nonetheless by timepoint 3 all aquaria in the 50 ng·L$^{-1}$ treatment were behaving similarly.

The variation in average diclofenac concentration in the 50 ng·L$^{-1}$ treatment may be due to variation in the ability of the trout population to bioaccumulate the available diclofenac pollution in the water. Until shortly before timepoint three 15L (12.5%) of aquaria water was being changed per day, and aquaria were being re-dosed with diclofenac solution in line with the amount of water changed to maintain concentrations at 50 ng·L$^{-1}$. From timepoint three onwards the volume of water changed per day was increased to 40L (33%), and the volume of diclofenac solution re-dosed daily was increased to match this. This change at timepoint three coincides with an increase in the measured diclofenac levels in the aquaria in exposed to diclofenac pollution. Trout are known to bioaccumulate and metabolise diclofenac in their environment (Kallio et al., 2010; Mehinto et al., 2010; Schwaiger et al., 2004). It is likely that trout were bioaccumulating and clearing the available diclofenac in the aquaria prior to timepoint 3, but increasing the volume of diclofenac solution redosed each day raised it beyond what the population could clear. Additionally, the decrease in the trout biomass in aquaria over time (from approximately 210g to 43g of biomass per aquarium from timepoints one to seven, unpublished data) to due to mortality and sampling likely further

reduced the populations' abilities to clear the available diclofenac. The elevated diclofenac concentrations in timepoint seven may be due to human error dosing too high a volume of diclofenac solution after timepoint five. Together these mechanisms likely resulted in the increase in measured diclofenac concentration in the nominally 50 $ng \cdot L^{-1}$ treatment aquaria from timepoint five onwards.

**Figure S3.** Scatterplot of measured diclofenac levels over the course of the experiment. Loess regression displaying confidence intervals is applied per treatment.



**Aquarium effects**

Unintended differences between replicate aquaria of the same treatment may result in differences in measured variables. These differences include density as well as unmeasured environmental differences such as potential differences in temperature, light, stress exposure, etc. Figure S4 plots (A) length and (B) $K_i$ in each replicate of each treatment, while Figure S5 plots the same against the number of fish in each aquaria at the end of the experiment.

**Figure S4.** Boxplots of the effect of treatment (Contr = control, DCF = exposed to diclofenac, PKD = exposed to *Tetracapsuloides bryosalmonae*, DCF:PKD = exposed to diclofenac and *T. bryosalmonae*) on (A) length and (B) $K_i$ in each aquarium of each treatment in the 2014-15 experiment. Median is shown, as are upper and lower quartiles, and variability outside the of those.

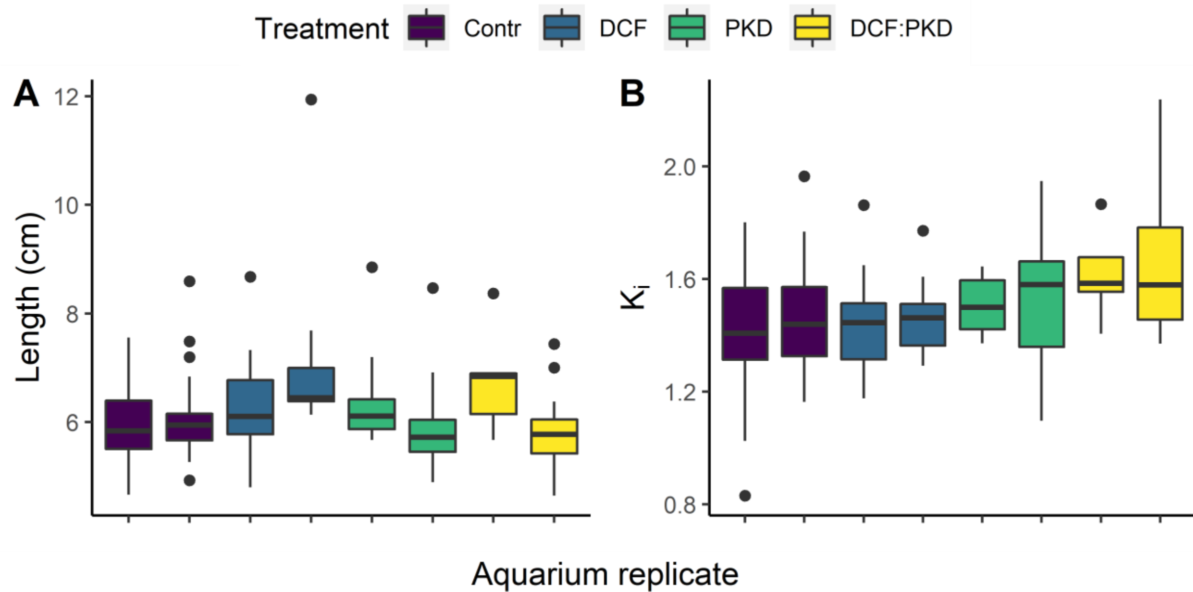
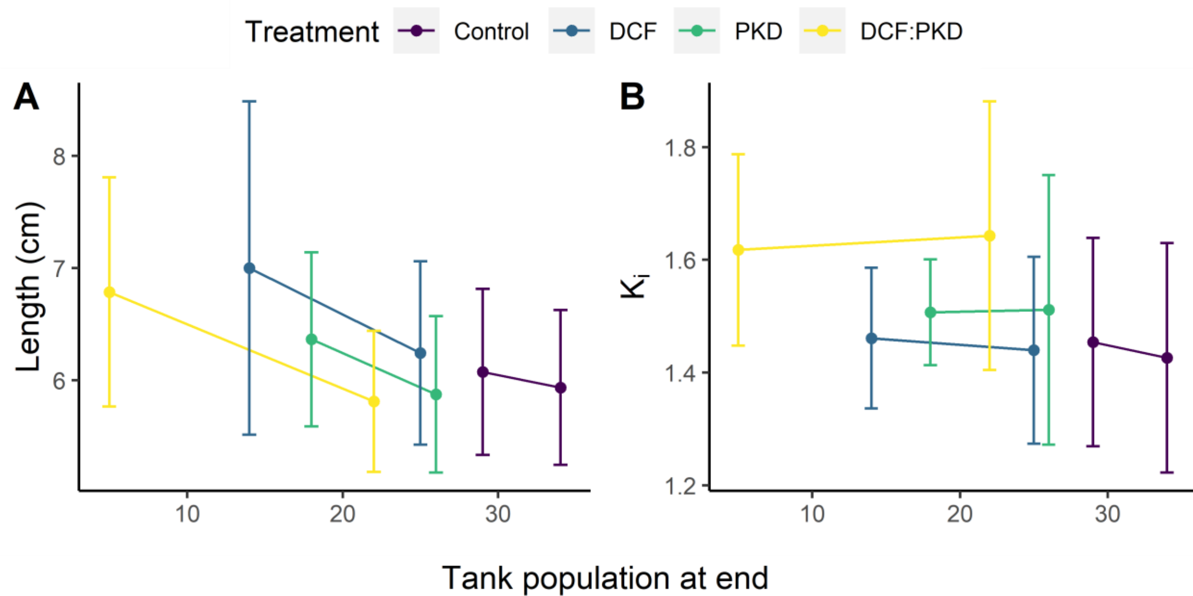


**Figure S5.** Aquarium population size at end of the experiment plotted against (A) length and (B) $K_i$ in the 2014-15 experiment measured in each of the aquaria of the four treatments (Control = control, DCF = exposed to diclofenac, PKD = exposed to *Tetracapsuloides bryosalmonae*, DCF:PKD = exposed to diclofenac and *T. bryosalmonae*). Mean and standard deviation are shown per tank. The two aquaria per treatment are connected for ease of comparison.



**Nephrosomatic index and swelling**

The effects of PKD on kidney inflammation, as measured by nephrosomatic index, become evident from approximately timepoint 4 onwards (Figure S2B). Prior to this point Figure S6 illustrates that nephrosomatic index and $K_i$ show a negative, often weak, correlation whereby individuals with a higher $K_i$, i.e. with more swollen bodies, have lower nephrosomatic index. This is likely due to uninflamed kidneys representing a smaller percentage of total mass of a fatter fish and a larger percentage of the total mas of a thinner fish. By timepoint 5 this relationship has begun to reverse and appears flatter. By timepoint 7 kidney inflammation is severe in infected fish and nephrosomatic index correlates with $K_i$ with an $R^2$ of 0.28. Furthermore, analysis of variance of the linear regression model of $K_i$ as a function of nephrosomatic index shows that nephrosomatic index significantly influences $K_i$ in timepoint 7 (df=1, F=7.6, p=0.013).

**Figure S6.** Scatterplots of the correlation between nephrosomatic index and $K_i$ over time in the 2018-19 experiment, each panel shows a different timepoint (1-7). Linear regression of X ~ Y with confidence intervals is applied per timepoint. $R^2$ is given for timepoints showing statistically significant correlations between nephrosomatic index and $K_i$.

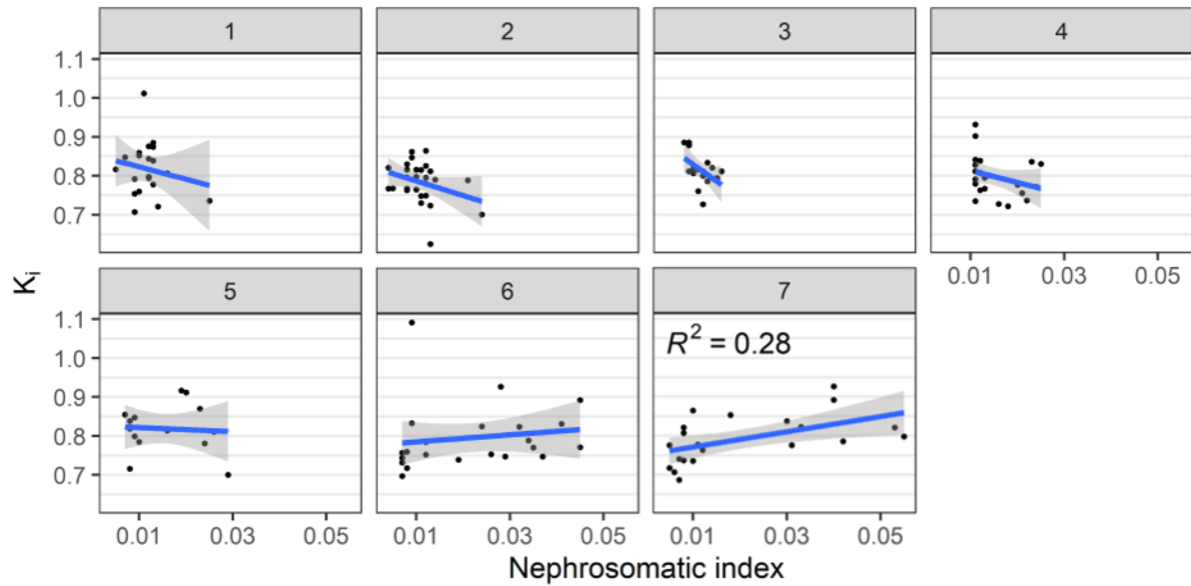
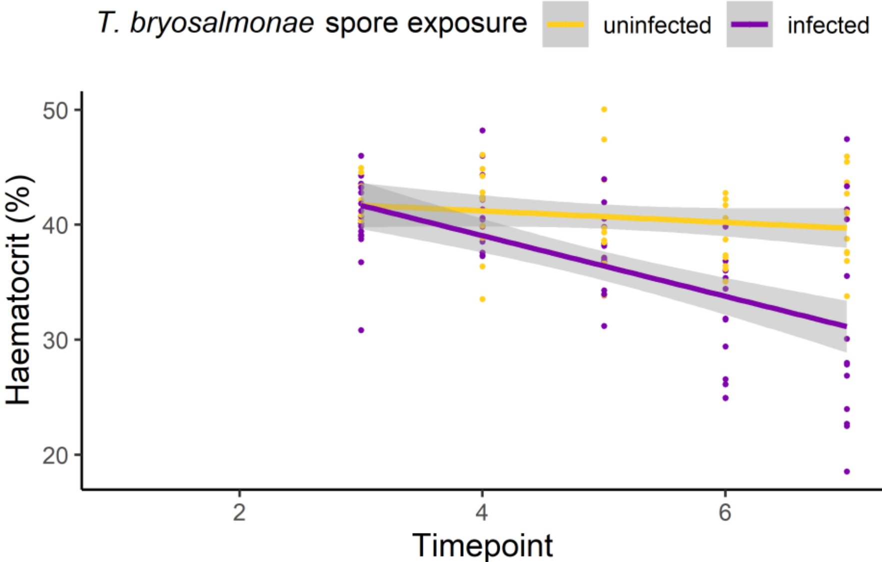
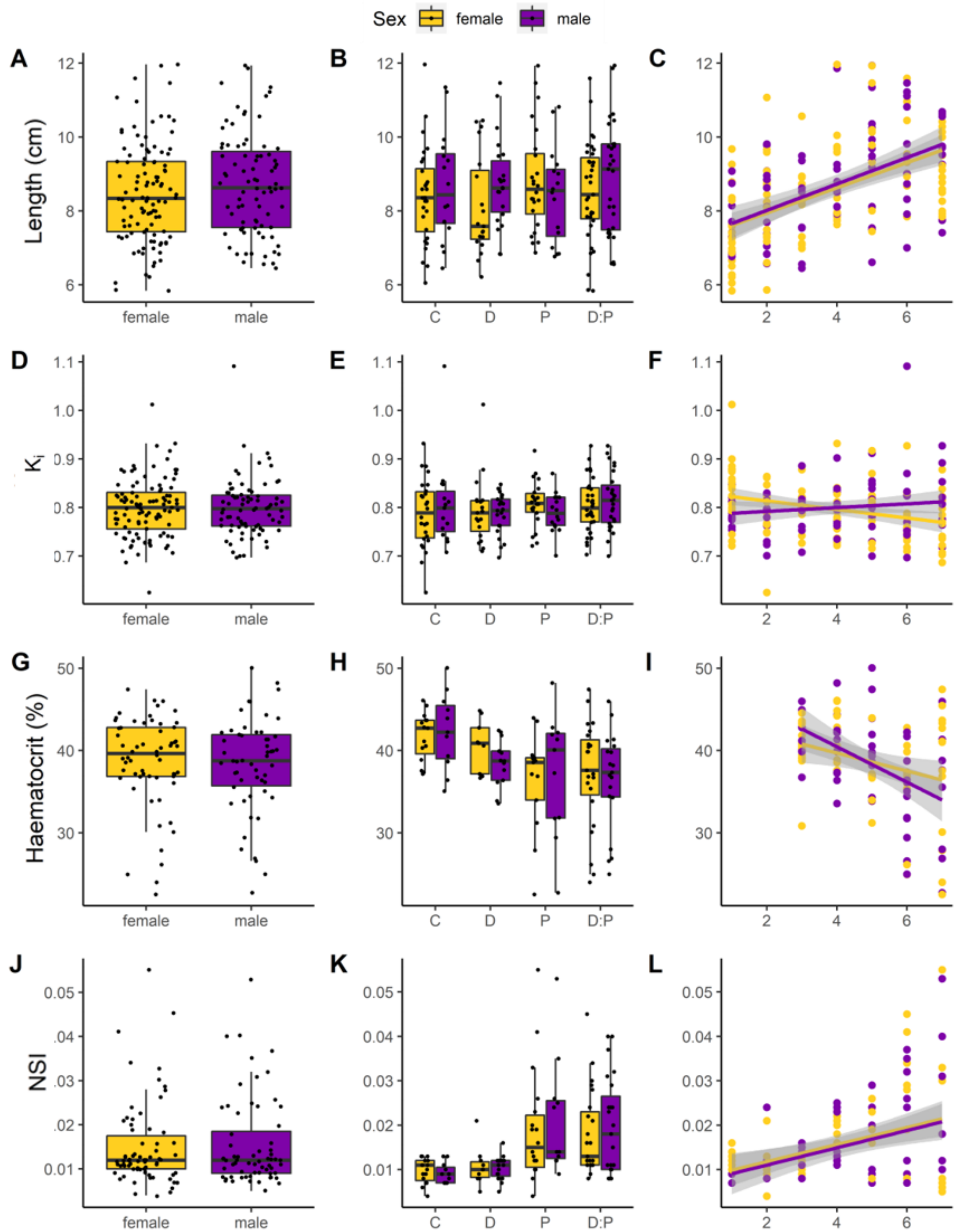


**Figure S7.** The effect of *T. bryosalmonae* exposure on haematocrit levels over time. Linear regression of X ~ Y with confidence intervals is applied per treatment.



**Sex effects**

**Figure S8.** Sex effects in the 2018-19 experiment on (A-C) length (cm), (D-F) $K_i$, (J-L) haematocrit (%), and (M-O) nephrosomatic index when considering (A,D,G,J,M) sex alone, (B,E,H,K,N) sex per treatment, or (C,F,I,L,O) sex over time. Treatments: C = control, D = diclofenac, P = PKD, D:P = diclofenac × PKD. In panels displaying the effect of sex alone and sex per treatment boxplots with median, upper and lower quartiles, and variability outside of those are shown. In panels displaying the effect of sex over time scatterplots are shown with linear regressions and confidence intervals per treatment are applied.



Sex
female
male
A
B
C
D
E
F
G
H
I
J
K
L
Length (cm)
Kᵢ
Haematocrit (%)
NSI
female
male
C
D
P
D:P

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# Chapter IV - Sex-specific life history affected by stocking in juvenile brown trout

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Chapter status: **published**

**Author contributions**

LM, DN, IC, AU, and CW managed the breeding, sampling, and data extraction for the field experiment. KM developed the genotyping protocol. LM and JB managed the genetic sexing and parental assignments. HP, LM, DN, and CW bred the fish for the laboratory study, which were then raised and sampled by HP and VA. VA conducted the histology for phenotypic sexing, supervised by SK. HP, JB, and CW analysed the data and wrote the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

frontiers | Frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 02 May 2022
doi: 10.3389/fevo.2022.869925

# Sex-Specific Life History Affected by Stocking in Juvenile Brown Trout

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OPEN ACCESS

**Edited by:**
Amanda Wilson Carter,
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**Specialty section:**
This article was submitted to
Population, Community,
and Ecosystem Dynamics,
a section of the journal
Frontiers in Ecology and Evolution

**Received:** 05 February 2022
**Accepted:** 22 March 2022
**Published:** 02 May 2022

**Citation:**
Palejowski H, Bylemans J, Ammann V, Marques da Cunha L, Nusbaumer D, Castro I, Uppal A, Mobley KB, Knörr S and Wedekind C (2022) Sex-Specific Life History Affected by Stocking in Juvenile Brown Trout.
Front. Ecol. Evol. 10:869925.
doi: 10.3389/fevo.2022.869925

Salmonids are a socioeconomically and ecologically important group of fish that are often managed by stocking. Little is known about potential sex-specific effects of stocking, but recent studies found that the sexes differ in their stress tolerances already at late embryonic stage, i.e., before hatchery-born larvae are released into the wild and long before morphological gonad formation. It has also been speculated that sex-specific life histories can affect juvenile growth and mortality, and that a resulting sex-biassed demography can reduce population growth. Here we test whether juvenile brown trout (*Salmo trutta*) show sex-specific life histories and whether such sex effects differ in hatchery- and wild-born fish. We modified a genetic sexing protocol to reduce false assignment rates and used it to study the timing of sex differentiation in a laboratory setting, and in a large-scale field experiment to study growth and mortality of hatchery- and wild-born fish in different environments. We found no sex-specific mortality in any of the environments we studied. However, females started sex differentiation earlier than males, and while growth rates were similar in the laboratory, they differed significantly in the field depending on location and origin of fish. Overall, hatchery-born males grew larger than hatchery-born females while wild-born fish showed the reverse pattern. Whether males or females grew larger was location-specific. We conclude that juvenile brown trout show sex-specific growth that is affected by stocking and by other environmental factors that remain to be identified.

**Keywords: sex, life history, genetic sexing, *Salmo trutta*, growth, survival, stock-enhancement, anthropogenic effects**

## INTRODUCTION

Intersexual differences in life history strategies exist across diverse unrelated taxa (Brooks and Garratt, 2017; Li and Kokko, 2019) and can affect population dynamics and extinction risks (Hendry et al., 2018; Cally et al., 2019). Despite this, intersexual differences in early life history strategies receive little attention when studying the fitness of individuals and/or assessing population viability (Tarka et al., 2018). Stock enhancement practices primarily focus on the release of early life stages but sex effects are only rarely considered [but see for example Huertas and Cerdà (2006), Lenz et al. (2007), and Svobodová et al. (2020)]. Sex-specific early life history strategies, and their relationship with stock enhancement practices, may thus be a hidden driver of population dynamics and need to be studied in more detail.

Sex can affect physiology (Grilo et al., 2018; Millington and Rideout, 2018; Selmoni et al., 2019), development (Maitre et al., 2017; Gurley et al., 2018) and behaviour (Magurran and Garcia, 2000; Klemetsen et al., 2003; Pearse et al., 2019) well before gonad formation (Tsai et al., 2009; Mousavi et al., 2021). Divergent selection pressures may thus act on the sexes even at early developmental stages (Bolund et al., 2013; Schroeder et al., 2013; Forsman, 2018) leading to the development of distinct life history strategies and sometimes even sex-specific mortality (Badyaev et al., 2002; Wiklund et al., 2003; Husby et al., 2006; but see Dietrich et al., 2003; Altwegg et al., 2007). Consequently, operational sex ratios within populations can be biassed, and this can affect population dynamics and extinction risks through a reduced effective population size (Donald, 2007; Geffroy and Wedekind, 2020), behavioural changes (Eberhart-Phillips et al., 2018) and altering the direction of evolution (Forsman, 2018; Gomes et al., 2018). While sex-specific effects can be profound, they are generally understudied in ecology, evolution and conservation biology (e.g., Einum and Fleming, 2001; Nislow and Armstrong, 2012; Skov et al., 2012; Tsuboi et al., 2013; Louison and Stelzer, 2016; Wilkins and Marsden, 2021).

Salmonid species exhibit diverse life-history strategies and thus form excellent models to study the importance and consequences of sex-specific life history strategies at early developmental stages (Birnie-Gauvin et al., 2021). Sex differences have been reported for growth (Yamamoto, 2004; Maitre et al., 2017), aggression (Rgen et al., 2001), migration strategies (Kelson et al., 2019; Nevoux et al., 2019; Eldøy et al., 2021) and size- and age-at-maturation (Young, 2005; McKinney et al., 2020; Mobley et al., 2021; Tréhin et al., 2021). During the embryonic phase, male lake char (*Salvelinus umbla*) were found to hatch earlier and be less susceptible to environmental stressors (Nusbaumer et al., 2021). In contrast, sex did not seem to influence emergence time, or morphological and physiological traits in newly emerged brown trout (*Salmo trutta*) (Régnier et al., 2015). However, male-specific mortality has been reported in the first month of development of brown trout leading to female-biassed sex ratios (Morán et al., 2016). Growth during the juvenile phase has been reported to be higher for males in masu salmon (*Oncorhynchus masou*) (Yamamoto, 2004), chinook salmon (*Oncorhynchus tshawytscha*) (Mizzau et al., 2013) and European grayling (*Thymallus thymallus*) (Maitre et al., 2017). The latter study showed that differences in growth rates in grayling can be attributed to a differential resource allocation between sexes. That is, gonadal differentiation is delayed in males which instead invest more in growth (Maitre et al., 2017). These intersexual differences could be large enough to cause female-biassed juvenile mortality and hence male-biassed adult sex ratios (Wedekind et al., 2013). Differences in sexual differentiation have also been reported for sea migrating brown trout but no sex-specific differences were recorded for growth (Dziewulska and Domagala, 2004). While sex-specific early life history strategies may be common in salmonids, they remain understudied which may partly be because of the challenges involved in sexing early life stages.

In Salmoninae and Thymallinae (two salmonid subfamilies), sex has been shown to be under the control of a conserved master sex-determining gene (i.e., sdY) (Yano et al., 2012, 2013). This allowed for the development of genetic sexing methods. Initially a simple PCR-based presence-absence test was proposed (Yano et al., 2013) which was later modified to a multiplex PCR allowing for high-throughput genetic sexing and genotyping (Quéméré et al., 2014). However, the use of a presence-absence of the sdY gene appears to be prone to false positive male identifications with the percentage of females misidentified as males ranging from 1 to 71% (Ueda et al., 2021). Various reasons for such misidentifications have been proposed (Ayllon et al., 2020). Firstly, mislabelling errors or low levels of between sample cross-contamination could result in a positive detection of the sdY gene in females. Second, discrepancies between genetic and phenotypic sex can arise due to the genetic silencing of the sdY gene (e.g., Yano et al., 2014) or environmentally driven sex reversal (e.g., Magerhans et al., 2009; Weber et al., 2020). Finally, non-functional copies of the sdY gene may be present in female salmonids and lead to the observation of high error rates in the genetic identification of phenotypic females (Ayllon et al., 2020; Bertho et al., 2021). While genetic sexing of early life stages of salmonid species is now feasible, improvements to genetic sexing methods are still needed to reduce misidentifications.

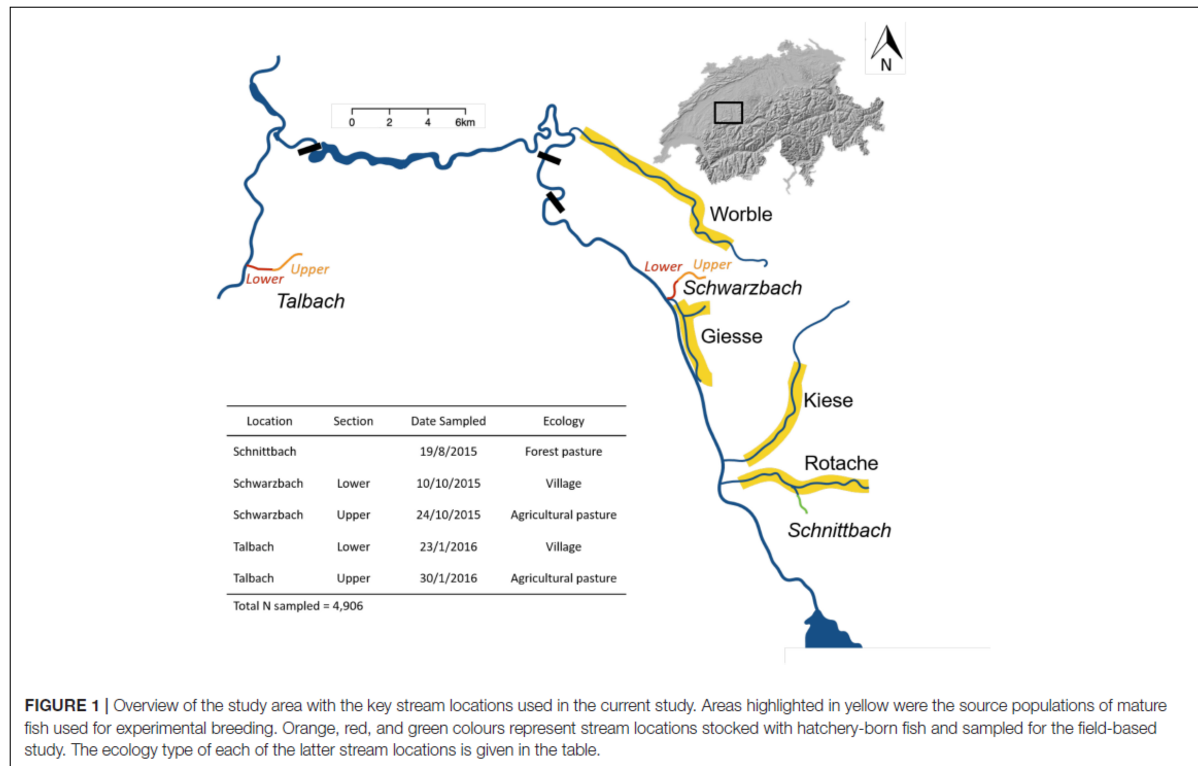
Brown trout are a keystone species within their natural range, have been translocated across the globe (Halverson, 2008; Hunt and Jones, 2018; Hasegawa, 2020), and have great socio-economical value (Elliott, 1989; Liu et al., 2019). Because of their value and often high exploitation rates, restocking and stock-enhancement are a common management practice for brown trout populations (Aas et al., 2018; Cucherousset et al., 2021). A large body of literature is thus available focussing on the effectiveness of these management practises (Pinter et al., 2018; Dieterman et al., 2020), the intraspecific competition between wild- and hatchery-born individuals (Einum and Fleming, 2001; Araki et al., 2008) and the overall population-level consequences (Meier et al., 2011; Leitwein et al., 2018; Klütsch et al., 2019). However, to the best of our knowledge, sex-specific effects have not been considered yet when evaluating the demographic and genetic consequences of restocking and stock-enhancement in this or any other salmonid fish.

Here we combine a large-scale field experiment with a controlled rearing of males and females under laboratory conditions to test for sex-specific growth and survival under various ecological conditions. The laboratory population also enabled continuous sampling for histological analyses to study possible sex differences in the timing of sex differentiation. The field experiment allows us to test whether sex-specific effects on growth and survival differ for hatchery- and wild-born fish that occupy the same habitat, and whether such differences are habitat specific.

## MATERIALS AND METHODS

### Study Populations

We focus here on different brown trout populations within the Aare catchment between Lake Thun and the city of Bern (Switzerland) (**Figure 1**). Within the study system, multiple




| Location | Section | Date Sampled | Ecology |
|---|---|---|---|
| Schnittbach | | 19/8/2015 | Forest pasture |
| Schwarzbach | Lower | 10/10/2015 | Village |
| Schwarzbach | Upper | 24/10/2015 | Agricultural pasture |
| Talbach | Lower | 23/1/2016 | Village |
| Talbach | Upper | 30/1/2016 | Agricultural pasture |
| Total N sampled = 4,906 | | | |

**FIGURE 1** | Overview of the study area with the key stream locations used in the current study. Areas highlighted in yellow were the source populations of mature fish used for experimental breeding. Orange, red, and green colours represent stream locations stocked with hatchery-born fish and sampled for the field-based study. The ecology type of each of the latter stream locations is given in the table.

genetically and phenotypically different brown trout populations occupy the different tributaries (Stelkens et al., 2012). Individuals used in this study are either wild-caught or originate from the yearly stock-enhancement programmes (F1 fish) conducted only by the Fishery Inspectorate of the Bern canton (continuously for more than 25 years). Stocking fish without their consent would be illegal and is unlikely in the study area, especially in the case of 0 + brown trout.

## Laboratory Study

For the laboratory study, breeding adults were collected around the beginning of the spawning season from the river Kiese through backpack electrofishing and kept in a hatchery (i.e., *Fischereistützpunkt* in Reutigen) until ovulation allowed the stripping of eggs. On the 28th November and 4th December 2018, 15 males and 20 females were haphazardly selected for block-wise full-factorial breeding. On the respective dates, one 8 × 10 breeding block (number of females crossed with number of males) and one 7 × 10 block were created. Adult breeders were narcoticized using Tricaine Methanesulfonate (i.e., 0.075 g/L buffered with 0.15 g/L $NaHCO_3$), stripped for gametes for full-factorial breeding, photographed, measured (i.e., standard length and wet weight) and fin clips were collected and stored in 70% ethanol. Fertilised eggs were held in the hatchery using typical hatchery-rearing methods while adult fish were released in their stream of origin after breeding. Post-hatching, fish were kept in a common environment in the hatchery in large tanks with a circulating water current, with a water temperature of 9°C, and fed *ad libitum* with size-appropriate commercial dry fish food. The peak of hatching occurred on 17th February 2019 (i.e., *ca.* 77 days post-fertilisation). Further reports of fish age are based on this hatching date.

A sample of 816 hatchery-raised fish were moved to the laboratory at the University of Lausanne at 190 days post-hatching to study the effects of sympatric diclofenac pollution and proliferative kidney disease infection (Palejowski et al., in preparation). They were randomly divided into 12 groups, with each group containing 68 fish in a 120 L aquaria filled with tap water. Each aquarium was constantly pump-filtered, the water was aerated, a water current was applied, and to reduce stress to the fish a structure was provided to hide under and the front of the aquarium was obscured by an opaque cover. Aquaria were kept in a temperature-controlled room with a 12:12 light-dark cycle at a water temperature of 9°C for 3 weeks to allow the fish to acclimatise to the laboratory environment. The temperature was then gradually raised from 9°C to a final mean of 14.6°C (SD = 0.49°C). Water was changed daily for the first 35 days in the laboratory and every weekday from then on, changing 15 L for the first 70 days then 40 L per change for the following 55 days. Fish were fed *ad libitum* with size-appropriate commercial dry fish food and water quality was monitored weekly using JBL Pro Scan® tests (JBL GmbH, Germany) and JBL Denitrol® was added to correct excessive nitrate levels if necessary. As population density is known to affect salmonid health, and differences in

densities may therefore result in differences in development strategy (Pickering and Pottinger, 1987; Mazur and Iwama, 1993) the number of fish was equalised between aquaria once a week if mortality occurred. Population densities were reduced to the level of the least dense aquaria. This was done by dividing the aquaria into a top-down two-dimensional grid, choosing a grid square using a random number generator and removing and euthanising the nearest individual.

The timing of sexual differentiation was studied by monthly sampling of 24 fish from 129 days post-hatching onward for a period of 5 months (**Table 1**). During each of timespoints 1–3, 24 fish were haphazardly taken from the fish that had been left at *Fischereistützpunkt Reutigen*, while all later samples were taken from the laboratory population (all originating from the same breeding experiment). For sampling timespoints 4 and 5, 2 fish were randomly selected from each of the 12 aquaria and euthanised. Fish were euthanised with an overdose of KoiMed® Sleep (KOI&BONAI, Bühlertann, Germany) at a concentration of 0.7 ml/L. For all fish in all timespoints, total length and weight measures were taken and fin clips were stored in 70% ethanol at −20°C for genetic sexing. The heads (caudal of the operculum) and tails (rostral of the anus) were removed, and the remainder of the bodies were immersed in histological tissue fixative (Hartmann's Fixative, H0290, Sigma-Aldrich, Germany) for 2 weeks to be used for phenotypical sexing and analysis of sexual differentiation. Fixative solution was changed after 1 week for better bone decalcification. After immersion for 2 weeks in histological tissue fixative the bodies were embedded in cassettes and dehydrated using a LEICA TP1020 Tissue Processor (LEICA, Tempe, AZ, United States). After 48 h of dehydration, the tissues were cast in paraffin wax (Histoplast P, Serva, Heidelberg, Germany) with a paraffin dispenser insert (Leica EG1150H, Leica, Tempe, AZ, United States) and cooled to obtain solid paraffin blocks. Using a microtome, 5μm cuts were made in a coronal plane, with a ventral to dorsal direction. Cuts were then floated in a 35°C water bath and collected on glass slides (Thermo Scientific™ SuperFrost Plus™). The sections were deparaffinised, using a xylene substitute (X-tra-solv, Medite, Burgdorf, Germany) dehydrated and stained with Mayer's haematoxylin and eosin in a two-stage procedure according to standard HE-staining protocols (Aescht et al., 2010). Sections were then covered by applying drops of glue (X-tra-Kit, Medite, Burgdorf, Germany) followed by a cover glass (Sail brand™). An average of 12 slides per fish were made and checked for the presence of gonads. All sections containing gonads were analysed for the presence and developmental stage of the gonads (**Supplementary Material**). Phenotypical sex was determined by analysing cellular composition of the gonads with fish being classified using four categories: (i) undeveloped genetic male (only immature germ cells are present in the gonad), (ii) phenotypically differentiated male, (iii) undeveloped genetic female, (iv) phenotypically differentiated female (**Supplementary Material**). One individual was removed before final analysis as phenotypic and genotypic sex were inconsistent likely due to a labelling error during histological slide preparation. To study sex-specific growth and mortality, from timespoints 4 onward this dataset was expanded, randomly sampling five fish per aquaria once every 2 weeks for a total of 3 months. Fish to sample were randomly selected *via* the method used to equalise aquaria densities as described above. All fish sampled were euthanised and fin clips were taken for genetic sexing, but histological analyses were only performed on two of the five fish per aquaria collected at timespoints 4 and 5. Over time, the number of aquaria reduced due to mortality making it impossible to continuously collect five fish per timespoints (see **Supplementary Material** for full details).

## Field Experiment

In autumn 2014, adult brown trout were caught and used to further optimise genetic sexing methods and create offspring used in the field experiment. Breeding males ($♂_n$ = 358) and females ($♀_n$ = 493) were collected around the beginning of the spawning season from different spawning tributaries representing six genetically different populations (Stelkens et al., 2012) in the context of a parallel study (Bylemans et al., in preparation). Adult fish were caught using backpack electrofishing and kept at the hatchery until ovulation allowed the stripping of eggs. All adults were narcoticised when they were ready to spawn and gametes were stripped for block-wise full-factorial *in vitro* fertilisations (Marques da Cunha et al., 2019). Photographs were taken for all adult fish as well as measurements (i.e., standard length and wet weight) and fin clips for molecular analyses. Offspring of the full factorial breeding designs from three different populations were used as the basis for the field experiment here. Breeders from the Giesse were used to create 7 full factorial breeding blocks (i.e., three 2 × 2, one 2 × 3, two 5 × 5 and one 6 × 6 block) producing 14,812 fertilised eggs. A total of 2,659 fertilised eggs were produced from 6 full factorial breeding blocks with breeders from the Rotache (i.e., three 2 × 2, one 3 × 2 and two 5 × 5 blocks). Breeders originating from the Worble were used in 13 full factorial breeding blocks (i.e., nine 2 × 2 and four 5 × 5 blocks) and generated a total of 13,643 fertilised eggs. Breeding was done on Nov 19th and 26th 2014 (Giesse and Worble) and on December 3rd 2014 (Rotache). A sample of 24 eggs per each sibgroup was taken to the laboratory for parallel studies (Marques da Cunha et al., 2019). The remaining eggs were incubated under standard hatchery conditions until hatching and released into the streamlets Schwarzbach (offspring from Giesse), Schnittlauch (offspring from Rotache) and Talbach (offspring from Worble) (**Figure 1**). No other brown trout were stocked into these streamlets in 2015. Stocking was done in early March 2015 about 17 to 18 days at 8°C after peak hatching, i.e., at the developmental stage that corresponds to the stage at emergence from gravel (yolk sacs are not fully used up yet and the larvae start to take up food items).

Between August 2015 and January 2016, juvenile fish were sampled from the three streams stocked with hatchery-born fish. Only one stream section was sampled for Schnittlauch while a lower and upper stream section was sampled for Schwarzbach and Talbach (**Table 1** and **Figure 1**). All five locations differ in ecology and anthropogenic pressures (**Figure 1**). Backpack electrofishing was used to sample locations and for all fish with a body length below 18 cm. Fish were narcoticized with Tricaine Methanesulfonate as described above, photographed on a scale

**TABLE 1** | Summary details of the fish sampled in the field-based study.

| Location | N stocked | Date sampled | N genotyped 0 + | Male (%) | Hatchery-born (%) |
|---|---|---|---|---|---|
| Schnittlauch | 2,659 | 19.08.15 | 181 | 49.2 | 61.1 |
| Schwarzbach lower | 14,812 (in all Schwarzbach) | 10.10.15 | 201 | 54.4 | 71.8 |
| Schwarzbach upper | | 24.10.15 | 174 | 46.6 | 62.9 |
| Talbach lower | 13,643 (in all Talbach) | 23.01.16 | 100 | 42.9 | 9.2 |
| Talbach upper | | 30.01.16 | 95 | 50.5 | 15.7 |

to later take weight and fork length, and fin clip were stored in 70% ethanol for molecular analyses. All fish were released into the wild after they had recovered from the narcosis. A subset of tissue samples was randomly selected and used for further molecular analyses (**Table 1**).

## Molecular Analyses

Tissue samples from both the laboratory and field experiments were used in further molecular analyses. For all samples, DNA was extracted using the BioSprint® 96 robot following the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). A total of 94 samples and two negative controls were included in each batch extraction to assess potential cross-contamination. DNA extracts were quantified using a HS dsDNA assay on a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States). DNA concentrations were standardised to 20 ng/μL where possible and samples with lower DNA concentrations were left undiluted for further analyses.

Standardised DNA extracts were sent to Ecogenics GmbH (Balgach, Switzerland) for microsatellite typing at 13 loci (i.e., Brun13, Brun25, BS131, MST-15, Mst543, MST-591, Ssa-197, Ssa-85, SSOSL438, Str12INRA, Str2INRA, Str58CNRS, and T3-13) using in house protocols (see **Supplementary Material** for full details). Amplification of microsatellite loci was conducted in three multiplex reactions. Multiplex one and two, each containing primers for the amplification of four microsatellites, was performed using 2X QIAGEN® Multi-plex PCR Master Mix (Qiagen GmbH, Hilden, Germany). Multiplex three included primers for the amplification of five microsatellite loci and was amplified using 2X HotStarTaq Mastermix (Qiagen GmbH, Hilden, Germany). Before thermal cycling, a prolonged denaturation step (95°C for 15 min) was included followed by 35 cycles with 94°C for 30 s, 58°C for 90 s, 72°C for 60 s and a final elongation step of 30 min at 72°C. Fragment analyses were performed on a 3730XL DNA Analyser (Applied Biosystems, Foster City, CA, United States) with a GeneScan LIZ500 size standard (Applied Biosystems) and allele calling was performed using the GeneMarker V2.6.4 software (SoftGenetics LLC, State College, PA, United States).

Genetic sexing of all samples was achieved through a modification of the protocol from Quéméré et al. (2014). As previously mentioned, presence-absence detections of the male specific sdY gene fragment can be prone to errors. While errors due to mislabelling, gene silencing and environmentally driven sex reversal are challenging to detect, the potential influences of small levels of cross contamination and the presence of pseudogenes could be mitigated through a more quantitative approach. In a XY sex-determination system, the abundance of the sdY fragment in male individuals should be approximately half that of any autosomal marker (i.e., a relative abundance of 0.5) while in females the sdY fragment should be absent (i.e., a relative abundance of 0). Small levels of cross contamination, especially DNA transfer from males to females, may result in the detection of the sdY fragment in females but the abundance of the sdY fragment relative to autosomal markers should stay well below 0.5 and close to 0. The presence of non-functional sdY pseudogenes, which can be amplified with the target specific primers but possibly with a reduced efficiency, will also result in shifts in the relative abundances of the sdY fragment. Nonetheless, in the majority of cases relative abundance measures of the sdY fragment should always be higher in phenotypic males (see Ayllon et al., 2020). Here we achieve a relative quantification of the sdY fragment by incorporating the target specific primers (Quéméré et al., 2014) into the first microsatellite multiplex assay (**Supplementary Material**). Genetic sexing was then achieved by calculating peak area ratios between the sdY fragment and the autosomal MST-591 microsatellite marker to obtain a more quantitative measure. Under a perfect XY sex-determining system it can be expected that peak area ratios are 0 for females and 0.5 for males. This protocol was applied to all 851 adult breeders collected in the context of this and a parallel study (Bylemans et al., in preparation), whose phenotypic sex was accurately determined through gamete collections, for validation and determining optimal threshold value for assigning genetic sexing. The validated and optimised protocol was subsequently used for the genetic sexing of all juvenile tissue samples from the laboratory study and field experiment.

## Data Analyses

Statistical analyses were performed in R version 3.6.1 (R Development Core Team, 2010). Individuals from the laboratory ($n$ = 1) and field experiment ($n$ = 17) whose sex could not be determined (e.g., due to a failed amplification of the MST-591RB reference) were removed prior to analyses. Statistical significance was evaluated using an alpha value of 0.05.

Validation of the modified genetic sexing protocol was performed by comparing genetic sexing results based on the presence-absence of the sdY fragment and the semi-quantitative analyses. For each approach, the replicability was determined based on 19 males and 13 females for which molecular analyses were performed twice and the false assignment rates were calculated based on all the data from the 351 males and 490 females (Bylemans et al., in preparation) returning high quality genotyping and sexing results (i.e., percentage of missing

loci <50% and the percentage of loci containing tri- and tetra-allelic loci <25%). The threshold value used for genetic sex determination using the peak area ratios was determined by evaluating a range (i.e., from 0.1 to 0.4 with steps of 0.025) of threshold values to minimise the false assignment rates.

Differences in the timing of sexual differentiation and growth rates in the laboratory study were assessed using a generalised linear regression model (glm) and a standard linear regression model (lm), respectively. Analyses of sexual differentiation included a total of 100 individual length measurements over five timespoints (i.e., 1–5) (**Supplementary Material**) with a binomial response variable indicating the differential status (i.e., one for individuals with differentiated gonads and zero for undifferentiated gonads). Sampling timespoints, genetic sex and the two-way interaction were included as categorical explanatory variables. Variable significance was assessed using the *Anova* function from the car package (Fox and Weisberg, 2019) conducting Kenwood–Roger *F*-tests with Satterthwaite degrees of freedom, applying a heteroscedasticity-corrected coefficient covariance matrix [based on Long and Ervin (2000)]. Type 3 sums of squares were calculated, then type 2 were calculated to interpret the final model if no significant effect of the interaction was found. Length was used as a proxy for growth rates as all sampled fish were approximately the same age. Total length data of 349 individuals (**Supplementary Material**) was included as continuous response variable with timespoints, genetic sex and the two-way interaction as categorical explanatory variables. Model assumptions were checked *via* visual inspection of residuals and quantile-quantile plots, and a heteroscedasticity-corrected coefficient covariance matrix was applied to correct for heteroscedasticity. Variable significance was tested as described above. Finally, sex-specific mortality in the laboratory study was assessed by testing for an association between sex ratios and sampling date, as a significant relationship would indicate a higher rate of mortality in one of the sexes. The data used consisted of all individual fish sampled as part of the laboratory study (**Supplementary Material**). A glm model with sampling date (converted to Julian date) as a continuous explanatory variable was used with the genetic sex (i.e., value of zero for females and one for males) as a binomial response variable, again conducting Kenwood-Roger *F*-tests. Model analysis was carried out with the *Anova* function from the car package as described above, calculating only type 2 sums of squares.

Prior to the analyses of the field experiment, juvenile fish representing the young-of-year (0 +) age class were selected based on density plots of the distribution of fork lengths, with all individuals with length less than a determined threshold value being characterised as 0 + (**Supplementary Material**). Individuals were further characterised as being hatchery- or wild-born through parental assignments using microsatellite data (see **Supplementary Material** for further details). The effects of sex and hatchery raising on growth and mortality were assessed using a standard linear regression model and Chi-squared goodness of fit tests, respectively. Fork length data from 748 individuals was modelled as a continuous response variable, including genetic sex, rearing origin, location, all two-way interactions and the three-way interaction as categorical explanatory variables. Model assumptions were checked *via* visual inspection of residuals and quantile-quantile plots, and a heteroscedasticity-corrected coefficient covariance matrix was applied to correct for heteroscedasticity. Variable significance was assessed using the *Anova* function from the car package, applying a heteroscedasticity-corrected coefficient covariance matrix. No evidence was found for a significant effect of the three-way interaction term so it was removed and the model was re-analysed. Type 3 sums of squares were calculated, reverting to type 2 to analyse the final model if no significant effect of the interaction was found. Analyses of sex-specific mortality were conducted testing for significant deviations from a 1:1 sex ratio in the entire study area, in each of the three streams, and in each of the five locations, using the *chisq.test* function from the stats package. Deviations from a 1:1 sex ratio were then also assessed within laboratory and wild populations within each of these groups using the same function.

# RESULTS

## Molecular Sexing

Presence-absence analyses of the sdY fragment showed two distinct peaks around a 166 and 177 base-pair (bp) length roughly corresponding to the previously reported two haplotypes [i.e., Quéméré et al. (2014) reported two haplotypes with a length of 167 and 177 bp]. Replicability of genetic sexing results based on the presence-absence approach was 100 and 61.5% for the phenotypic males and females, respectively. False assignment rates for the presence-absence approach were 0 and 42.4% for phenotypic males and females, respectively. Of all cases testing positive for the sdY fragment, the majority (i.e., 98.1%) showed only a positive signal for the 177 bp haplotype. In the phenotypic males, 2.5% of the cases showed a positive detection for the 166 bp fragment with 1.9% showing only a signal for this haplotype and 0.5% testing positive for both haplotypes. All phenotypic females testing positive for the 166 bp haplotype (i.e., 0.9% of cases) did not show a signal at a 177 bp length. For the modified protocol, an initial evaluation of a range of threshold values (i.e., from 0.1 to 0.4) for the peak area ratio revealed that an intermediate value of 0.25 (i.e., between the 0 and 0.5 values expected for females and males, respectively) reduced the false assignment rate to 0 and 0.2% for the phenotypic males and females, respectively (**Supplementary Material**). Finally, replicability of the genetic sexing using the semi-quantitative approach was 100% for both phenotypic sexes.

## Laboratory Study

Histological analysis showed that females started sex differentiation earlier than males (**Table 2A**). This was evident at timespoints 1, while from timespoints 2 on both sexes showed similar and high rates of sex differentiation that gradually increased to 100% at timespoints 4 (220 days post-hatching) (**Figure 2**). There was no significant sex difference in growth during this period of sex differentiation (**Table 2A** and **Supplementary Figure 3**). Despite some non-explained

**TABLE 2 |** Summary table of the statistical analyses for both the laboratory and field experiments, conducting analysis of variance on linear regression models.

| Response variable | Predictor variable | *DF* | *F* | *p* |
|---|---|---|---|---|
| ***(A) Laboratory study*** | | | | |
| Differentiated | Timespoints | 4 | 10.8 | **<0.001** |
| | Sex | 1 | 5.5 | **0.021** |
| | Timespoints × Sex | 4 | 2.0 | 0.10 |
| Length | Timespoints | 9 | 173 | **<0.001** |
| | Sex | 1 | 0.0 | 0.87 |
| | Timespoints × Sex | 9 | 0.4 | 0.9 |
| ***(B) Field experiment*** | | | | |
| Length | Sex | 1 | 0.5 | 0.47 |
| | Rearing origin | 1 | 0.3 | 0.59 |
| | Location | 4 | 72.4 | **<0.001** |
| | Sex × Rearing origin | 1 | 8.4 | **0.004** |
| | Sex × Location | 4 | 4.6 | **0.001** |
| | Rearing origin × Location | 4 | 7.5 | **<0.001** |

*Timespoints = sampling timespoints, Sex = genetic sex, Rearing origin = whether a fish was born in the hatchery or the wild, Location = in which section (upper/lower) of which stream a fish was caught. For each combination of response and predictor variables the degree of freedoms (DF), the F statistic (F) and the p-value (p) are given. Significant p-values are highlighted in bold.*

mortality in the laboratory population, sex ratio did not change over time ($F = 1.7$, d.f. = 1, $p = 0.20$).

## Field Experiment

A total of 4,906 juvenile fish were sampled from the Schnittlauch, Schwarzbach, and Talbach streams. Of these 4,367 were assessed as being 0 + fish based on length thresholds as detailed above. A total of 762 0 + fish could be categorised as hatchery- or wild-born of which 748 fish could be genetically sexed (**Table 1**).

As expected, the samples differed in average length (see factor "location" in **Table 2B** that also includes effects of the timing of sampling). Overall, there seemed to be no effects of sex or rearing origin on growth (main effects in **Table 2B**). Importantly, however, the sexes grew differently depending on whether they were hatchery-born or wild born (interaction sex × rearing origin in **Table 2B**). Hatchery-born males were on average larger than hatchery-born females, while wild-born females were on average slightly larger than wild-born males (**Figure 3A**).

Sexes also grew differently in the different locations (interaction sex × location in **Table 2B**). Females tended to be larger than males in the Schnittlauch sample, while males tended to the larger than females in the Schwarzbach and Talbach samples (**Figure 3B**). And while hatchery-born fish grew larger in the Talbach and upper Schwarzbach, wild-born ones were larger in the lower Schwarzbach and the Schnittlauch (**Table 2B** and **Figure 3C**). See **Supplementary Material** for the respective pairwise comparisons.

There seemed to be no sex-biassed mortality in the total studied area, in each stream, or in each location (no significant deviations from a 1:1 ratio). There was also no significant sex-biassed mortality in either hatchery- or wild-born fish. All relevant pairwise comparisons are given in the **Supplementary Material**.

## DISCUSSION

We modified an existing protocol to improve the accuracy of genetic sexing and used it to test for sex-specific life histories in early life stages of brown trout. We found that females start sex differentiation on average earlier than males, as previously observed in grayling, another salmonid (Maitre et al., 2017). However, the period of sex differences in differentiation seemed much shorter in brown trout than what was observed in grayling. Moreover, while Maitre et al. (2017) found that males grew larger than females during these juvenile stages, suggesting a trade-off between the investment into gonad formation and growth, we found no such sex-specific differences under laboratory conditions. This was different from what we observed in the field: Females were on average larger than males in two locations

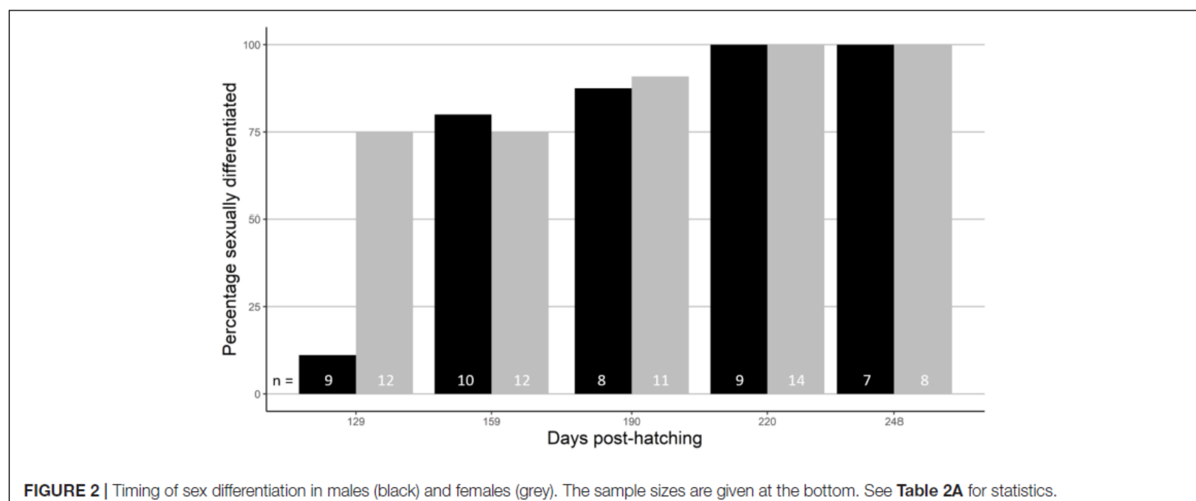



**FIGURE 2 |** Timing of sex differentiation in males (black) and females (grey). The sample sizes are given at the bottom. See **Table 2A** for statistics.

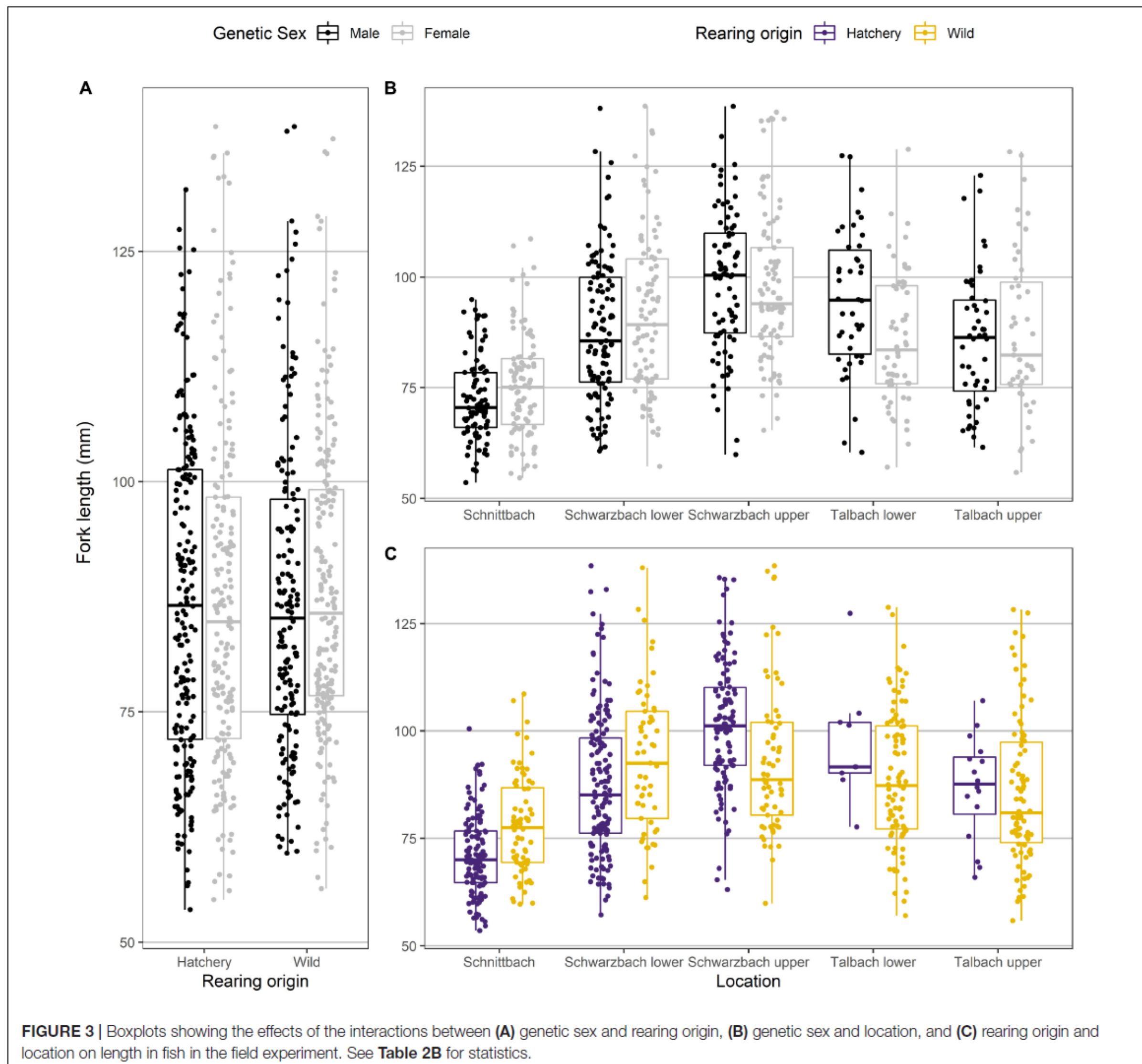



**FIGURE 3 |** Boxplots showing the effects of the interactions between **(A)** genetic sex and rearing origin, **(B)** genetic sex and location, and **(C)** rearing origin and location on length in fish in the field experiment. See **Table 2B** for statistics.

that were sampled first, while males were larger than females in the three locations that were sampled last. It remains to be shown whether these sex differences reveal effects of time or effects of environmental differences between the locations. The location-specific sex differences in growth seemed to not be dependent on whether fish were hatchery-born or wild-born (the corresponding three-way interaction was not significant). We conclude that there are sex-specific life histories in juvenile brown trout that affect the timing of sex differentiation and also growth.

The rate of hatchery-born fish varied between the different locations, ranging from 9.2 to 71.8%. These rates of success may reveal location-specific mortalities of hatchery-born fish, but if so, the mortalities did not seem to be sex-specific as sex ratios remained about equal in all locations (including the laboratory population). We found that hatchery-born 0 + trout were smaller than wild-born ones in the two locations that were sampled first, and tended to be larger than wild-born ones in the locations that were sampled later. Moreover, wild-born females grew on average slightly larger than wild-born males while in hatchery-born trout females grew less than males (even if **Figure 3A** suggests only a marginal effect, the model that takes all effects into account revealed a significant interaction between offspring sex and rearing origin ($p = 0.004$). We conclude that the origin of fish (hatchery or wild) affects growth and sex-specific life histories in brown trout. It is therefore likely that growth finally depends on the interplay between origin, sex, and location, and that we missed the corresponding three-way interaction due to a of lack of statistical power. The nature of potential environmental effects

on sex-specific life histories will require data from more diverse sampling sites that differ, for example, in temperature, river slope, or riparian land use.

In salmonids, female length is tightly linked to fecundity (Lobon-Cervia et al., 1997; Einum and Fleming, 1999; Olsen and Vøllestad, 2003; Gregersen et al., 2009) and sometimes to offspring fitness (Hutchings, 1991; Ojanguren et al., 1996). Furthermore, early life female growth rates have been reported to influence reproduction in brown trout populations, with slower growing females generally delaying maturation and producing smaller clutches with overall larger eggs (Lobon-Cervia et al., 1997; Olsen and Vøllestad, 2003) that affect the life history of the offspring (Taborsky, 2006; Burton et al., 2013; Carim et al., 2017) and may eventually influence population viability (Carim et al., 2017). Our field experiment demonstrates that stocking can affect female growth relative to male growth. Reduced early growth rates in hatchery-born females may thus reveal different life history strategies compared to their wild conspecifics (e.g., mature later and produce smaller clutches with overall larger eggs). The overall effects of these observation on the viability of the studied brown trout population need to be better understood. We conclude that sex can be a factor that affects the impacts of restocking and stock-enhancement programmes (e.g., affects female life history and hence fecundity per age class).

Higher growth rates have often been reported in juvenile males relative to females of various salmonid fishes (Yamamoto, 2004; Mizzau et al., 2013; Maitre et al., 2017), and wild-born fish often differ in growth rates to hatchery-born fish (Einum and Fleming, 2001; Skov et al., 2012; Wilkins and Marsden, 2021). We found that such sex and origin effects are not a general rule but are context dependent in the case of brown trout. In our study, the context was defined by the location and the origin of fish. It is also possible that what we observed is partly the result of stocking-induced evolution, i.e., the long stock-enhancement history (several decades) of the study population could have caused evolutionary shifts in early growth rates.

Maitre et al. (2017) proposed that sex-specific life histories in early life-stage European grayling could be strong enough to result in sex-biassed mortality and may explain the highly male-biassed sex ratio seen in a pre-Alpine population of European grayling (Wedekind et al., 2013). The water temperature that juveniles are exposed to during their first summer best explained these unequal sex ratio (Wedekind et al., 2013). Because temperature effects on sex determination could be excluded (Pompini et al., 2013; Maitre et al., 2017), sex-specific sensitivity to temperature-related stress, and subsequent sex-biassed mortality, caused by sex-specific life history strategies has been proposed to be a likely cause for male-biassed sex ratios in this population and its subsequent decline. Similar drivers may potentially explain the comparable declines in other salmonid populations such as brown trout (Burkhardt-Holm et al., 2002). However, no sex-biassed mortality was observed in either the laboratory and or different streamlets we studied.

Restocking and stock enhancement is a taxonomically and geographically widespread. For conservation purposes, stocking is frequently done at larval or early juvenile stages (Halverson, 2008; Hunt and Jones, 2018), and a growing body of work provides evidence for sex-specific life histories in salmonid larvae and juveniles. We demonstrated that this is also true in the case in brown trout, with females starting sex differentiation earlier than males when raised under laboratory conditions. It is therefore possible that stocking affects sex-specific life histories in this species. We found that growth in the field was indeed sex-specific and depended on further factors that remain to be identified. One contributing factor that we identified here is whether fish were hatchery- or wild-born. We therefore conclude that stocking affects sex-specific life histories. This is a further aspect that needs to be considered when discussing the potential impacts of stocking on population viability and of hatchery-induced evolution.

## DATA AVAILABILITY STATEMENT

The data used in this study have been deposited on the Dryad repository: https://doi.org/10.5061/dryad.7h44j0zwj and https://doi.org/10.5061/dryad.pzgmsbcpj.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Veterinary Office of the Bern Canton and Veterinary Office of the Vaud Canton.

## AUTHOR CONTRIBUTIONS

LM, DN, IC, AU, and CW managed the breeding, sampling, and data extraction for the field experiment. KM developed the genotyping protocol. LM and JB managed the genetic sexing and parental assignments. HP, LM, DN, and CW bred the fish for the laboratory study, which were then raised and sampled by HP and VA. VA conducted the histology for phenotypic sexing, supervised by SK. HP, JB, and CW analysed the data and wrote the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

## FUNDING

The project was funded by grants from the Swiss Federal Office for the Environment and the Swiss National Science Foundation (31003A_159579 and 31003A_182265).

## ACKNOWLEDGMENTS

We thank U. Gutmann and B. Bracher (Fischereistützpunkt in Reutigen) who collected the fish from the wild, assisted in the experimental breeding, raised the fish in the hatchery, and stocked them into the wild. We also thank L. Cornetti, O. Darbellay, J. Ehinger, L. Garaud, F. Glauser, E. Longange, D. Maitre, S. Mandra, E. Pereira-Alvarez, L. Wilkins, and D. Zeugin,

and the members of the fishery associations FV Aaretal, FV Rotache, FV Saane/Sense and FI Bern for their assistance in the field experiments, T. Bösch, J. Kast, and the ecogenics team for genotyping the fish, and T. Braunbeck, A. Hertig, C. Küng, and T. Vuille for support and discussion.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.869925/full#supplementary-material

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# Supplementary information for: Sex-Specific Life History Affected by Stocking in Juvenile Brown Trout

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# Supplementary Material

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# 1 Molecular analyses

## 1.1 Microsatellite genotyping

**Table S1.** Summary table of the three multiplex panels for microsatellite genotyping and genetic sex determination. The latter was achieved through the amplification of a fragment of the brown trout (*Salmo trutta*) sdY gene (i.e. Salmo-sdY).

| Panel | Name | Primers (5'-3') | Core sequence | Reference |
|---|---|---|---|---|
| 1 | MST-591 | CTGGTGGCAGGATTTGA<br>CACTGTCTTTCGTTCTT | $(CT)_{11}$ | (Presa and Guyomard, 1996) |
| | MST-543 | ATTCTTCGGCTTTCTCTTGC<br>ATCTGGTCAGTTTCTTTATG | $(CT)_{13}$ | (Presa and Guyomard, 1996) |
| | SSOSL438 | GACAACACACAACCAAGGCAC<br>TTATGCTAGGTCTTTATGCATT | $(AC)_{26}AT(AC)_6$ | (Slettan et al., 1996) |
| | BS131 | CACATCATGTTACTGCTCC<br>CAGCCTAATTCTGAATGAG | $(TG)_6\ldots(TG)_{18}$ | (Estoup et al., 1998b) |
| | Salmo-sdY | GGGCCTATGAATTTCTGATG<br>ACAGATTTGCGACATGAACA | | (Quéméré et al., 2014) |
| 2 | Str58CNRS | AACAATGACTTTCTCTGAC<br>AAGGACTTGAAGGACGAC | $(GT)_{40}$ | (Poteaux et al., 1999) |
| | Str2INRA | GGTGGCCTGGGTATAGCC<br>GGTGTCGTTCAGCTGTAGCG | $(CT)_4(TG)_{31}$ | (Estoup et al., 1998a) |
| | T3-13 | CCAGTTAGGGTTCATTGTCC<br>CGTTACACCTCTCAACAGATG | $(TG)_{10}CG(TG)_{40}$ | (Estoup et al., 1998b) |
| | Brun25 | TGATGGGATGGTGTTTCC<br>ACATTTGCACACGGGTAG | not reported | (Sønstebø et al., 2007) |
| 3 | Ssa-85 | AGGTGGGTCCTCCAAGCTAC<br>ACCCGCTCCTCACTTAATC | $(GT)_{14}$ | (O'Reilly et al., 1996) |
| | MST-15 | TGCAGGCAGACGGATCAGGC<br>AATCCTCTACTGAAGGGATTTGC | $(CT)_{13}$ | (Estoup et al., 1993) |
| | Ssa-197 | GGGTTGAGTAGGGAGGCTTG<br>TGGCAGGGATTTGACATAAC | $(GT)_5C(TG)_4TC(TG)_3A(GTGA)_{15}$ | (O'Reilly et al., 1996) |
| | Str12INRA | GTTCAGCCTAAGCTTCATGG<br>CTGGGTAAAGAATTCATCTGG | not reported | (Gharbi et al., 2006) |
| | Brun13 | TGAAACTAGGATGCCTGG<br>TCTGACCCACACACAAGC | not reported | (Sønstebø et al., 2007) |

## 1.2 Determination of threshold values for genetic sex determination

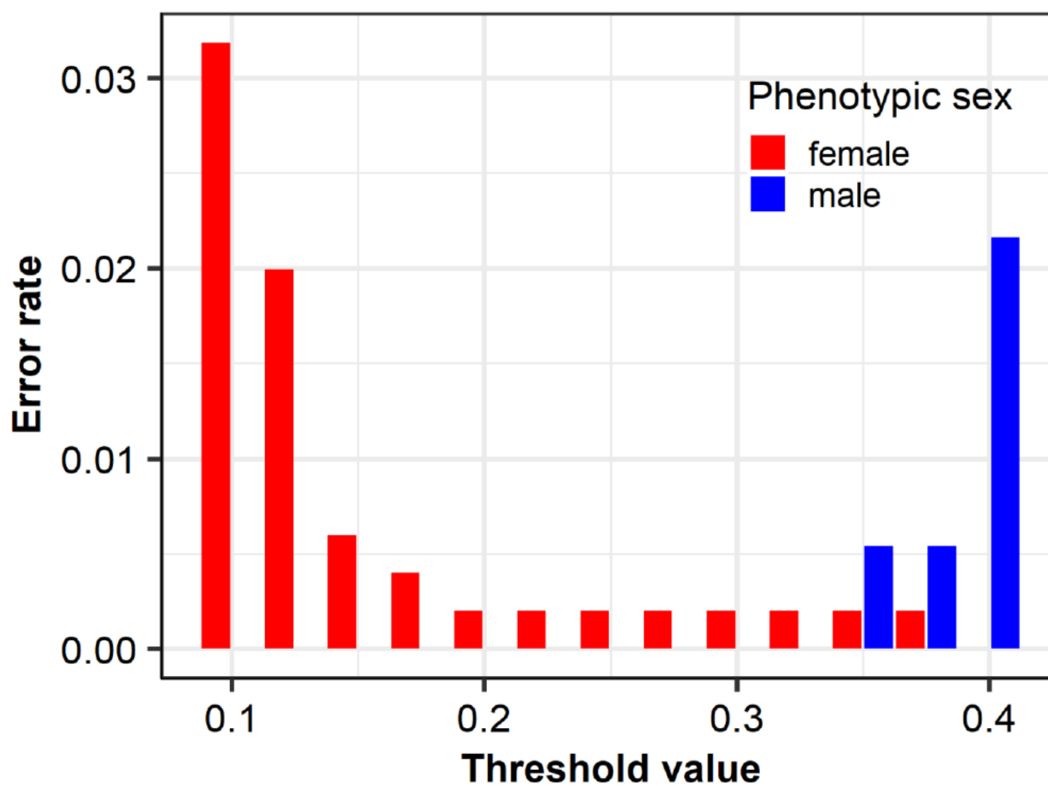


**Figure S1.** Evaluation of range of threshold values (i.e. from 0.1 to 0.4 with steps of 0.025) for the genetic sex determination of the adult brown trout from the 2014 sampling season. Threshold values are given on the x-axis while the false assignment rate (i.e. error rate) is given on the y-axis for both phenotypic males and females.

# 2 Laboratory study

## 2.1 Phenotypic sexing

**Table S2**. The number of fish sampled per timepoint to study sex differentiation and sex-specific effects on growth and mortality ($N_{total}$ = 363).

| | **Timepoint** (days post-hatching) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **1** (129) | **2** (159) | **3** (190) | **4** (220) | **4.5** (234) | **5** (248) | **5.5** (262) | **6** (276) | **6.5** (290) | **7** (304) |
| Total sampled | 24 | 24 | 23 | 58 | 53 | 41 | 34 | 35 | 34 | 37 |
| Sex differentiation | 21 | 22 | 19 | 23 | | 15 | | | | |
| Growth | 24 | 24 | 19 | 55 | 53 | 36 | 33 | 34 | 34 | 37 |

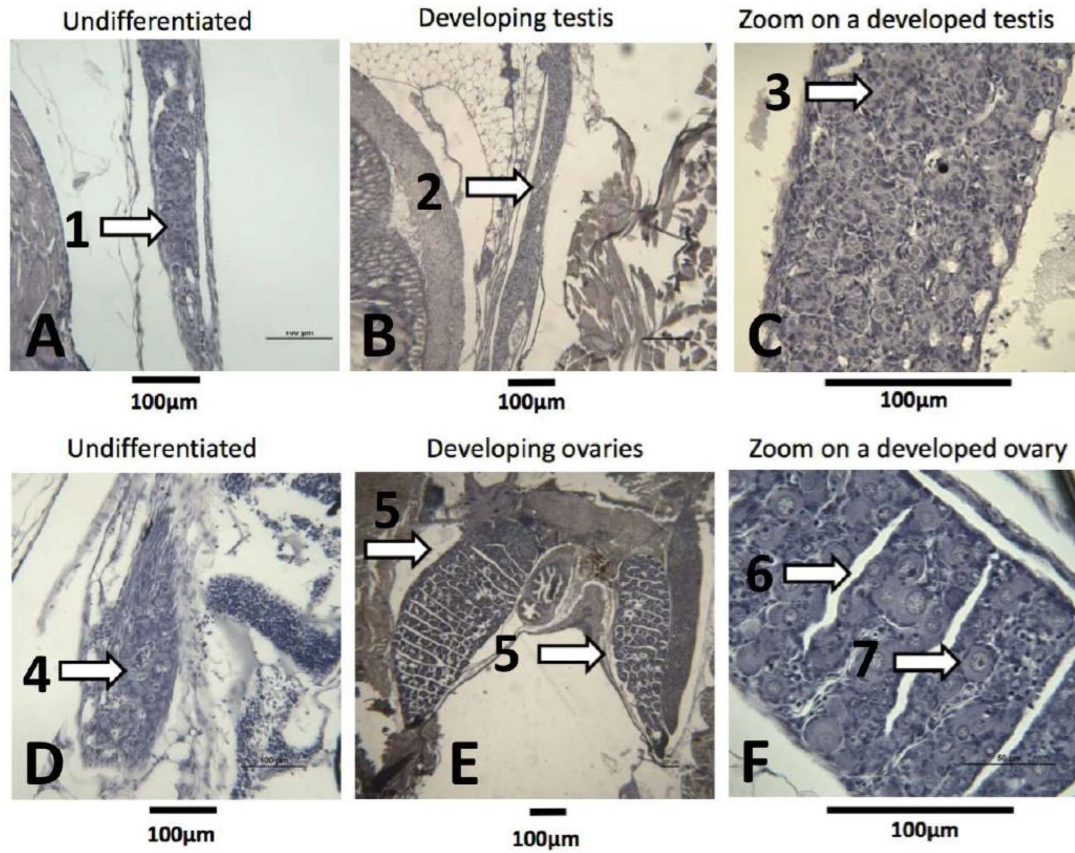


**Figure S2.** Gonad differentiation. (A) Undifferentiated (juvenile) gonad with primordial germ cell (labelled 1) of a genetic male; (B) developing testis (2); (C) undeveloped testis containing only spermatogonia (3). (D) undifferentiated gonad with primordial germ cells (4) of a genetic female; (E) developing ovaries (5); (F) undeveloped ovary with perinucleolar follicles (6) and oocytes (7).

**Table S3.** Different classification categories and indicators for the phenotypic sexing of juvenile brown trout used in the experimental study.

| Phenotypical sex category | Indicator |
|---|---|
| Undifferentiated | Gonad not found<br>Small gonad size<br>Primordial germ cell |
| Differentiated male | Small gonad size<br>Spermatogonia<br>Presence of Sertoli cells |
| Differentiated female | Large gonad size<br>Presence of perinucleolar oocyte or oogonia<br>Presence of ovarian lumen |

## 2.2 Growth analyses

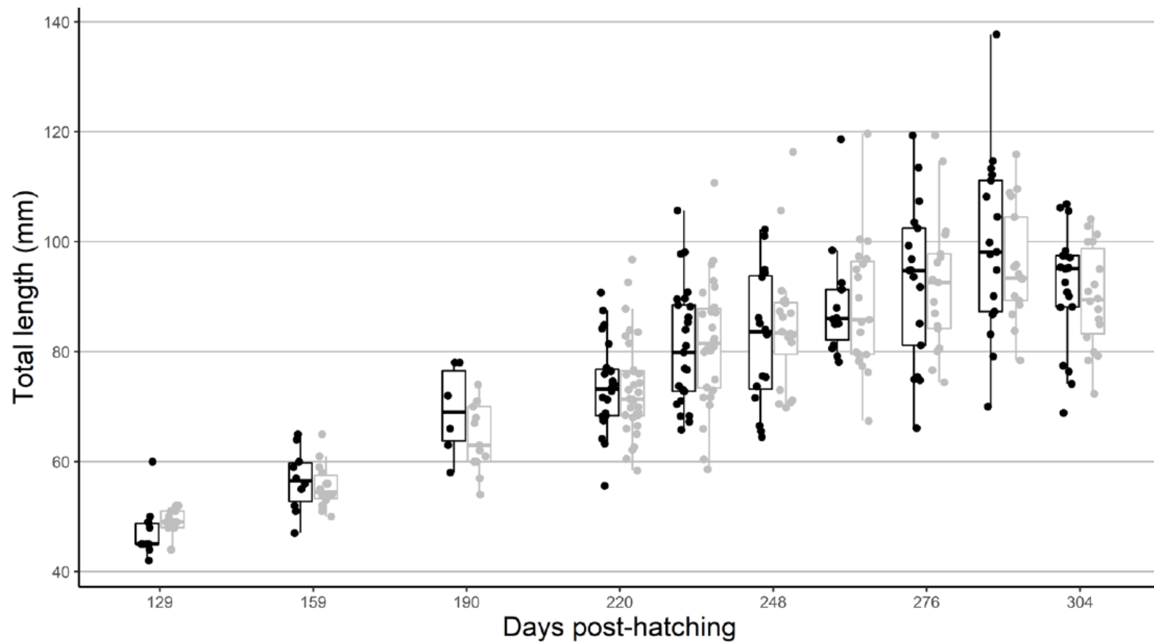


**Figure S3.** Sex-specific length of 0+ fish in the laboratory study. Black represents males, grey represents females. Mean, quartiles, and whiskers indicated. Length measurements plotted over the boxplots. See Table 3 for statistics.

## 3 Field experiment

### 3.1 Characterizing young-of-year fish

Prior to the analyses, juvenile fish representing the young-of-year (0+) age class were selected and 0+ individuals were characterised as being either hatchery- or wild-born. To select 0+ individuals, density plots were constructed to visualise the distribution of the fork length for each of the five sampling locations and a length threshold value was visually determined to only retain the 0+ age class (i.e. the first and largest peak in the density plot, see Figure S3). Classification of 0+ individuals as hatchery- or wild-born was done using parental assignments based on the microsatellite genotypes of the juveniles and the 2014 breeders as potential parents. Parental assignments were performed using the full-likelihood approach implemented in Colony V2.0.6.5 (Jones and Wang, 2010). In the genotyping data, tri- and tetra-allelic loci were recorded at low frequencies (ca. 0.2 %) which could have arisen due to low levels of cross-contamination, the presence of pseudogenes and/or stuttering. To utilise all the available data during the parental assignments, the genotypic data was first transformed to a dominant marker style data which is an approach typically used in polyploid species (Wang and Scribner, 2014; Liu et al., 2017). Although this approach may reduce assignment power, simulations have indicated that the assignment accuracy remains high (i.e. above 90%) when data from more than 10 microsatellite loci is considered (Wang and Scribner, 2014). Other simulation studies have demonstrated that the full-likelihood parentage assignment method as implemented in COLONY outperforms other available approaches (Harrison et al., 2013). Standard parameters were used except for allowing for female and male polygamy with the possibility for inbreeding. The allelic dropout rate and genotyping error rates per locus were estimated from a subset of re-genotyped breeders and were set to 0.0021 and 0.06, respectively. Individuals were characterised as hatchery-raised when they were assigned back to the parental breeding pairs used during the 2014 breeding season with a probability higher than 0.95 while all other individuals were classified as wild-raised.

Previous studies have achieved high assignment accuracy with lower numbers of microsatellite loci, for example, >95% accuracy with 8 microsatellites in Atlantic salmon *Salmo salar* (Norris et al., 2000), 85-100% accuracy with 4 microsatellites in freshwater prawn *Macrobrachium rosenbergii* (Karaket and Poompuang, 2012) and >98% accuracy with 10 microsatellites in giant grouper *Epinephelus lanceolatus* (Weng et al., 2021).

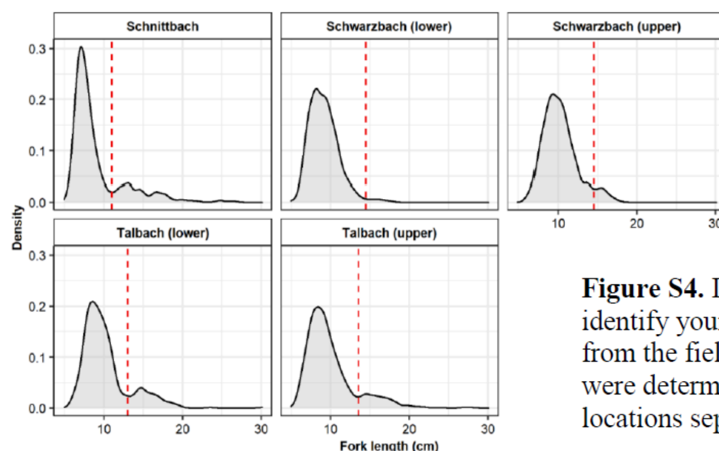


**Figure S4.** Density plots used to visually identify young-of-year (0+) brown trout from the field-based study. Threshold values were determined visually for each of the locations separately.

## 3.2 Statistical results

**Table S4.** Post-hoc testing of pairwise differences in the fork length data between different variable levels. Between group comparisons were conducted using the TukeyHSD function in R with the reported p-values being adjusted using the default method. Statistically significant p-values (< 0.05) are highlighted in bold.

| Comparisons | | Difference | Lower | Upper | adj *p* |
|---|---|---|---|---|---|
| ***A. Between sexes*** | | | | | |
| female | male | 0.65 | -1.50 | 2.80 | 0.552 |
| ***B. Between rearing origins*** | | | | | |
| wild | hatchery | 0.62 | -1.53 | 2.77 | 0.573 |
| ***C. Between locations*** | | | | | |
| Schwarzbach lower | Schnittbach | 15.56 | 11.35 | 19.76 | **<0.001** |
| Schwarzbach upper | Schnittbach | 24.38 | 20.03 | 28.73 | **<0.001** |
| Talbach lower | Schnittbach | 15.42 | 10.28 | 20.56 | **<0.001** |
| Talbach upper | Schnittbach | 12.31 | 7.12 | 17.51 | **<0.001** |
| Schwarzbach upper | Schwarzbach lower | 8.83 | 4.58 | 13.07 | **<0.001** |
| Talbach lower | Schwarzbach lower | -0.14 | -5.19 | 4.91 | 1.000 |
| Talbach upper | Schwarzbach lower | -3.24 | -8.35 | 1.86 | 0.412 |
| Talbach lower | Schwarzbach upper | -8.97 | -14.14 | -3.79 | **<0.001** |
| Talbach upper | Schwarzbach upper | -12.07 | -17.30 | -6.84 | **<0.001** |
| Talbach upper | Talbach lower | -3.10 | -9.00 | 2.80 | 0.603 |
| ***D. Between sexes within rearing origins*** | | | | | |
| female:hatchery | male:hatchery | -0.72 | -4.64 | 3.21 | 0.966 |
| male:wild | male:hatchery | -0.78 | -4.80 | 3.24 | 0.959 |
| female:wild | male:hatchery | 1.27 | -2.62 | 5.16 | 0.835 |
| male:wild | female:hatchery | -0.06 | -4.17 | 4.04 | 1.000 |
| female:wild | female:hatchery | 1.99 | -1.99 | 5.96 | 0.572 |
| female:wild | male:wild | 2.05 | -2.02 | 6.12 | 0.565 |
| ***E. Between sexes within locations*** | | | | | |
| female:Schnittbach | male:Schnittbach | 3.41 | -3.66 | 10.48 | 0.880 |
| male:Schwarzbach lower | male:Schnittbach | 15.53 | 8.77 | 22.30 | **<0.001** |
| female:Schwarzbach lower | male:Schnittbach | 19.38 | 12.26 | 26.51 | **<0.001** |
| male:Schwarzbach upper | male:Schnittbach | 26.62 | 19.32 | 33.92 | **<0.001** |
| female:Schwarzbach upper | male:Schnittbach | 25.71 | 18.66 | 32.76 | **<0.001** |
| male:Talbach lower | male:Schnittbach | 22.90 | 14.00 | 31.80 | **<0.001** |
| female:Talbach lower | male:Schnittbach | 12.91 | 4.80 | 21.02 | **<0.001** |
| male:Talbach upper | male:Schnittbach | 13.64 | 5.12 | 22.15 | **<0.001** |
| female:Talbach upper | male:Schnittbach | 14.44 | 5.87 | 23.02 | **<0.001** |
| male:Schwarzbach lower | female:Schnittbach | 12.13 | 5.42 | 18.83 | **<0.001** |
| female:Schwarzbach lower | female:Schnittbach | 15.98 | 8.91 | 23.05 | **<0.001** |
| male:Schwarzbach upper | female:Schnittbach | 23.22 | 15.97 | 30.46 | **<0.001** |
| female:Schwarzbach upper | female:Schnittbach | 22.30 | 15.31 | 29.29 | **<0.001** |
| male:Talbach lower | female:Schnittbach | 19.49 | 10.64 | 28.34 | **<0.001** |
| female:Talbach lower | female:Schnittbach | 9.50 | 1.45 | 17.56 | **0.007** |
| male:Talbach upper | female:Schnittbach | 10.23 | 1.77 | 18.70 | **0.005** |
| female:Talbach upper | female:Schnittbach | 11.04 | 2.51 | 19.56 | **0.002** |
| female:Schwarzbach lower | male:Schwarzbach lower | 3.85 | -2.91 | 10.62 | 0.731 |
| male:Schwarzbach upper | male:Schwarzbach lower | 11.09 | 4.14 | 18.04 | **<0.001** |
| female:Schwarzbach upper | male:Schwarzbach lower | 10.17 | 3.49 | 16.86 | **<0.001** |
| male:Talbach lower | male:Schwarzbach lower | 7.36 | -1.25 | 15.98 | 0.170 |
| female:Talbach lower | male:Schwarzbach lower | -2.62 | -10.42 | 5.17 | 0.988 |
| male:Talbach upper | male:Schwarzbach lower | -1.89 | -10.11 | 6.32 | 0.999 |
| female:Talbach upper | male:Schwarzbach lower | -1.09 | -9.36 | 7.19 | 1.000 |
| male:Schwarzbach upper | female:Schwarzbach lower | 7.24 | -0.06 | 14.54 | 0.054 |
| female:Schwarzbach upper | female:Schwarzbach lower | 6.32 | -0.73 | 13.37 | 0.123 |
| male:Talbach lower | female:Schwarzbach lower | 3.51 | -5.39 | 12.41 | 0.963 |

| | | | | | |
|---|---|---|---|---|---|
| female:Talbach lower | female:Schwarzbach lower | -6.47 | -14.58 | 1.64 | 0.253 |
| male:Talbach upper | female:Schwarzbach lower | -5.75 | -14.26 | 2.77 | 0.499 |
| female:Talbach upper | female:Schwarzbach lower | -4.94 | -13.51 | 3.63 | 0.717 |
| female:Schwarzbach upper | male:Schwarzbach upper | -0.92 | -8.14 | 6.31 | 1.000 |
| male:Talbach lower | male:Schwarzbach upper | -3.73 | -12.77 | 5.31 | 0.952 |
| female:Talbach lower | male:Schwarzbach upper | -13.71 | -21.97 | -5.45 | **<0.001** |
| male:Talbach upper | male:Schwarzbach upper | -12.98 | -21.64 | -4.32 | **<0.001** |
| female:Talbach upper | male:Schwarzbach upper | -12.18 | -20.90 | -3.46 | **<0.001** |
| male:Talbach lower | female:Schwarzbach upper | -2.81 | -11.65 | 6.03 | 0.992 |
| female:Talbach lower | female:Schwarzbach upper | -12.80 | -20.84 | -4.75 | **<0.001** |
| male:Talbach upper | female:Schwarzbach upper | -12.07 | -20.52 | -3.62 | **<0.001** |
| female:Talbach upper | female:Schwarzbach upper | -11.26 | -19.77 | -2.75 | **0.001** |
| female:Talbach lower | male:Talbach lower | -9.98 | -19.69 | -0.28 | **0.038** |
| male:Talbach upper | male:Talbach lower | -9.26 | -19.30 | 0.79 | 0.101 |
| female:Talbach upper | male:Talbach lower | -8.45 | -18.55 | 1.64 | 0.194 |
| male:Talbach upper | female:Talbach lower | 0.73 | -8.62 | 10.08 | 1.000 |
| female:Talbach upper | female:Talbach lower | 1.53 | -7.87 | 10.94 | 1.000 |
| female:Talbach upper | male:Talbach upper | 0.81 | -8.95 | 10.56 | 1.000 |
| ***F. Between rearing origins within locations*** | | | | | |
| wild:Schnittbach | hatchery:Schnittbach | 6.56 | -0.70 | 13.82 | 0.117 |
| hatchery:Schwarzbach lower | hatchery:Schnittbach | 16.41 | 10.39 | 22.42 | **<0.001** |
| wild:Schwarzbach lower | hatchery:Schnittbach | 22.11 | 14.36 | 29.86 | **<0.001** |
| hatchery:Schwarzbach upper | hatchery:Schnittbach | 30.03 | 23.62 | 36.44 | **<0.001** |
| wild:Schwarzbach upper | hatchery:Schnittbach | 21.69 | 14.26 | 29.11 | **<0.001** |
| hatchery:Talbach lower | hatchery:Schnittbach | 24.35 | 7.87 | 40.83 | **<0.001** |
| wild:Talbach lower | hatchery:Schnittbach | 17.66 | 10.90 | 24.43 | **<0.001** |
| hatchery:Talbach upper | hatchery:Schnittbach | 16.19 | 3.11 | 29.27 | **0.004** |
| wild:Talbach upper | hatchery:Schnittbach | 14.94 | 7.96 | 21.91 | **<0.001** |
| hatchery:Schwarzbach lower | wild:Schnittbach | 9.85 | 2.91 | 16.78 | **<0.001** |
| wild:Schwarzbach lower | wild:Schnittbach | 15.55 | 7.07 | 24.03 | **<0.001** |
| hatchery:Schwarzbach upper | wild:Schnittbach | 23.47 | 16.19 | 30.75 | **<0.001** |
| wild:Schwarzbach upper | wild:Schnittbach | 15.13 | 6.94 | 23.32 | **<0.001** |
| hatchery:Talbach lower | wild:Schnittbach | 17.79 | 0.96 | 34.63 | **0.029** |
| wild:Talbach lower | wild:Schnittbach | 11.10 | 3.51 | 18.70 | **<0.001** |
| hatchery:Talbach upper | wild:Schnittbach | 9.63 | -3.89 | 23.16 | 0.417 |
| wild:Talbach upper | wild:Schnittbach | 8.38 | 0.60 | 16.16 | **0.023** |
| wild:Schwarzbach lower | hatchery:Schwarzbach lower | 5.70 | -1.74 | 13.15 | 0.309 |
| hatchery:Schwarzbach upper | hatchery:Schwarzbach lower | 13.62 | 7.57 | 19.67 | **<0.001** |
| wild:Schwarzbach upper | hatchery:Schwarzbach lower | 5.28 | -1.83 | 12.39 | 0.354 |
| hatchery:Talbach lower | hatchery:Schwarzbach lower | 7.95 | -8.39 | 24.29 | 0.874 |
| wild:Talbach lower | hatchery:Schwarzbach lower | 1.25 | -5.16 | 7.67 | 1.000 |
| hatchery:Talbach upper | hatchery:Schwarzbach lower | -0.21 | -13.12 | 12.69 | 1.000 |
| wild:Talbach upper | hatchery:Schwarzbach lower | -1.47 | -8.11 | 5.17 | 0.999 |
| hatchery:Schwarzbach upper | wild:Schwarzbach lower | 7.92 | 0.15 | 15.69 | **0.042** |
| wild:Schwarzbach upper | wild:Schwarzbach lower | -0.42 | -9.05 | 8.21 | 1.000 |
| hatchery:Talbach lower | wild:Schwarzbach lower | 2.24 | -14.81 | 19.30 | 1.000 |
| wild:Talbach lower | wild:Schwarzbach lower | -4.45 | -12.51 | 3.62 | 0.766 |
| hatchery:Talbach upper | wild:Schwarzbach lower | -5.92 | -19.71 | 7.88 | 0.938 |
| wild:Talbach upper | wild:Schwarzbach lower | -7.17 | -15.42 | 1.07 | 0.151 |
| wild:Schwarzbach upper | hatchery:Schwarzbach upper | -8.34 | -15.79 | -0.89 | **0.015** |
| hatchery:Talbach lower | hatchery:Schwarzbach upper | -5.67 | -22.16 | 10.82 | 0.985 |
| wild:Talbach lower | hatchery:Schwarzbach upper | -12.37 | -19.16 | -5.57 | **<0.001** |
| hatchery:Talbach upper | hatchery:Schwarzbach upper | -13.83 | -26.93 | -0.74 | **0.029** |
| wild:Talbach upper | hatchery:Schwarzbach upper | -15.09 | -22.09 | -8.09 | **<0.001** |
| hatchery:Talbach lower | wild:Schwarzbach upper | 2.66 | -14.25 | 19.57 | 1.000 |
| wild:Talbach lower | wild:Schwarzbach upper | -4.03 | -11.78 | 3.73 | 0.824 |
| hatchery:Talbach upper | wild:Schwarzbach upper | -5.50 | -19.12 | 8.12 | 0.958 |
| wild:Talbach upper | wild:Schwarzbach upper | -6.75 | -14.69 | 1.19 | 0.176 |
| wild:Talbach lower | hatchery:Talbach lower | -6.69 | -23.32 | 9.94 | 0.959 |
| hatchery:Talbach upper | hatchery:Talbach lower | -8.16 | -28.21 | 11.89 | 0.955 |
| wild:Talbach upper | hatchery:Talbach lower | -9.42 | -26.13 | 7.30 | 0.743 |
| hatchery:Talbach upper | wild:Talbach lower | -1.47 | -14.74 | 11.80 | 1.000 |
| wild:Talbach upper | wild:Talbach lower | -2.73 | -10.05 | 4.60 | 0.975 |
| wild:Talbach upper | hatchery:Talbach upper | -1.26 | -14.63 | 12.12 | 1.000 |

**Table S5.** Summary statistics for the Chi-squared goodness-of-fit tests to assess deviations from equal sex ratios in the specific groups. Adjusted p-values are correction for multiple testing using the Holm-Bonferroni method, correcting for 24 tests.

| Test | N | $\chi^2$ | DF | p | adj p |
|---|---|---|---|---|---|
| ***A. Within entire study system*** | | | | | |
| all | 748 | 0.05 | 1 | 0.83 | 1 |
| hatchery-raised | 387 | 0.75 | 1 | 0.39 | 1 |
| wild-raised | 361 | 1.47 | 1 | 0.23 | 1 |
| ***B. Within streams*** | | | | | |
| Schnittbach | 181 | 0.05 | 1 | 0.82 | 1 |
| hatchery-raised | 111 | 0.44 | 1 | 0.51 | 1 |
| wild -raised | 70 | 1.43 | 1 | 0.23 | 1 |
| Schwarzbach | 374 | 0.27 | 1 | 0.61 | 1 |
| hatchery-raised | 252 | 0.14 | 1 | 0.71 | 1 |
| wild-raised | 122 | 0.13 | 1 | 0.72 | 1 |
| Talbach | 193 | 0.88 | 1 | 0.35 | 1 |
| hatchery-raised | 24 | 0.67 | 1 | 0.41 | 1 |
| wild-raised | 169 | 1.71 | 1 | 0.19 | 1 |
| ***C. Within locations*** | | | | | |
| Schnittbach | 181 | 0.05 | 1 | 0.82 | 1 |
| hatchery-raised | 111 | 0.44 | 1 | 0.51 | 1 |
| wild-raised | 70 | 1.43 | 1 | 0.23 | 1 |
| Schwarzbach lower | 200 | 2.42 | 1 | 0.12 | 1 |
| hatchery-raised | 143 | 1.57 | 1 | 0.21 | 1 |
| wild-raised | 57 | 0.86 | 1 | 0.35 | 1 |
| Schwarzbach upper | 174 | 0.83 | 1 | 0.36 | 1 |
| hatchery-raised | 109 | 0.74 | 1 | 0.39 | 1 |
| wild-raised | 65 | 0.14 | 1 | 0.71 | 1 |
| Talbach lower | 98 | 2.00 | 1 | 0.16 | 1 |
| hatchery-raised | 9 | 0.11 | 1 | 0.74 | 1 |
| wild-raised | 89 | 2.53 | 1 | 0.11 | 1 |
| Talbach upper | 95 | 0.01 | 1 | 0.92 | 1 |
| hatchery-raised | 15 | 0.60 | 1 | 0.44 | 1 |
| wild-raised | 80 | 0.05 | 1 | 0.82 | 1 |

**Table S6.** Summary table of the analyses of fish growth (i.e. approximated by fork length) using standard linear regression models.

| Response variable | Predictor variable | *DF* | *F* | *p* |
|---|---|---|---|---|
| ***A. Original model with location (i.e. stream name + within stream location)*** | | | | |
| Fork length | Sex | 1 | 0.5 | 0.47 |
| | Rearing origin | 1 | 0.3 | 0.59 |
| | Location | 4 | 72.4 | **<0.001** |
| | Sex * Rearing origin | 1 | 8.4 | **0.004** |
| | Sex * Location | 4 | 4.6 | **0.001** |
| | Rearing origin * Location | 4 | 7.5 | **<0.001** |
| ***B. Modified model with stream (i.e. only stream name)*** | | | | |
| Fork length | Sex | 1 | 0.1 | 0.79 |
| | Rearing origin | 1 | 0.9 | 0.34 |
| | Population | 2 | 127.9 | **<0.001** |
| | Sex * Rearing origin | 1 | 5.4 | **0.021** |
| | Sex * Population | 2 | 4.3 | **0.014** |
| | Rearing origin * Stream | 2 | 6.5 | **0.002** |

Note: Timepoint = sampling timepoint, Sex = genetic sex, Rearing origin = whether a fish was hatchery- or wild-born, Location = stream name and within stream location, Stream = only the stream name where a fish was caught

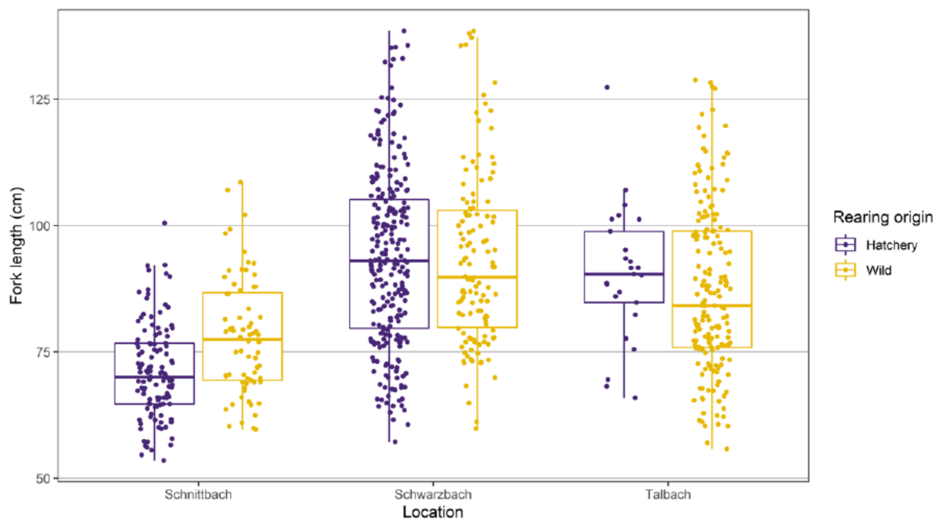


**Figure S5.** Interaction between rearing origin and stream on fork length.

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# Chapter V – Testing for inbreeding depression in juvenile brown trout (*Salmo trutta*)

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Chapter status: unpublished

**Author contributions**

HP and CW conceived the study. HP raised and sampled the fish. HP and CdG produced the RAD-seq libraries. CdG called the SNPs. HP and CdG analysed the data. HP and CW wrote the manuscript with input from CdG. All authors contributed to the article and approved the submitted version.

**Abstract**

Supportive breeding, whereby wildlife managers breed fish in captivity and release the offspring back into the origin population, is a widespread wildlife management practice used to bolster the numbers of fish species for sport and conservation purposes. Despite increasing census size, this practice can induce inbreeding in the supported population if the breeders are related and due to reductions in the effective population size. If inbreeding levels reach thresholds high enough to induce inbreeding depression supportive breeding may counterintuitively contribute to the decline of the population. Salmonid populations in Switzerland are declining and have received supportive breeding for generations, to the point where some populations are now believed to be entirely reliant on management to maintain census size. The worry over inbreeding depression in these populations is therefore great and the consequences of stocking are not well understood. Here we assess whether inbreeding has significant effects on fitness in a population of Swiss brown trout (*Salmo trutta*), a species of conservation concern in Switzerland, that has been exposed to supportive breeding for at least 10 generations. We genotyped 359 3+ juvenile F1 offspring of wild breeders bred using standard hatchery practices and calculated individual inbreeding coefficient ($F_\beta$). We found high inbreeding coefficient, including offspring of breeding between full siblings, parent-offspring, or grandparent-grandoffspring pairings. We tested for associations with 18 fitness-linked traits and found that $F_\beta$ was 0.028 higher in fish that had died from accidental disease infection than in fish that survived in our tanks, indicating immune resistance-mediated viability selection. We also found a positive association between body condition and $F_\beta$; however, we argue that the evidence for this is unconvincing. No other $F_\beta$-trait phenotype association was found, and after multiple testing correction the effect on survival was also not significant. We conclude that hatchery breeding may be inducing inbreeding in Swiss brown trout and that inbreeding depression may be increasing disease susceptibility, but stress that this should be confirmed experimentally.

## Introduction

Populations of salmonids in Switzerland experience widespread intense restocking (Cooke and Cowx, 2006; Wedekind et al., 2022) with over 170 million fish stocked into Swiss waters in 2019 (down from a peak of over 840 million in 1989) (OFEV, 2022). Despite this populations are declining (Burkhardt-Holm et al., 2005; Wedekind and Küng, 2010; Wedekind et al., 2022). In supportive breeding, the method most employed in Switzerland, hatchery-bred offspring of wild-caught parents are released back into their parents' origin waters; restocking with non-local fishes has been prohibited in Switzerland since 1991. This attempts to minimise the negative effects of genetic diversity that stocking can have if interpopulation translocations occur. These include homogenisation of genetically distinct gene pools and their introgression by exotic, potentially maladaptive, alleles (Mills and Allendorf, 1996). In some cases, supportive breeding practices have successfully allowed census size to recover (Berejikian et al., 2011). However, supportive breeding carries other risks to the genetic diversity of the supported population that may not be initially reflected in the census size (Araki and Schmid, 2010; Wedekind et al., 2022), including increasing inbreeding (Ryman and Laikre, 1991). This could lead to inbreeding depression, reducing average fitness in the population. Research over recent decades highlights that hatchery breeding has the potential to induce inbreeding in salmonid populations in as little as a single generation (Wang et al., 2002; Fraser, 2008). The intensity of supportive breeding in Swiss salmonids of conservation concern makes it important to determine whether evidence of inbreeding or inbreeding depression exists in these populations.

Inbreeding arises from mating by related individuals and increases the homozygous and identical by descent fraction of the genome. It is therefore closely linked to genome wide heterozygosity (Alemu et al., 2020). While some level of inbreeding can be advantageous to populations (reviewed in Wang et al., 2002) theory states that it gives rise to inbreeding depression, as recessive deleterious alleles are unmasked in their homozygous state and become open to selection (Charlesworth and Charlesworth, 1987). The traditional method of estimating individual

inbreeding coefficient is based on pedigrees (Wright, 1922). This predicts the identical by descent proportion of the genome based on known common ancestors and assumes that pedigree founders are outbred and unrelated. Pedigree-based methods require detailed breeding records over many generations and are therefore difficult when studying captive populations and impossible when working with cryptic or elusive species such as fishes. Modern methods make use of large-scale molecular genetic data to more precisely estimate the proportion of the genome in isolation by descent (Kardos et al., 2015), even in difficult-to-study species. They therefore estimate realised inbreeding coefficients, while pedigrees calculate expected inbreeding coefficients. The improvement of methods over time has now revealed that inbreeding is widespread in both plants and animals (Crnokrak and Roff, 1999; Keller and Waller, 2002; Angeloni et al., 2011). Genetic methods are also showing it to have an even larger effect than had previously been believed (Hedrick and Garcia-Dorado, 2016), at both individual and population scales (Bozzuto et al., 2019). In salmonids, negative effects of inbreeding are now well-documented, having been found in traits including life history traits (Gjerde et al., 1983a; Thrower and Hard, 2009), growth (Gjerde et al., 1983a; Thrower and Hard, 2009), reproductive success (Su et al., 1996), body development (Aulstad and Kittelsen, 2011), and disease resistance (Eszterbauer et al., 2015). A growing debate amongst fisheries scientists is whether common management practices may in fact be contributing to inbreeding in wild salmonid populations, thereby possibly inducing inbreeding depression in them (Wang et al., 2002; Fraser, 2008).

Despite being widely used as a conservation tool, supportive breeding can induce inbreeding into a population, a fact that is little-appreciated by fisheries managers. Managers remove a small proportion of individuals from the wild, frequently fewer than 50 (Fraser, 2008), breed them in sheltered conditions, where they typically suffer lower rates of predation and therefore have higher recruitment than would be found in the wild, and release the offspring back into the wild. Whether new breeders are caught each season, or a breeding population is held in the hatchery varies (Fraser, 2008). One mechanism by which inbreeding may be induced is through

size-selective breeding. In salmonids female size is tightly linked to fecundity (Lobon-Cervia et al., 1997; Einum and Fleming, 1999) and sometimes to offspring fitness (Hutchings, 1991; Ojanguren et al., 1996), while the growth-predation theory states that in both sexes larger fish have higher survivorship (Meekan and Fortier, 1996; Raventos and Macpherson, 2005). Therefore, managers typically select larger breeders from available stock. In salmonids size has high heritability (Berejikian et al., 2016; Marques da Cunha et al., 2019), thus this size-selective breeding may breed closely related individuals. A second mechanism is via the Ryman-Laikre effect (Ryman and Laikre, 1991). If hatchery breeding is successful then the offspring of the relatively small number of breeders used will make up a relatively large proportion of the next generation (Wedekind et al., 2022). Many of these offspring will be closely related. When they breed in the next season the average offspring will be more inbred than it would have been in the absence of supportive breeding, causing a reduction in the effective population size ($N_e$). The consequences of this effect are large 1) when species have high reproductive variance, as salmonids do (Araki et al., 2007); 2) when supportive breeding is applied over many generations, as is the case in Switzerland (OFEV, 2022); 3) when the population is small, as is likely to be common considering Switzerland's declining salmonid populations (Burkhardt-Holm et al., 2005; Wedekind and Küng, 2010); and 4) when stocked fish represent a large proportion of the breeders, as has been shown in some populations (Schmid and Lundsgaard-Hansen, 2016; Wedekind et al., 2022). The possibility that inbreeding and inbreeding depression may be common in Swiss salmonids is therefore well-supported, making the assessment of evidence for both in species of conservation concern highly pertinent.

We therefore assess evidence for effects of inbreeding on fitness in 359 3+ brown trout (*Salmo trutta*), the F1 offspring of wild-caught breeders, based on genomic sequence data. Brown trout are an ecologically important species that have been translocated around the world (Halverson, 2008; Hunt and Jones, 2018; Hasegawa, 2020). Due to their socioeconomic value restocking practices such as supportive breeding are common (Aas et al., 2018; Cucherousset et al.,

2021). In Switzerland such management actions serve a conservation function as the population has declined by 80% since the 1980s (Burkhardt-Holm et al., 2005; OFEV, 2022). As a result, the parents' population has now seen supportive breeding for more than 10 generations, as our records of stocking in this population network go back to the 1980s, although it is known to have occurred for longer than this without good record keeping. They are therefore an ideal model for salmonids of conservation concern. We tested whether inbreeding coefficient significantly predicts variation in 18 fitness-linked traits to assess evidence for inbreeding depression in a heavily managed salmonid population.

**Methods**

*Study population*

Fish were bred at the *Fischereistützpunkt Reutigen* by communally stripping and mixing the gametes of selected breeders using standard hatchery breeding practices in November 2018. All breeders are believed to originate from the same population, likely the Kiese river (7°37'11,27'' 46°50'55,85'') (Stelkens et al., 2012), and were captured via backpack electrofishing and held in the hatchery until ovulation allowed stripping of the eggs. Fertilised eggs were held in the hatchery using typical hatchery-rearing methods while adult fish were released back to their stream of origin after breeding. After hatching, fish were kept in a common environment in large tanks filled with river water, with a circulating water current applied, fed *ad libitum* with size-appropriate commercial dry fish food, until use in this study.

On the 11$^{th}$ June 2020 900 trout were transported to the University of Lausanne and held in 1800L tanks filled with water pumped from a depth of 50m from Lake Geneva, with a circulating water current applied. Fish were first equally divided into two tanks, then into six on the 16$^{th}$ October 2020, taking care to divide the most and least evasive individuals equally. Water temperature was 8.4°C when fish were introduced to the tanks and varied seasonally with ambient temperature, but remained fairly stable due to the depth of the water source. Regular temperature

data was collected from the 28th April 2021, and ranged from 3.68°C to 12.79°C with a mean of 7.65°C (standard deviation of 0.71°C). Fish were held in these conditions until the end of the experiment.

Tanks were filled with uncleaned unfiltered water pumped from Lake Geneva, thus were exposed to environmentally-relevant pathogens. Mortality occurred in all tanks during the experiment. Salt (Reosel, Switzerland) was provided for trout to treat themselves against skin parasites. Three dead fish were taken to the Institute for Fish and Wildlife Health in Bern on the 22nd February 2021 and evidence was found for Coldwater Disease caused by Flaviobacteria and a bacterial mixed infections involving *Aeoromonas hydrophila*, with secondary infections by *Saprolegnia* sp. Tanks were then treated with the disinfectant Chloramine-T (Halamid, Axcentive SARL, France) treating twice a day, 3 times per week, for one week starting on the 1st March 2021 and the 15th March 2021. On the 13th July 2021 moribund fish were examined by Dr. Ralph Knüsel who diagnosed furunculosis infection caused by *Aeromonas salmonicida* and concurred with Bern's findings of secondary *Saprolegnia* infection. On the 26th July 2021 fish in two tanks were treated with the broad-spectrum antibiotic Shotaflor® (Virbac S.A., Carros Cedex) due to high mortality rates once a day for 10 days, mixed with vegetable oil and administered via food. On the 8th November 2021 a further 2 fish were taken to the Institute for Fish and Wildlife Health and diagnosed with a non-specific bacterial mixed infection and fungal infection in the skin caused by opportunistic pathogens, further confirming the previous findings of *Saprolegnia* infection. From the 1st October 2021 Chloramine-T treatment restarted treating once a week every week.

*Trait measurement*

Fish were sampled on six sampling days: the 19th, 20th, and 30th of April 2021, 10th May 2021, 8th December 2021, then all remaining fish were sampled on 18th January 2022. To sample fish, individuals were collected using hand nets, anaesthetised using a 0.3ml/L solution then euthanised using a 0.7ml/L overdose solution of KoiMed® Sleep (KOI&BONAI, Bühlertann,

Germany). All fish were photographed, total length (cm), fork length (cm), total weight (g), liver weight (g), and gonad weight (g) were measured. All were categorised based on visual inspection of the gonads as male, female, or juvenile (undifferentiated gonad not visibly distinguishable as teste or ovary tissue). A tissue sample was taken from the anal fin from all and stored in 70% ethanol at -20°C for molecular analysis. From these we calculated body condition index, a commonly used proxy for individual fitness (Irons et al., 2007; Arismendi et al., 2011), following Musseau et al. (2018), as $K_i = (\frac{W_i}{L_i^3}) \times 100$ where *W* is the weight (g) and *L* is the total length (cm) for each fish. We also calculated hepatosomatic index (liver weight divided by total weight), gonadosomatic index (gonad weight divided by total weight), and gonad weight disparity (the difference in weight between the two lobes divided by the weight of the smaller lobe). On the 19$^{th}$, 20$^{th}$, and 30$^{th}$ of April kidney and gill weights (g) were also measured. On the 8$^{th}$ December males were stripped for milt and both ovary lobes were extracted from sexually mature females. Males were stripped into 150mm diameter petri dishes soon after euthanasia. Twenty µl of ejaculate was collected, avoiding droplets contaminated with blood or faeces, then transferred to a 1.5 ml microtube and placed on ice. 180 µl of StorFish (imv Technologies, France) was added to dilute the milt sample to 10% concentration, then mixed by quickly vortexing. All samples were kept on ice then at 4°C until analysis. From these samples we measured sperm longevity in seconds, concentration (number of cells in millions ml$^{-1}$), speed (average path velocity in µm second$^{-1}$), and the proportion of fast progressive, slow progressive, non-progressive, and immotile sperm in the ejaculate. Measurements were made using the CASA QualiSperm software (Biophos AG, Switzerland) and recorded under a phase contrast microscope at ×20 magnification 30 seconds after activation. All measurements of a given variable were conducted by one observer on between 5 and 11 sperm samples, except for one male for which just 3 samples were available. Sexually mature females were defined based on visual inspection of ovaries as being in gonad stage 3 or 4, with most follicles in late vitellogenic or spawning stages. For these females we extracted both lobes of the gonad and estimated average egg weight by weighing 10 haphazardly selected eggs to

1 mg precision then dividing by 10. The total number of eggs per female was then estimated as weight of both gonad lobes divided by the weight of one egg.

All dead or moribund fish were also removed, euthanised in the case of moribund fish, and sampled. Weight (g) and fork and total lengths (cm) were measured, and a tissue sample was taken and stored in 70% ethanol at -20°C. For fish with known dates of death longevity in days was also calculated as the number of days between hatching and death. Hatching date per individual was not available so was estimated as 17$^{th}$ February 2019 (the peak hatching date of this cohort; Ulrich Gutmann, personal communication) for all fish.

DNA was extracted from all tissue samples using the BioSprint® 96 robot following the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). DNA extracts were quantified using a HS dsDNA assay on a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States). DNA concentrations were standardised to 20 ng/mL where possible and samples with lower DNA concentrations were left undiluted for further analyses. A subset of extracted DNA samples were then chosen for further molecular analysis.

*Molecular Analysis*

Full details of the molecular analyses are reported in the supplementary information, repeated here in brief. DNA extracts from 359 fish were chosen for sequencing using Restriction site Associated DNA Sequencing (RAD-Seq) and sequenced with an Illumina NovaSeq 6000. DNA extracts were sequenced from 107 randomly selected trout with DNA concentrations over 10ng/µl and that died due to disease infection prior to the 8$^{th}$ December 2021, the 60 trout that were sampled and euthanised on the 8$^{th}$ December 2021, and 192 randomly-selected trout with DNA concentrations over 20ng/µl and that were sampled and euthanised on the 19$^{th}$, 20$^{th}$, and 30$^{th}$ April 2021. Using the VCF obtained after quality checks and SNP filtering, we estimated inbreeding coefficient ($F_{\beta}$) using an allele dosage matrix based method (Goudet et al., 2018). This metric was

chosen as it does not require the allelic frequencies of the overall population to calculate it. All analyses are conducted on these 359 sequenced fish.

*Statistical Analysis*

In total we measured 18 variables related to survival, morphology, and gamete characteristics. A multiple linear regression model of morphological traits was built to assess correlations between $F_\beta$ and morphological traits including total length, $K_i$, hepatosomatic index, gonadosomatic index, gill weight, kidney weight as fixed effects. Associations between $F_\beta$ and lifespan and survival (whether a fish died prior to the 8$^{th}$ December 2012, hereafter "naturally dying fish", or was euthanised on that day, hereafter "surviving fish") could not be analysed with multivariate statistical models as for some individuals lifespan is NA. These would therefore be removed by the modelling function preventing the estimation of an association between $F_\beta$ and survival. Multivariate models were not possible when analysing the remaining variables due to low sample sizes (see below). Linear regression models of $F_\beta$ as a function of a trait of interest were thus built for survival, gonad disparity, sperm longevity, concentration, and speed, and the percentage composition of fast progressive, slow progressive, non-progressive, and immotile sperm in the ejaculate. A linear mixed effects model of $F_\beta$ as a function of lifespan included infection window (a factor describing whether the individual died before or after July 2021) as a random intercept factor. Two linear models of $F_\beta$ as a function of a trait of egg weight and another of egg number both included total length as a covariate as both traits are known to vary with female size (Jonsson and Jonsson, 1999). Three surviving fish showed mild symptoms; we never observed a symptomatic fish recover and assume that these fish would have died soon after sampling. Analysis of survival is therefore run again removing these fish. All linear regression models and linear mixed effects models were built using the lm and lmer functions from the *stats* and *lme4* packages, respectively (Bates et al., 2015; R Core Team, 2020). Model assumptions were checked via visual inspection of residuals and quantile-quantile plots. One outlier that died 163 days before

the next-dying fish was removed from the model of longevity to satisfy model assumptions. Variable significance was assessed using the Anova function from the *car* package (Fox and Weisberg, 2019), with an alpha value of 0.05, conducting F-tests. Adjusted $p$ values were then calculated correcting for multiple testing correction using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) for all models except the multiple linear regression model of morphological traits. Models had final sample sizes of 152 (morphological traits), 74 (longevity), 125 (survival: 75 died, 52 euthanised, 13 (egg weight, egg number), 11 (sperm longevity), 12 (sperm concentration, sperm speed, and the percentage composition of fast progressive, slow progressive, non-progressive, and immotile sperm in the ejaculate).

Finally, we test whether average $F_{\beta}$ differed between the sampling dates with a linear model of sampling date as a function of $F_{\beta}$ using the lm function in the *stats* package. Significance was estimated with the Anova function from the *car* package. All effect sizes were extracted from the summary function in the *stats* package. All statistical analyses were performed in R version 4.0.2 (R Core Team, 2020).

## Results

### *Breeder origin and population genetic statistics*

After filtering for data quality, including sequencing depth, missing loci across individuals and loci out of Hardy-Weinberg equilibrium (see Supplementary Material), a total of 6189 SNPs with a mean coverage of 56.24X were retained with a genotyping rate of 100% and shared between 314 individuals. The $F_{\beta}$ ranged from -0.22 to 0.28 with an overall mean of -0.01. $F_{\beta}$ values surpassed 0.18 in three individuals (1%) and 0.0625 in 32 individuals (10.2%).

### *Associations between inbreeding coefficient and fitness*

Naturally dying fish had $F_{\beta}$ 0.028 higher than surviving fish (Table 1). Removing the three symptomatic fish from the group of surviving fish did not qualitatively change the results (see supplementary information). After multiple testing correction this difference was non-significant.

$K_i$ positively correlated with $F_\beta$ (Table 1). $F_\beta$ did not significantly influence variation in any other trait (Table 1). Variation in average $F_\beta$ was not explained by sampling date (df=3, F=0.5, $p$=0.70) (Figure S3).

## Discussion

We raised 900 F1 offspring of wild-caught brown trout, from a population that has seen supportive breeding for at least 10 generations in six large outdoor tanks until age 3+. We sequenced 359 individuals with RAD-seq and estimated $F_\beta$. After filtering $F_\beta$ was estimated for 314 individuals with $F_\beta$ values ranging from -0.22 to 0.28. We found evidence of high, widespread, inbreeding including between full siblings or grandparent-grandchild. We then tested for associations between $F_\beta$ and 18 fitness-linked traits related to mortality, organ morphology, and gamete characteristics in order to assess evidence for effects of inbreeding on fitness. We found that $F_\beta$ was 0.028 higher in 75 naturally dying fish than in 52 surviving fish, and that $F_\beta$ was positively correlated with $K_i$. We found no other significant associations. After multiple testing correction the effect on survival became non-significant. We conclude that hatchery breeding in this system produces highly inbred individuals, and that there is evidence for inbreeding-fitness correlations including a positive correlation between inbreeding and disease susceptibility. This may increase mortality rates in inbred trout; however, this hypothesis will need to be confirmed.

Population genetic analysis of the genomic sequence data confirms that all individuals came from one population, the Kiese river. Assuming random mating in a large population we would expect realised inbreeding coefficients centred around 0 and with a variance of ±0.03 in the wild, while values of 0.25 ±0.07 would indicate breeding between full siblings or a grandparent and a grandchild (Zhang et al., 2021). We found 3 individuals with inbreeding coefficients in that range, and 10.2% of the population had inbreeding coefficient values above the theoretically expected value for breeding between first cousins (0.0625). Our results indicate that some individuals in this system are highly inbred. Salmonid populations in Switzerland suffered severe population

bottlenecks during the 20th century due to eutrophication which reduced genetic diversity and might have increased inbreeding levels (Vonlanthen et al., 2012). Alternatively inbreeding in this system may be being induced by hatchery breeding practices due to size-selective breeding and the Ryman-Laikre effect, as is predicted by theory (Ryman and Laikre, 1991). Selection is expected to act strongly at embryo and larval stages especially in an externally fertilising species with no parental care (Hendry and Stearns, 2004). In the wild, maladaptive phenotypes will likely be purged at this stage. Relaxed selection in hatchery facilities that feed *ad libitum* and suffer no predation pressure will drastically reduce purging of maladaptive phenotypes, instead releasing fish at a stage where they can survive in the wild. The 'unpurged' maladaptive alleles of these more inbred fish are therefore allowed to mix into the recipient population with consequent effects on population fitness (McDermid et al., 2011). Reviews by Fraser (2008) and Araki and Schmid (2010), however, argue that empirical data showing negative genetic effects of hatchery breeding on stocked populations is mixed (but see Tessier et al. (1997) as an example of increased susceptibility to inbreeding due to stocking). Fraser concludes that hatchery breeding does at least have the potential to substantially reduce fitness in wild populations after as little as one generation, especially if little consideration has been taken to minimise these genetic effects. We conclude that the inbreeding observed in this population likely does stem from the hatchery breeding practices used to stock this system for more than 10 generations.

Significantly higher $F_\beta$ in naturally dying than in surviving fish indicates that inbreeding increases susceptibility to disease, either by increasing susceptibility to infection, or if all fish are equally infected then by increasing mortality due to infection. Our tanks were filled with uncleaned unfiltered lake water piped from Lake Geneva from a depth of 50m and were therefore exposed to environmentally relevant pathogens. Mortality occurred in two peaks around the 24th April and the 14th November 2021. Necropsies performed by the Institute for Fish and Wildlife Health in Bern, Switzerland and Dr Ralph Knüsel diagnosed Coldwater Disease, Furunculosis, both of which are major causes of loss in cultured salmonids (Nikoskelainen et al., 2001; Loch and Faisal, 2015),

and secondary infection by *Saprolegnia*. All naturally dying fish showed clear symptoms of one or more of these diseases and no symptomatic fish was ever observed to recover. Removal of the three mildly symptomatic surviving fish from the model does not qualitatively change results (see supplementary information). No other surviving fish was symptomatic. Evidence of inbreeding depression in salmonid immune response has previously been shown in brown trout infected by the Myxozoan parasite *Myxobolus cerebralis* which causes the ecologically and economically important whirling disease (Eszterbauer et al., 2015). In other groups, evidence for similar effects have been seen in deer mice (*Peromyscus maniculatus*) where genomic diversity negatively correlates with severity of coccidian infection (Budischak et al., 2022), in Soay sheep (*Ovis aries*) where inbreeding level predicts parasite susceptibility (Coltman et al., 1999), and in American crows (*Corvus brachyrhynchos*) where inbred nestlings mounted weaker microbiocidal immune responses (Townsend et al., 2010). The trout that died in our experiment did so prior to their first spawning and are thus juveniles. Juvenile mortality due to inbreeding as we suggest here represents viability selection, where homozygosity has a negative effect on survival to adulthood. Examples of this have been seen in birds and mammals, but few exist in fishes. An early study to suggest such an effect was Coltman et al., (1998) who showed that harbour seals (*Phoca vitulina*) with higher genetic variation had higher birth weight and neonatal survival. Since then, viability selection has been proposed in golden eagle (*Aquila chrysaetos*) where high homozygosity resulted in chick mortality (Doyle et al., 2016), and in fur seals (*Arctocephalus gazelle*) where breeding females were significantly more heterozygous than nonbreeders due to viability selection against homozygous offspring (Forcada and Hoffman, 2014). Evidence for genome-wide heterozygote advantage consistent with viability selection has also been found in the imperial eagle (*Aquila heliaca*) (Doyle et al., 2019). Thus, we suggest that trout in this system may show evidence of viability selection mediated by disease susceptibility, examples of which are already known in the literature (Hoffman et al., 2014). However, we stress that this should be confirmed with further experiments. Importantly, high individual-level inbreeding coefficients do not necessarily (Johnson et al., 2011), but can (Bozzuto

et al., 2019), translate into reduced population viability. Confirmation of an inbreeding depression effect on immune response would be especially noteworthy as the decline of Swiss trout populations has been partially attributed to the parasitic disease Proliferative Kidney Disease (PKD) (Burkhardt-Holm, 2008). Inbreeding-induced reduction in disease resistance may therefore help explain the increase in PKD virulence that started in the 1980s (Burkhardt-Holm et al., 2005).

In addition to the sampling in December 2021 symptomatic and non-symptomatic fish were sampled on other days; it is therefore possible that this could have biased our results by inflating $F_\beta$ in the fish that died or reduced it in the fish that survived. We reject this possibility for the following reasons. As above, we assume all sampled symptomatic fish would have died. During our sampling we removed fish that were visibly symptomatic when observed from outside the tanks, therefore probably removing the fish with the worst symptoms. To have inflated the $F_\beta$ of fish that died we would have had to remove symptomatic fish with lower-than-average $F_\beta$, therefore sampled symptomatic fish would have to be more outbred than average. Firstly, outbreeding can increase disease susceptibility if parents from genetically distinct populations are interbred (Goldberg et al., 2005), but the hatchery that supplied our study population only breeds fish from the same population. Secondly, to have removed more outbred than average symptomatic fish during prior sampling fish showing symptoms on those sampling dates would have to be more outbred than symptomatic fish that died before and after those dates. We find both these possibilities highly unlikely. To have reduced $F_\beta$ of the fish that survived we would have had to remove the more inbred fish from the pool of survivors. Fish were sampled from the tanks haphazardly using hand nets. It's therefore possible that more inbred fish were sampled on previous days if they were less able to avoid the net due to poor swimming performance (Duthie, 1987). However, this mechanism would be expected to depress $F_\beta$ of all fish equally, not just the survivors, thus we also find it highly unlikely. Finally, if sampling prior to December 2021 had affected $F_\beta$ then we would predict a significant difference in the average $F_\beta$ of the different sampling days. We found no such effect (Figure S3). We therefore conclude that this result is not due to a sampling

artefact. Indeed, if fish that die are more inbred than those that survive then by removing fish that would have died and not sequencing them the reported difference in $F_\beta$ may underestimate the true effect size.

We found that higher $F_\beta$ was positively associated with higher $K_i$, a commonly used measure of individual fitness in fishes (Irons et al., 2007; Arismendi et al., 2011). This is contrary to most published literature that either finds that inbreeding has negative (Kincaid, 1983a, 1983b, 2011; Townsend et al., 2010) or no (Gjerde et al., 1983b; Su et al., 1996; Ketola and Kotiaho, 2009; Aulstad and Kittelsen, 2011; Aulstad et al., 2011) effects on condition. While the effect was significant the amount of variation in $K_i$ explained by $F_\beta$ was small (<0.01%). Furthermore, following the terminology of Muff et al. (2021) we found only moderate evidence for this effect. This association should be investigated experimentally in the future.

Prior to multiple testing correction no significant effect was found in 16 of 18 variables that might be expected to respond to inbreeding depression (Falconer and Mackay, 1996; DeRose and Roff, 1999). Life history traits show larger effects of inbreeding than morphological traits (DeRose and Roff, 1999), so finding significant inbreeding depression on a life history trait and not on the majority of morphological traits is expected. Alternatively, the lack of significant effects could result from type II (false negative) errors. These arise from large measurement error, low sample size, or low effect sizes. Sample sizes are large for 9 of 18 variables, those related to survival or morphology, with between 238 and 41 replicates per model. Metrics of survival and organ weight were measured with low error. Tanks were checked every weekday so the day a dead fish was found reflects true date of death closely, although as tanks were not checked on the weekend the distribution of collections throughout the week is biased towards Monday (Figure S4). Organ weights on a given sampling day were all measured in one location on one set of scales therefore within-sample repeatability is high. In contrast, sample sizes for gamete characteristics were low, with between 11 and 13 samples per variable. When measuring both sperm and egg characteristics the protocol was carried out by one person to minimise observer effects. Furthermore, as there is large variation in

$F_\beta$ values (range -0.22 to 0.28) and in survival and organ morphology traits we would have detected an effect if it existed. Thus we argue that a type II error is unlikely in models of mortality and morphology, but acknowledge that these may be possible in the models of gamete characteristics.

Supportive breeding is an internationally widespread practice, and is intensely used in Switzerland. Well-accepted theory predicts that these practices will increase average inbreeding levels in the population, and may therefore lower population fitness due to inbreeding depression. We therefore tested F1 offspring of wild brown trout bred in a hatchery breeding program to see whether variation in individual inbreeding coefficients predicted variation in fitness-linked traits. Increased knowledge of if and how inbreeding depression affects the population may aid the management of populations of conservation concern. We found highly inbred individuals in our population, with values indicating mating between full siblings, parent-offspring, or grandparent-grandoffspring. Disease susceptibility was higher in more inbred fish, however this effect was non-significant after multiple testing correction. We argue that this represents viability selection mediated through disease resistance, but experimental confirmation of this hypothesis is needed. We conclude that hatchery breeding in this system produces inbred fish which may be increasing disease susceptibility in the supported population when hatchery-produced fish breed, thereby contributing to observed declines in brown trout and other salmonids.

**Data availability**

All data will be published on the Dryad Digital Repository.

**Ethics statement**

The study was approved by the Fishery Inspectorate of the Bern canton. Handling of adult fish happened in the context of the cantonal stocking program.

**Funding**

Swiss National Science Fondation (31003A_182265).

**Acknowledgements**

We thank B. Bracher, U. Gutmann, and C. Rolli for breeding and rearing the fish in Reutigen, D. Nusbaumer, L. Marques da Cunha, L. Garaud, G. Brazzola, L. Novovitch, J. Bylemans, D. Rodriguez, G. Perroud, and H. Cayuela for assistance and discussions, and R. Knüsel, H. Schmidt-Posthaus and staff at the Institute for Fish and Wildlife Health for veterinary help.

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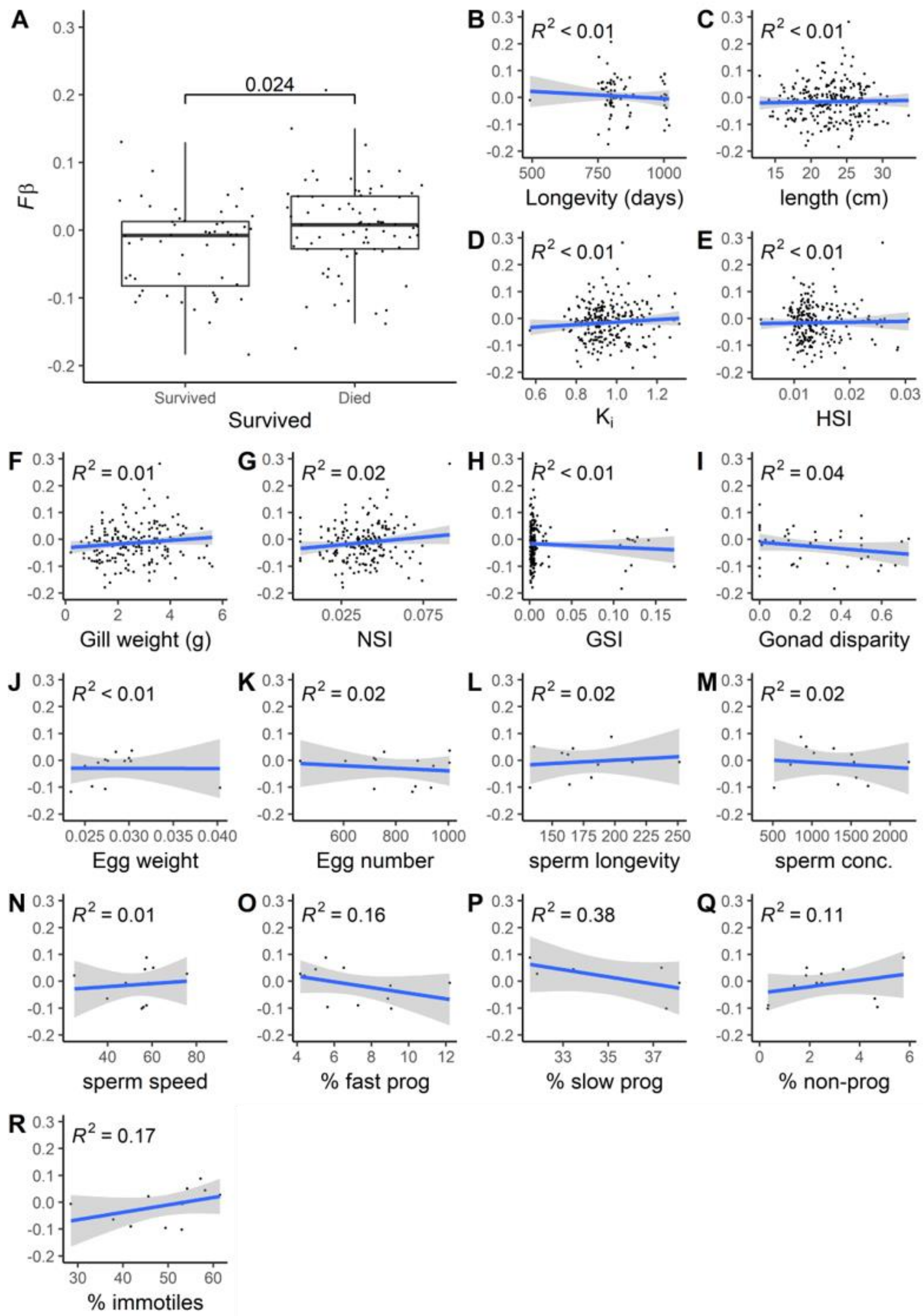
**Tables and figures**

**Table 1.** Associations between inbreeding coefficient and traits of interest. Significant *p* values are highlighted in bold and given to 3 decimal places. adj *p* are *p* values adjusted for multiple testing correction using the Benjamini-Hochberg method, correcting for all univariate and bivariate analyses.

| Trait of interest | DF | F | *p* | adj *p* | Trait of interest | DF | F | *p* | adj *p* |
|---|---|---|---|---|---|---|---|---|---|
| *Model of morphology* | | | | | *Uni- and bivariate statistics* | | | | |
| Length | 1 | 0.2 | 0.65 | | Egg weight | 1 | 0.0 | 0.99 | 0.99 |
| **$K_i$** | **1** | **6.1** | **0.015** | | Egg number | 1 | 2.6 | 0.14 | 0.71 |
| Hepatosomatic index | 1 | 0.1 | 0.70 | | Sperm longevity | 1 | 0.2 | 0.67 | 0.91 |
| Gill weight | 1 | 2.5 | 0.12 | | Sperm concentration | 1 | 0.2 | 0.68 | 0.91 |
| Kidney weight | 1 | 2.1 | 0.15 | | Sperm speed | 1 | 1.6 | 0.24 | 0.71 |
| Gonadosomatic index | 1 | 0.1 | 0.72 | | % Fast progressive | 1 | 0.4 | 0.55 | 0.91 |
| *Uni- and bivariate statistics* | | | | | % Slow progressive | 1 | 0.2 | 0.66 | 0.91 |
| Lifespan | 1 | 0.0 | 0.98 | 0.99 | % Non progressive | 1 | 0.0 | 0.83 | 0.99 |
| **Survival** | **1** | **5.2** | **0.024** | 0.28 | % Immotile sperm | 1 | 0.4 | 0.55 | 0.91 |
| Gonad disparity | 1 | 1.8 | 0.19 | 0.71 | | | | | |

**Figure 1.** Associations between inbreeding coefficient ($F_\beta$) and traits of interest. Y axis is $F_\beta$ for all panels. (A) boxplot (displaying median, upper and lower quartiles, and variation outside of those) of $F_\beta$ in surviving fish and those that died (*p* value of the difference is given), scatterplots with linear regression (of model Y ~ X) and confidence intervals of the association between $F_\beta$ and (B) longevity in days, (C) total length in cm, (D) $K_i$, (E) hepatosomatic index, (F) gill weight in grams, (G) nephrosomatic index, (H) gonadosomatic index, (I) gonad disparity index, (J) egg weight, (K) egg number, (L) sperm longevity in seconds, (M) sperm concentration in million cells $ml^{-1}$, (N) sperm speed, and the % of (O) fast progressive, (P) slow progressive, (Q) non-progressive, and (R) immotile sperm in the ejaculate. $R^2$ values are given in all scatterplots.



A
0.024
Fβ
Survived
Died
Survived
B $R^2$ < 0.01
Longevity (days)
C $R^2$ < 0.01
length (cm)
D $R^2$ < 0.01
$K_i$
E $R^2$ < 0.01
HSI
F $R^2$ = 0.01
Gill weight (g)
G $R^2$ = 0.02
NSI
H $R^2$ < 0.01
GSI
I $R^2$ = 0.04
Gonad disparity
J $R^2$ < 0.01
Egg weight
K $R^2$ = 0.02
Egg number
L $R^2$ = 0.02
sperm longevity
M $R^2$ = 0.02
sperm conc.
N $R^2$ = 0.01
sperm speed
O $R^2$ = 0.16
% fast prog
P $R^2$ = 0.38
% slow prog
Q $R^2$ = 0.11
% non-prog
R $R^2$ = 0.17
% immotiles

# Supplementary information for: Inbreeding depression increases disease susceptibility in juvenile brown trout (Salmo trutta)

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**Content**

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**Figure S1.** Detection of genetic clusters using cross-entropy

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**Figure S3.** Boxplot of Inbreeding coefficient per sampling date.

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**Double-digested RAD sequencing genotyping and SNP calling**

DNA was extracted from 20 mg tissue from the anal fin using the DNAeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA concentration was measured using Qubit 4.0 (Thermo Fisher Scientific, Waltham**,** Massachusetts) while its integrity was verified on a 1% agarose gel. DNA was subsequently standardized to 20 ng/µl to avoid preferential sequencing of individuals with high DNA concentration. Four sequencing libraries were produced with the 359 individuals following the Brelsford et al., (2016) protocol adapted from Parchman et al. (2012) and modified for the Illumina NovaSeq 6000 (Illumina, Cambridge, United Kingdom). Briefly, DNA was digested using the enzymes EcoRI-HF and MspI (New England Biolab, Ipswich, MA, USA). When preparing for the PCR, a UDI (Unique Double Index) was added for each individual. DNA fragments were size-selected in between 400-600bp using a BluePippin 2% DF Marker V2 cassette (Sage Science, MA, USA). Libraries were then single-end genotyped on 4 lanes of Illumina NovaSeq 6000 (SP flowcell) with fragments of 100 bp length at Lausanne Genomic Technologies Facility (University of Lausanne).

After the quality control done with FASTQC v0.11.7 (Andrews, 2010), the heads of the reads were trimmed of 11bp and mapped to the brown trout reference genome (Hansen et al., 2021) using BWA. Individuals for which genotyping has failed (n=16) were remove from further analysis. The resulting SAM file were converted in BAM and sorted by coordinates using Samtools (Danecek et al., 2021). Sorted reads were then processed using Stacks, v.2.53 (Catchen et al., 2013) using the default parameters unless otherwise specified.

SNPs were identified with *Gstacks* (Stacks 2.53) and a first round of filtering was done using *Populations* (Stacks v. 2.52) to generate a VCF file including only loci present in at least 80% of the individuals and markers heterozygosity of 0.5 (*--max_obs_het* 0.50). After quality control on the VCF file we did not find a significant correlation between heterozygosity and coverage ($p=0.859$; $r^2=0$) yet an additional 30 individuals who had more than 30% of missing data where excluded from further analysis. Further filtering was done using *vcftools*, v0.1.15 (Danecek et al., 2011). To reduce

incorrect heterozygosity call, loci were filtered for a minimum coverage of 10X (*--minDP* 10) and a mean maximum coverage of 80X *(--max-meanDP* 80). Only loci under Hardy-Weinberg equilibrium were retained with a P-value threshold of 0.05 (*--hwe* 0.05). Finally the loci not shared between all individuals were discarded (*--max-missing* 1). For more accurate estimates of inbreeding, no filtering was made based on minor allele frequencies.

The realized inbreeding coefficient was estimated using the *beta.dosage* function of the package *Hierfstat* (Goudet, 2005). This function generate a genomic matrix based on allele dosage used to estimate inbreeding coefficients considering the proportion of alleles shared between individuals (Goudet et al., 2018). The presence of a potential genetic structure was tested by further filtering the VCF for maf (*--maf* 0.01), linkage disequilibrium *(--indep-pairwise* 50 10 0.05) and by removing related individuals at the first-cousin degree (*--rel-cutoff* 0.125) using Plink v1.9 (Purcell et al., 2007). With this VCF a principal component analysis (PCA) was done using SNPrelate (*snpgdsPCA;* (Zheng et al., 2012)) and the genetic structure was investigates using LEA (Frichot and François, 2015) by setting *K*=10 with 100 repetitions for each. Population structure analysis were performed in R v.3.6.2 (R Core Team, 2020).

Concerning population structure, a total of 774 SNPs were retained, and 275 individuals were excluded after applying the relatedness cut off. The lowest observed K was 1 (Figure S1) suggesting the presence of a single genetic cluster. The plot obtained from the PCA similarly suggests the presence of only a single genetic cluster as the variance explained by the two principal components is very low (PC1 = 0.06; PC2 = 0.05) (Figure S2).

**Figure S1.** Detection of genetic clusters using the cross-entropy criterion, that evaluate the fit of a model for a given K (number of ancestral populations) where the lowest K is the number of populations that better represent the data, from R package LEA version 3.7.0. The selected value is K=1 for which the cross-entropy is at its minimum.

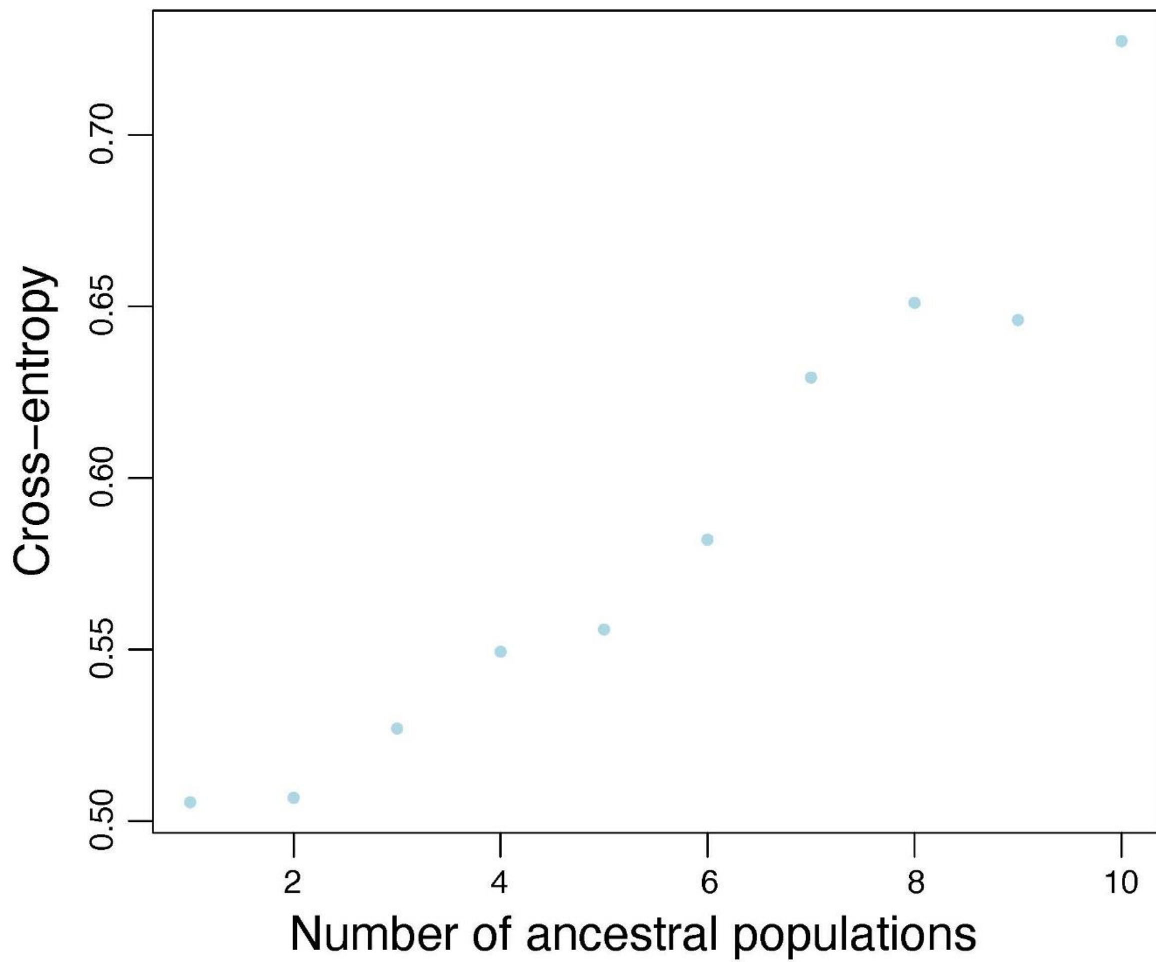
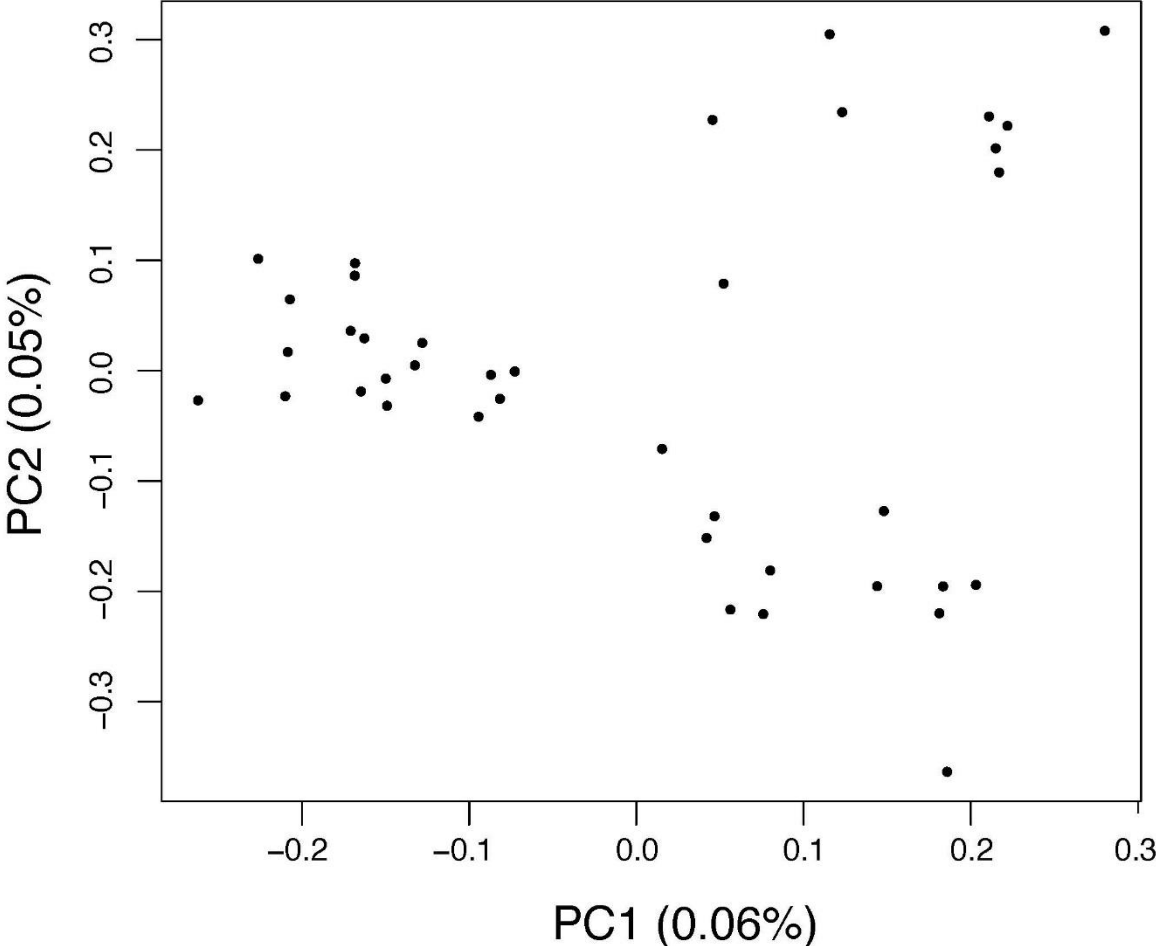


**Figure S2.** Principal component analysis of brown trout from the river Kiese based on 774 SNPs and 39 individuals retained after filtering. Within brackets, the proportion of variance explained by each axis is reported.



**Analysis of survival model including the symptomatic surviving fish**

When sampling on the $8^{th}$ December 2021 three symptomatic fish were noted and sampled. Along with all other fish that were sampled on this day they were selected for sequencing, $F_\beta$ values were calculated, and they could therefore be considered as part of the surviving fish in the model of survival. Throughout the experiment we never observed a symptomatic fish recover: all symptomatic fish died within at most a week. We therefore assume that these three fish would also have died soon after sampling. They are removed from the model of survival and significance is recalculated. Removal from the model of survival does not qualitatively affect the results: naturally dying fish have $F_\beta$ values 0.031 higher than surviving fish and this difference is statistically significant (ANOVA: df=1, F=6.0, $p$=0.015).

**Figure S3.** Boxplot of inbreeding coefficients on the various sampling dates (dd/mm/yy date format).

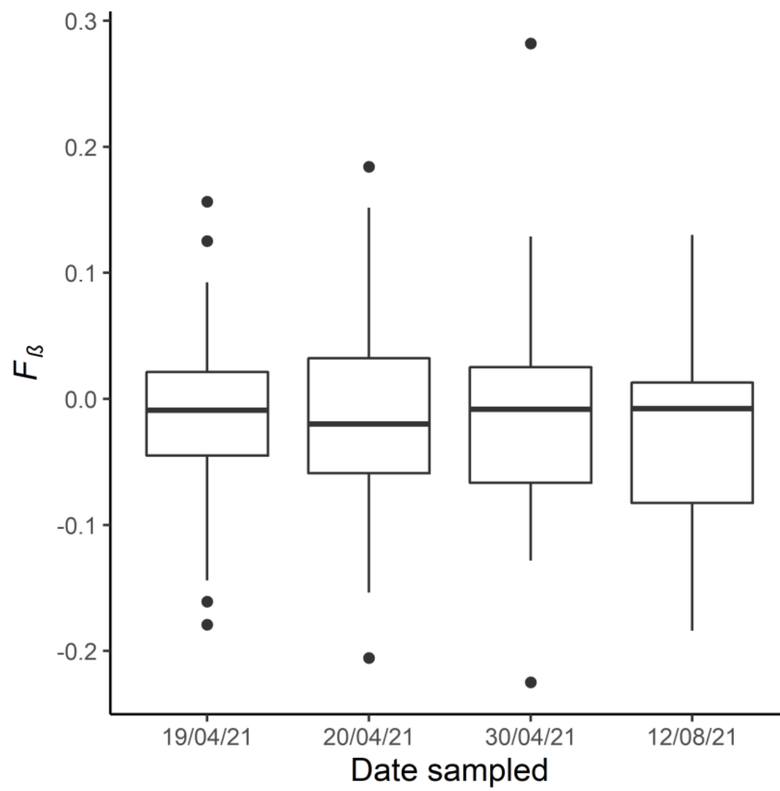
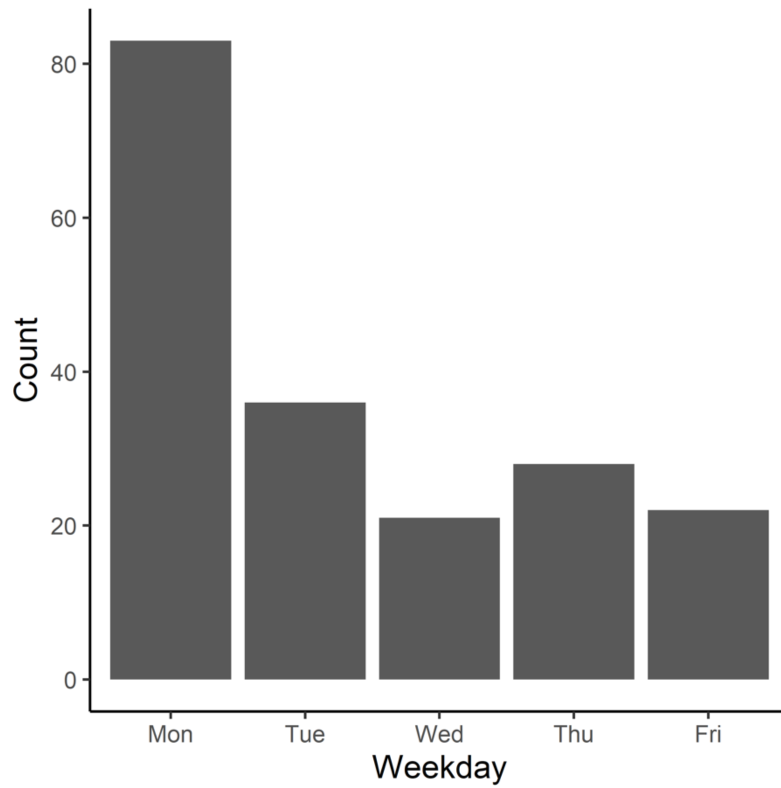


**Figure S4.** Barplot of count of collected dead fish on each week day. Tanks were only checked on weekdays. Fish that had died on Saturday or Sunday were marked as Monday.



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# Summary of results

**Chapter I - Nonlinear growth patterns and predicting end of the larval period in European grayling (*Thymallus thymallus*)**

We found that growth until the end of the larval stage in grayling shows a logarithmic shape, while yolk sac volume declined linearly or with a nearly-linear logistic shape. We developed a modelling tool that predicts the timing of and length at the end of the larval stage in grayling based on hatching characteristics and published this on GitHub. We showed the effect of *Pseudomonas fluorescens* infection on development pattern varied by dam and sire. *P. fluorescens* infection caused earlier hatching at smaller size but with larger yolk sacs.

**Chapter II - Sublethal toxicity and low adaptive potential for tolerance to diclofenac pollution in a wild brown trout population (*Salmo trutta*)**

We found that diclofenac had no effect on hatching success, growth over 14 days, or longevity. Diclofenac-exposed embryos hatched 0.3% and 0.6% faster (50 and 250 $ng \cdot L^{-1}$ diclofenac, respectively) in 2018 and 0.07% faster in 2020, although the latter was not statistically significant. Embryos exposed to 250 $ng \cdot L^{-1}$ diclofenac hatched 9.5% shorter in 2020. Exposure to 50 $ng \cdot L^{-1}$ diclofenac increased long-term growth; however, the evidence in support of this was unconvincing when weight of evidence and directed testing is considered. No significant additive genetic variance for tolerance to diclofenac was found in any measured variable (no diclofenac × sire effects). Brown trout growth patterns were shown to be logarithmic in shape, and the variables significantly affected short- and longer-term growth patterns were not identical.

**Chapter III - Micropollution by diclofenac increases virulence of PKD infection in brown trout**

Neither diclofenac nor PKD had sex-specific effects on any measured variable. Alone, diclofenac had no effect on length, haematocrit, or nephrosomatic index, but did raise weight-length ratio by 0.6% in one experiment. PKD infection reduced haematocrit, increased

nephrosomatic index in the one experiment in which it was measured, and increased weight-length ratio in the other experiment. At the number of days post-infection at which sampling occurred nephrosomatic index significantly predicted weight-length ratio and explained 28% of variation in it. This suggests that PKD was increasing nephrosomatic index, therefore kidney swelling, in both experiments. In *T. bryosalmonae*-infected fish diclofenac increased kidney swelling in the experiment with higher temperature and had no effect on the other. Diclofenac also alleviated PKD-induced reduction in haematocrit.

**Chapter IV - Sex-specific life history affected by stocking in juvenile brown trout**

We published a modified genetic sexing protocol in brown trout that reduces false assignments. We showed that sex-specific life history strategies occur in juvenile brown trout, as females sexually differentiated prior to males, but found no evidence of sex-specific mortality under field or laboratory conditions. Growth rates were not sex-specific in the laboratory but were in the field. They also depended on other factors, including hatchery origin as overall wild-born females grew more than wild-born males, while hatchery-born females grew less than wild-born males. Growth patterns were also affected by other, undetermined, environmental conditions.

**Chapter V - Hatchery breeding induces inbreeding and increases disease susceptibility in juvenile brown trout (*Salmo trutta*)**

Artificial breeding with standard hatchery facility techniques produced highly inbred fish, including offspring of breeding between full siblings or grandparent-grandchild. Inbreeding coefficient was significantly higher in fish had died in our tanks as a result of accidental disease infection than in those that survived. Body condition was positively associated with inbreeding coefficient; however, evidence for this was unconvincing when weight of evidence and directed testing is considered. Inbreeding coefficient did not signficantly explain variation in 16 other traits, and after multiple testing correction the effect on mortality was also non-significant.

# General Discussion

Our understanding of global salmonid population declines is slowly increasing, but still incomplete. We do know that drivers of salmonid losses are multifactorial and species- and site-specific so assessments should be done on a population by population basis (Limburg & Waldman, 2009). In Switzerland there is considerable will to preserve salmonid populations, yet losses, especially of brown trout, continue. Potential drivers of the decline of Swiss brown trout have now been identified, including habitat degradation and disease (Burkhardt-Holm et al., 2005). Considerable literature exists on the effect of acute stressor exposure on adult fish, but early life stages may respond differently due to increased sensitivity (Mohammed, 2013) and changes in the developmental environment. A major focus of this thesis was therefore to experimentally investigate how pollution, an important factor in habitat quality, and disease act and interact to affect fitness in early life-stage trout. We thus assessed the effect of chronic exposure to diclofenac, a common anthropogenic pollutant, on larval trout fitness, and discussed the population's capacity to rapidly adapt to this. We modelled larval growth of brown trout exposed to diclofenac and grayling, a close relative, exposed to a common pathogen to better understand the early-stage salmonid growth patterns and the effects of stressors on them. We then investigated whether diclofenac pollution increases virulence in juvenile trout infected with proliferative kidney disease (PKD), the disease identified as a likely driver of Swiss trout losses. Hatchery stocking is widely implemented to halt the loss of trout and other salmonids in Switzerland, but appreciation of its potential negative effects on the stocked population is growing (Claussen & Philipp, 2022). Thus we investigated two possible mechanisms through which hatchery breeding may be reducing fitness in trout. We used the brown trout population of the river Aare catchment between Lake Thun and the city of Bern as a study population. This population has been exposed to treated sewage effluent for decades and has been heavily stocked since at least the 1980s. It is therefore an ideal population with which to study questions relating to anthropogenic pollution and hatchery management.

## Synthesis

*Effect of diclofenac pollution*

The toxicity of environmentally-relevant diclofenac concentrations to non-target species has been debated extensively in the literature (see reviews by Sathishkumar et al., 2020; Wolf, 2021). In this thesis we found little direct evidence of diclofenac's toxicity at environmentally-relevant concentrations, but did find indirect evidence of toxicity. We found no convincing evidence for a significant effect of diclofenac alone on larval growth or mortality in chapter II, or on three measures of toxicity in juveniles in chapter III. However, in chapter II we argued that we found evidence for precocious hatching in diclofenac-exposed embryos. Environmentally-relevant diclofenac pollution has previously been shown to have diverse sublethal effects (e.g. Bio & Nunes, 2020; Hong et al., 2007). We argued that our results therefore suggest that trout are behaviourally responding to diclofenac toxicity on traits we did not measure. Interestingly, sensitivity to diclofenac may be higher in earlier life stages, as significant effects of diclofenac declined from chapter II (testing diclofenac on larvae) to III (testing diclofenac on juveniles). Due to differences in variables tested and experimental conditions these results should be considered preliminary, but declining sensitivity over ontogeny has been shown in other aquatic species (Mohammed, 2013). The debate over diclofenac toxicity has focused on its effects when alone, despite the fact that stressors co-occur in the wild and can interact (reviewed in Segner et al., 2014). In chapter III we found that environmentally relevant diclofenac pollution increases PKD-associated kidney swelling under certain conditions, probably hotter water temperatures. Ectotherms are sensitive to changes in temperature (Little, 2021) so temperature-stressor interactive effects are to be expected (e.g. Wu et al. 2022). However, we also found that diclofenac alleviated PKD-reduced haematocrit. We found no evidence for diclofenac or PKD causing sex-specific mortality. Resulting demographic shifts and subsequent reductions in effective population size have been suggested as a cause of the decline of the grayling population of Lake Thun, Switzerland (Maitre et al., 2017; Pompini et al.,

2013; Ryman & Laikre, 1991; Wedekind et al., 2013). Our results suggest that neither stressor tested in chapter III is driving such an effect in trout.

Taken together our results indicate that anthropogenic diclofenac pollution, either alone or in synergy with PKD, can have significant negative effects on trout fitness. It may therefore be a driver of trout declines, supporting the findings of Borsuk et al. (2006) that diluted wastewater inputs are contributing to loss of Swiss trout. Furthermore, in chapter II we showed that rapid adaptation to diclofenac stress is unlikely in our study population, although we were unable to determine whether this was due to past adaptation or whether such a response had never been possible. Our study population has been exposed to treated waste water for decades, thus inability to rapidly adapt may be expected in other similarly exposed populations and possibly more broadly. The findings of this thesis contribute to the ongoing debate regarding the effects of diclofenac (e.g. Sathishkumar et al., 2020; Wolf, 2021) and we argue that diclofenac pollution likely can have significant negative ecological effects. However, our results were mixed. We also found no effects of diclofenac under certain environmental conditions, and that it can have positive effects on fitness-linked traits, in chapter III. We conclude that diclofenac likely can have negative effects on trout fitness under some environmental conditions. Diclofenac's occurrence is widespread (aus der Beek et al., 2016) and concentrations are predicted to increase (Schröder et al., 2016). Thus, the frequency and magnitude of negative effects of diclofenac internationally are likely to rise.

*Unintended effects of hatchery breeding*

This thesis suggests that hatchery breeding programs in the Aare may be having diverse, including highly negative, effects on stocked populations. Firstly, in chapter V we showed that hatchery practices may be producing highly inbred progeny for stocking, including offspring of full siblings or grandparent-grandchild breeding, and that disease resistance may be reduced in inbred individuals (e.g. Coltman et al., 1999; Eszterbauer et al., 2015). As discussed in chapter III and above, PKD has been identified as a driver of trout declines in Switzerland. High PKD severity

may be at least partly explained by inbreeding-induced reduction in disease resistance. Secondly, in chapter IV we provided evidence that hatchery breeding affects sex-specific life histories. We demonstrated the existence of sex-specific strategies in juvenile trout as females sexually differentiated before males under laboratory conditions, as in grayling (Maitre et al., 2017). Growth of juveniles in the field was also sex-specific and, importantly, affected by whether a fish was hatchery- or wild-born. Thus we concluded that sex-specific life history strategies in juvenile brown trout are affected by hatchery breeding. Thirdly, our findings suggest that hatchery breeding may be inducing evolution in the stocked populations, as might be expected in small populations stocked intensively for several decades (Fraser, 2008). In chapter IV we found no intersexual differences in growth rates or between hatchery and wild fish, despite these being common in the literature (Einum & Fleming, 2001; Maitre et al., 2017). We argued that this may at least partly result from stocking-induced evolution. We did not, however, find any evidence for sex-biased mortality, which could have threatened population viability as discussed above, and in chapter III. The population-level consequences of the observed hatchery effects are unknown but may well be contributing to Swiss trout population declines.

*Growth patterns in juvenile salmonids*

Though not the primary focus, in this thesis we studied many aspects of salmonid development. Larval growth until emergence (when salmonid larvae emerge from the substrate into which eggs are laid) is rarely studied despite emergence being a crucial period in a fish's life (Einum & Fleming, 2000; Rønnestad et al., 1999). Larval growth patterns may also be inaccurately described by commonly-used growth functions (discussed in Vasbinder & Ainsworth (2020)). In chapter I we modelled grayling growth until emergence and created a modelling tool that predicts timing of and length at emergence, both highly fitness-linked traits (Einum & Fleming, 2000), based on hatching characteristics. We make this available for other researchers. We then applied a similar modelling approach to trout in chapter II. Both chapters highlight that salmonid larval growth

patterns are logarithmic in shape. This serves as a reminder that the simplification of growth into a linear relationship, inherent when growth is calculated between two points, is incorrect in juvenile salmonids as is not the case in juveniles of all fishes (Vasbinder & Ainsworth, 2020). In chapter II we also found that the variables significantly explaining short-term growth and growth until emergence were not identical. We argued that size at emergence is likely to more tightly correlate with fitness than size 14 days post-hatching. Analysis of growth until emergence may have suffered from low statistical power, and to the best of the authors knowledge has not been tested before, therefore this result should be considered preliminary. Nonetheless, this suggests that caution should be taken when using short-term growth patterns to assess the effects of variables on fitness.

**Future directions**

This work has opened new avenues for research which should be explored further. Few studies assess the effect of chronic pollutant exposure on early life stages which this thesis and Mohammed (2013) highlight are likely to be more sensitive. This is necessary to better predict the ecological effects of pollutants including diclofenac. PKD is strongly believed to be a major driver of trout losses in Switzerland (Borsuk et al., 2006; Burkhardt-Holm & Scheurer, 2007), yet we don't know how trout will respond to it evolutionarily. We now need a better understanding of the evolutionary potential of Swiss trout populations to quickly adapt to PKD. In chapter III we weren't able to test for sex-specific effects of the interaction between diclofenac and PKD. Sex-specific susceptibility to stressors have been identified in lake char (*Salvelinus umbla*) (Nusbaumer et al., 2021), and implicated in the decline of a Swiss grayling population (Maitre et al., 2017), as previously discussed. Whether diclofenac and PKD are interacting in a similar way in trout populations should, therefore, be investigated. In chapter V we showed that typical hatchery methods had produced highly inbred fish, and may be inducing inbreeding in a stocked population, a possibility that is well-supported in the literature (Fraser, 2008). However, we did not have a control group that would allow us to conclude that hatchery breeding is inducing inbreeding while

natural breeding is not. This could be experimentally tested in the future to confirm the action of hatchery breeding in increasing inbreeding coefficients. Furthermore, the Aare population has been stocked since at least the 1980s, thus a thorough assessment of the effects of stocking should be undertaken. This will have implications for management decisions in this population, in Switzerland more generally, and will contribute to the ongoing debate over the effectiveness of stocking (Rytwinski et al., 2021). In chapter V we also suggest that inbreeding increases disease susceptibility. After multiple testing this effect was not significant, but is well-supported in the literature (Coltman et al., 1999; Eszterbauer et al., 2015). As disease is believed to be a major driver of trout declines (Borsuk et al., 2006; Burkhardt-Holm & Scheurer, 2007) the veracity of this hypothesis should be experimentally tested. Finally, our study of salmonid development patterns can be built upon. In chapter I we developed a modelling tool to predict emergence in grayling, which can aid research in this species by generating values for two highly fitness-linked variables (timing of, and length at, emergence) based on hatching characteristics. Growth until emergence should be modelled in other salmonids, and predictive tools should be developed for these species. Chapter IV highlights that growth depends on, amongst other things, sex; therefore, genetic sex should be incorporated into these predictive tools.

**Conclusion**

In this thesis we explored one small part of the global biodiversity crisis, but one that is important for Switzerland and Europe generally. Brown trout have fascinated us for thousands of years, first appearing in text in 200CE in Aelian's *De Natura Animalium*. This was, of course, a treatise on how to fish them. Unfortunately, their ongoing population decline in Switzerland (Burkhardt-Holm et al., 2005; OFEV, 2022) is not yet fully understood but probably driven by a combination of overharvesting, habitat degradation, and disease (Burkhardt-Holm et al., 2005; Limburg & Waldman, 2009). This has not been reversed despite a century of hatchery stocking. In this thesis we identified the potential role of anthropogenic diclofenac pollution as a contributor

to the decline of brown trout populations in Switzerland. We stress that large gaps remain in our understanding of how diclofenac affects salmonids, especially at early life stages and at chronic exposures. Through a combination of field and laboratory experiments we highlighted that hatchery stocking practices may be having important impacts on the stocked populations of the Aare, including affecting life history strategies, and inducing inbreeding and inbreeding depression. Such effects could potentially undermine positive conservation effects of stocking. Further study is called for regarding how at least four decades of hatchery stocking, and a similar length of exposure to diclofenac pollution, has affected trout populations of the river Aare.

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