Ecological relevance of laboratory studies with brown trout embryos: hatchling size, carotenoids, and sex predict juvenile performance in the wild

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Abstract

Laboratory studies on embryos of salmonids, such as the brown trout (Salmo trutta), have been used extensively to study factors influencing offspring performance. These studies are based on the implicit assumption that early life-history traits are relevant for growth and survival in the wild. Here we test this assumption. We used gametes of wild-caught brown trout to perform a block-wise full-factorial breeding experiment, with some of the resulting offspring we carried out controlled laboratory experiments, and released the remaining offspring into a natural streamlet. We recaptured 0+ juveniles after their first summer in the wild and used microsatellite and sex-linked markers to determine sex-specific growth and survival for each of the 60 experimental families. We also recorded mean egg size and carotenoid content per female to evaluate their importance for juvenile growth and survival. We found strong correlations between laboratory performance and growth, but not survival, of 0+ juveniles in the wild. Egg size positively influenced juvenile growth. We also found that male juveniles grew faster than females. Juvenile survival seemed not influenced by egg size, but we observed a significant male-biased survival for eggs with a high zeaxanthin content (a carotenoid). This first observation suggests that female reproductive strategies can have sex-specific effects that need to be better understood. We conclude that embryo performance in controlled environments (laboratory or hatchery) can be useful predictors of offspring growth in a natural setting.

Keywords

Salmonids, life-history, growth, survival, maternal environmental effects, trout, embryo, 0+ juvenile

Highlights

• 60 full-sib families were raised under experimental conditions and in the wild
• Hatchling size in the laboratory predicted juvenile growth in the wild
• Evidence for sex-specific effects of egg carotenoids on juvenile survival

1 Introduction

Salmonids are charismatic fish of high ecological and socio-economical value. Understanding the various drivers of phenotypic variation in salmonids has thus been a very active area of research in aquaculture and evolutionary ecology. A multitude of studies under controlled laboratory conditions have been performed to gain insights into environmental, genetic, and maternal environmental
factors that may affect life history, growth, and survival. However, the relevance of such studies for natural populations is still poorly understood.

Salmonids are excellent models for experimental research at early developmental stages. External fertilisation and the lack of parental care allow performing full-factorial \textit{in vitro} fertilisations under controlled conditions. Embryos can be raised singly to generate large numbers of independent replicates. This allows for full-factorial treatments and testing for interesting 2-way or 3-way interactions between parental and environmental factors. Full-factorial crosses can also be used to disentangle the different variance components that contribute to important commercial traits (Colihueque, 2010; Ødegård et al., 2011) and they give insights into, for example, the potential of ‘good-genes’ sexual selection (Wedekind et al., 2008, 2001), the evolutionary potential of populations to react to chemical pollutants (Marques da Cunha et al., 2019; Nusbaumer et al., 2021b), pollution by nanoparticles (Clark et al., 2016; Yaripour et al., 2021), reactions to climate change (Muñoz et al., 2015), or to pathogens (Ødegård et al., 2011). Pathogens might influence hatching time, survival and growth of embryos (Pompini et al., 2013; von Siebenthal et al., 2009; Wilkins et al., 2017), and maternal environmental effects (egg size and content, including various carotenoids) might affect embryonic tolerance (Wilkins et al., 2017). Experiments have also provided insights into alternative female life-history strategies such as the trade-off between maternal egg size and fecundity (Einum and Fleming, 2000). Larger eggs produce larger hatchlings which likely have a competitive advantage over smaller conspecifics (Einum and Fleming, 1999; Ojanguren et al., 1996), and egg size can be a key driver for within and among population differences in early life-history traits (Thorn and Morbey, 2018). Finally, recent experimental work combined with genetic sexing protocols have provided insights into sex-specific timing of sex differentiation (Maitre et al., 2017), mortality (Moran et al., 2016), and stress tolerance (Nusbaumer et al., 2021a). However, experimental studies on embryo and larval characteristics rely on the assumption that these are fitness-relevant traits. This assumption is still poorly backed up, and the ecological relevance of controlled laboratory studies has been questioned (Conover and Baumann, 2009).

Here, we link growth and survival under laboratory conditions with performance in the wild using brown trout (\textit{Salmo trutta}) as our focal species. We produced 60 full-sib families for laboratory-based experiments (Marques da Cunha et al., 2018; Wilkins et al., 2017) and for release into a natural streamlet. We recaptured 0+ juveniles after their first summer in the wild and used molecular markers for sexing and parental assignments. This allowed us to evaluate the ecological relevance of measurements taken on embryos and larvae in the laboratory. We also recorded key maternal environmental variables to assess their importance on growth and survival in the wild.

2 Material and methods

2.1 Experimental breeding and raising of offspring

Adult brown trout of the Aare river catchment in Switzerland were caught as part of the annual stock-enhancement program by the canton fisheries authorities. Twenty-two fish from the Rotache tributary (Supplementary Material) were stripped (Nov 19\textsuperscript{th}, 2014) and their gametes used in two full-factorial breeding blocks consisting of six females crossed with five males each as described in Wilkins et al. (2017). Fin clips were stored in 70 % ethanol at -20 °C for further molecular analyses.

Prior to fertilization, total clutch weight per female was determined and four eggs per female were frozen in liquid nitrogen for later analyses of the carotenoids astaxanthin, capsanthin, lutein, and zeaxanthin as described in Wilkins et al. (2017). After fertilisation and egg hardening (\textit{ca.} 2 hours), photos of each clutch were taken to later count the eggs and determine the average egg weight per female as total clutch weight divided by total egg counts.
A subset of twenty-four freshly fertilized eggs per full-sib family was transported to the laboratory and raised singly under experimental conditions to assess the effects of egg carotenoid content on the tolerance of embryos to the mildly virulent bacterium *Pseudomonas fluorescens* (Marques da Cunha et al., 2018; Wilkins et al., 2017). Recorded response variables during laboratory studies included hatching time, hatchling length, hatchling yolk sac volume and hatchling growth over a fourteen days period post hatching. Remaining eggs were incubated under routine hatchery conditions at the cantonal Fischereistützpunkt Reutigen at around 8.5°C and stocked into the Mülibach streamlet (Supplementary Material) in early March 2015 at a late yolk-sac stage when emergence from gravel would usually happen and larvae would start exogenous feeding.

On August 27th, 2015 (281 days after fertilization and about 5.5 months after release into the wild), electrofishing was used to sample juvenile brown trout (< 18 cm) from the Mülibach stream. The fish were narcotized (0.075 g/L tricaine methanesulfonate buffered with 0.15 g/L NaHCO3) and photographed on a weighting scale with a length ruler to later extract fork length and weight using ImageJ v.1.49u (Schneider et al., 2012). Fin clips were collected and stored in 70 % ethanol at 4 °C for further molecular analyses. All wild-caught fish were returned into the wild.

### 2.2 Molecular analyses

Fin clips of adult breeders and a random subset of captured juveniles (i.e. 376 out of the 518 juveniles) were used for microsatellite genotyping and genetic sexing. DNA was extracted using the BioSprint® 96 workstation following the manufacturer’s protocol (Qiagen GmbH, Hilden, Germany) with 94 samples and two negative controls included in each batch extraction. DNA was quantified using a HS dsDNA assay on a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and DNA concentrations were standardized to 20 ng/µL when possible (i.e. samples with lower concentration were left undiluted or were concentrated when needed) and send to Ecogenics GmbH (Balgach, Switzerland) for genotyping at 13 microsatellite loci and genetic sex determination (see Palejowski et al. (2022) for full protocol details).

Identification of juvenile fish originating from the captive breeding program was achieved through parental assignment analyses using the full-likelihood approach implemented in Colony v2.0.6.5 (Jones and Wang, 2010). Parental assignments were performed using the microsatellite genotypes from the current breeders and their potential offspring, but also included additional genotypes from 16 additional streams, which were also stocked with juvenile brown trout (Bylemans et al., in prep.). Offspring were identified as coming from the captive breeding program when they were assigned to one of the parental crosses used with a probability higher than 0.98. Full details on the parentage assignment analyses can be found in the Supplementary Material.

### 2.3 Statistical analyses

All statistical analyses were performed in R version 4.0.2 (R Development Core Team, 2010) using the packages lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017). Significance of all test statistics was determined using an alpha value of 0.05.

Juvenile brown trout captured from the Mülibach stream include 0+ fish but may also include older age classes. Therefore, a 2-component Bayesian mixture model based on fish fork length was used to estimate the probability of each fish belonging to the 0+ age class and those with a probability higher than 0.25 were considered as ‘true’ 0+ fish (see Supplementary Material). In the case of the Mülibach samples, this resulted in a fork length threshold < 12.5 cm to identify ‘true’ 0+ fish. None of the offspring identified as coming from captive breeding origin exceed this threshold value.
Young-of-year fork length was used as a proxy for growth. Young-of-year survival analyses were performed using a binomial response variable. For each parental breeding pair, it was assumed that from all offspring released (i.e. the total number of fertilized eggs, n) sex ratios were equal (i.e. n/2 for each breeding pair). Total recaptured 0+ fish for each sex were thus considered as the number of successes (i.e. a value of 1) while remaining entries were considered fails (i.e. value of 0). This allowed modelling the probability of each egg resulting in a recaptured fish while taking into consideration sex-specific effects and unequal contributions between parental breeding groups.

Linear and generalized linear mixed models were used to assess the relative contributions of egg size and carotenoid content on the growth and survival of 0+ in the wild, respectively. Sib-group ID (i.e. female by male combination) was included in all models as a random effect to account for between family variances. Fixed-effect predictor variables considered were egg size, astaxanthin content, lutein content, and zeaxanthin content. While the capsanthin content of eggs was also determined, quantifications often fell below detection thresholds (Wilkins et al., 2017) and thus this variable was excluded from all analyses. Prior to fitting models, collinearity between predictor variables was evaluated using Pearson correlation coefficients. Three alternative models describing growth and survival of 0+ in the wild could be identified while avoiding the inclusion of highly correlated predictor variables (i.e. |r| > 0.5) within the same model (Supplementary Material) (Dormann et al., 2013). Firstly, predictor variables were centred and scaled to improve the fit of the models. Second, alternative models were fit with fixed effects including egg weight and lutein content; astaxanthin and lutein content or zeaxanthin and lutein content. Offspring sex was also included in the models as well as the two-way interactions between sex and the other predictor variables. The best fitting model was selected based on second order Akaike Information Criterion values (AICc) (Burnham and Anderson, 2002). Finally, a stepwise model selection approach was used to evaluate the relevance of the two-way interaction terms and select the most parsimonious model for final interpretation (i.e. when absolute differences between AICc-values (ΔAICc) were below 2 little support for either models is expected, and the most complete model was retained).

3 Results

3.1 Identifying and sexing experimental fish in the wild

The Bayesian mixture model identified 497 of 518 juvenile fish that were sampled as ‘true’ 0+ fish. The parental assignment analyses identified 303 of them (155 females, 147 males) as stocked, i.e. originating from our experimental breeding. One captive-bred 0+ fish could not be sexed and two of the males were assigned to breeding parents used to create the stocking material for a nearby stream (Supplementary Material). These fish were excluded from all further analyses.

3.2 Predicting growth and survival in the wild from embryo development

While day of hatching in the laboratory never predicted size of 0+ in the wild (Figure 1, A-B; p always > 0.05; Supplementary Material), hatchling length and yolk sac volume at hatching in both
The experimental conditions were always good predictors of the size of their 0+ full-sibs in the wild (Figure 1, C-F; p always < 0.001; Supplementary Material). The experimental conditions in the laboratory (control versus infected) did not seem to affect these first correlations (Figure 1). However, hatching growth during the control treatment was positively correlated to size of 0+ in the wild (slope: 13.71 ± 3.09, p < 0.001) while this correlation was absent for hatching growth under the infection treatment (slope: 0.49 ± 3.43, p = 1.0). When evaluating the relative larval performance under both treatments, only one significant positive correlation was observed between the difference in hatching growth and 0+ growth (Supplementary Material). Survival of 0+ in the wild could not be predicted from any of the traits measured in the laboratory (Supplementary Material).

3.3 Predicting growth and survival in the wild from egg characteristics

The most parsimonious model explaining 0+ growth included egg weight and egg lutein content, offspring sex and the two-way interaction between sex and egg lutein content (AICc = 748.91, R² = 0.246) (Table 1). Female egg weight was significantly positively correlated with 0+ growth and on average male 0+ offspring where 0.23 cm larger than their female conspecifics (Table 1 and Figure 2A). Young-of-year offspring survival was best described by a model including egg zeaxanthin and lutein content, offspring sex and the two-way interactions between sex and the egg carotenoid measures (AICc = 1483.16, conditional R² = 0.028). The interactive effect between egg zeaxanthin content and 0+ sex was significant (Table 1). Eggs with a relative low zeaxanthin content produced offspring with a female biased survival while eggs containing high levels of zeaxanthin produced offspring with a male biased survival (Figure 2B). Finally, given that the offspring originating from the eggs with the highest relative zeaxanthin content also had the highest skew in sex ratios (i.e. 22 male versus 4 female offspring), the robustness of this results was evaluated by refitting the model while excluding this “extreme” observation.

4 Discussion

Measuring family-specific fitness is notoriously difficult but relevant for applied and basic science (Carlson and Seamons, 2008; Viblanc et al., 2022). Laboratory studies usually focus on embryo growth and survival under stress- and non-stress conditions, typically assuming that these are components of fitness that have sufficient ecological relevance. This implicit assumption needs to be validated under natural conditions. Here we provide such a validation. We found that embryo development and growth as measured under experimentally controlled conditions predict juvenile growth during the first spring and summer in the wild. We also found that maternal environmental effects (egg size and carotenoid content) significantly affected juvenile performance, and that the effects of carotenoids on juvenile survival may sometimes be sex-specific.

While the links between embryo and juvenile size were strong even when measured under very different environmental conditions, juvenile survival in the wild could not be predicted by embryo growth and survival in the laboratory. It remains to be shown whether such a non-significant finding reveals a true lack of correlations between the traits. Alternatively, our analyses on juvenile survival could suffer from a lack of statistical power, for example, because of insufficient sample size. Measurement errors and insufficient sample size were arguable not a problem for the laboratory studies that were based on 3,278 singly raised embryos (Wilkins et al. 2017) and well-established protocols (von Siebental et al., 2009). However, although we were able to identify in total 303 wild-caught juveniles as originating from our breeding experiments, such a number may still be too small for reliably estimating the mortalities of in total 60 experimental families (more than half of these families, i.e., 31 out of 60, were represented by less than 5 juveniles). It may therefore not be surprising that when we tested whether embryo susceptibility to a
microbial infection would predict survival of their siblings in the wild, we found no significant correlations between this susceptibility and juvenile mortality. It therefore remains to be shown whether the microbial challenge we applied in the laboratory can sufficiently represent the pathogens that juveniles are exposed to in the wild.

Egg size has previously been linked to increased offspring performance such as larger hatchling size and increased larval survival and growth (Brooks et al., 1997; Einum and Fleming, 1999). Environmental influences sometimes overwrite such effects (Régnier et al., 2013; Thorn and Morbey, 2018) and sometimes not (Einum et al. 2002). Egg size is of primary importance during early embryo development while genetic and environmental factors often dominate during late embryo and juvenile stages (Clark et al., 2014; Einum and Fleming, 1999; Houde et al., 2015). We found that egg size was positively correlated to juvenile size in the wild. This finding corroborates many of the earlier findings on the significance of egg size.

Some salmonids show sex-specific growth during their first year of life. In European grayling (Thymallus thymallus), for example, males grow faster than females during their first summer, probably because gonad development starts earlier in females than in males of this species (Maitre et al. 2017). Palejowski et al. (2022) compared male and female size in 0+ brown trout sampled at 5 different locations and 5 different time points. They found significant sex differences in size, but these sex differences varied with time and/or location. It also seemed to matter in this context whether a 0+ was wild-born or stocked. Overall, hatchery-born males grew larger than hatchery-born females (Palejowski et al., 2022). Moreover, histological analyses of laboratory-reared juveniles revealed that females started sex differentiation earlier than males (Palejowski et al., 2022). We found that the male juveniles in our sample were significantly larger than the females. We conclude from these combined observations that male and female 0+ brown trout differ in their life histories. These differences can result in sex-specific growth, but whether males or females grow faster seem to depend on various factors that still need to be better understood.

In captive breeding, relaxed selection on embryos could give females with many small eggs an evolutionary advantage over females with few large eggs. This could lead to an evolutionary response and thereby decrease in mean egg size over time (Heath et al., 2003; Johnston, 2018). Our results suggest that such an evolutionary response would lead to smaller juveniles over time. Smaller juveniles are also predicted in response to reduced embryo growth and hatching size as a result of environmental stress during incubation (Clark et al., 2013). If such affects are to be avoided, adequate management is needed in hatchery environments, especially those producing offspring for conservation purposes, to avoid hatchery selection for smaller hatchlings.

Animals cannot synthesise carotenoids but have to obtain them through their diet (Svensson and Wong, 2011). Females may allocate carotenoids to developing offspring either based on their health and vigour (Hörak et al., 2001; Mccraw, 2005) or their life history (Svensson et al., 2006; Wilkins et al., 2017). In salmonids, egg carotenoid content influences embryonic susceptibility to oxidative stress and pathogenic infections (Tyndale et al., 2008; Wilkins et al., 2017). We therefore predicted that carotenoid content would also have a positive influence on juvenile growth and survival in the wild. We found no significant effects of astaxanthin and lutein on juvenile performance. However, zeaxanthin content of the eggs was statistically linked to sex-specific survival of juveniles: Males seemed to profit more from zeaxanthin than females. It remains to be tested whether this sex-specific effect of zeaxanthin is due to, for example, sex-specific life histories combined with the specific environmental conditions that the juveniles were exposed to in our study streamlet. Alternatively, zeaxanthin could generally be more important to male than female 0+ brown trout. If the latter is true, female allocation of zeaxanthin to eggs could reveal sex-specific reproductive strategies that may potentially show some analogies to the sex-specific reproductive
strategies in some mammals (Trivers & Willard 1973), potentially in response to expected sex-specific juvenile mortality in some environments (Moran et al., 2016).

Studies on embryos raised under controlled conditions have become a popular means to study life history and to determine the factors that affect salmonid performance. We found that embryo and larval performance measured under such experimental conditions can predict the size of juveniles that survived and grew under ecologically relevant conditions over a period of several months. We also found that maternal environmental effects affect juvenile performance. It remains to be shown how these maternal environmental effects interact with the specific challenges that males and females experience in a given natural environment.

Data availability statement

All data and R scripts used here will be made available through the Dryad Repository upon acceptance of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author statement

JB, LMC, LW & CW designed the project. LMC, LW, DN, AU & CW did the experimental breeding, LMC & LW raised the embryos in the laboratory, and LMC, DN & AU sampled the juveniles from the wild. JB & LMC were responsible for the genotyping and parental assignments. JB & CW performed the analyses and wrote the manuscript. All authors revised and approved the final manuscript for publication.

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Tables

**Table 1.** Summary statistics of the most parsimonious models describing size of young-of-year (0+) in the wild (A) and 0+ survival (B). Predictor variables included mean egg weight, egg lutein content, egg zeaxanthin content and offspring sex.

<table>
<thead>
<tr>
<th>Term</th>
<th>Estimate</th>
<th>SD</th>
<th>Statistic*</th>
<th>DF†</th>
<th>P</th>
</tr>
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<td></td>
<td></td>
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<tr>
<td>(Intercept)</td>
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<td>90.2</td>
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<td>4.0</td>
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<tr>
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<td>-0.8</td>
<td>67.5</td>
<td>0.441</td>
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<tr>
<td>sex</td>
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<td>0.11</td>
<td>2.0</td>
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<tr>
<td>lutein:sex</td>
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<td>0.12</td>
<td>1.7</td>
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<tr>
<td><strong>B. Survival</strong></td>
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<td></td>
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<tr>
<td>(Intercept)</td>
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<td>0.701</td>
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</tbody>
</table>

* t- and z-statistics for the fork length and survival model, respectively; † degrees of freedom are not reported for generalized linear mixed-effect models.
**Figure 1.** Summary figure showing the correlations between young-of-year growth under natural conditions and laboratory-based fitness traits of their full-sibs. Laboratory-based fitness traits were measured under control conditions (green) and when developing embryos were exposed to the pathogenic bacterium *Pseudomonas fluorescens* (orange). Regression lines are shown only when the slope estimate was significantly different from zero. Points show mean values along both axes and the point size is proportional to the total number of observations.
Figure 2. Illustrating the significant effects in the most parsimonious models describing survival and growth of 0+ in the wild (Table 1): Regressions between (A) mean egg weight and sex-specific growth in the wild, and (B) egg zeaxanthin content and sex-specific survival in the wild. Note that egg traits measures were centred and scaled prior to model fitting. Points show mean values along both axes, and the point size is proportional to the total number of observations. Lines show the best fitting regressions for male (blue) and female (red) offspring and shading shows the 95% confidence intervals. Regression lines for 0+ survival are given as dashed lines as the regression is not significant when removing offspring survival from the female producing eggs with the highest zeaxanthin content and also showing a strong male offspring bias.