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Pathogen-induced hatching and population-specific life-history response to water-borne cues in brown trout (*Salmo trutta*)

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Abstract

Hatching is an important niche shift, and embryos in a wide range of taxa can either accelerate or delay this life-history switch in order to avoid stage-specific risks. Such behavior can occur in response to stress itself and to chemical cues that allow anticipation of stress. We studied the genetic organization of this phenotypic plasticity and tested whether there are differences among populations and across environments in order to learn more about the evolutionary potential of stress-induced hatching. As a study species we chose the brown trout (*Salmo trutta*; Salmonidae). Gametes were collected from five natural populations (within one river network) and used for full-factorial in vitro fertilizations. The resulting embryos were either directly infected with *Pseudomonas fluorescens* or were exposed to water-borne cues from *P. fluorescens*-infected conspecifics. We found that direct inoculation with *P. fluorescens* increased embryonic mortality and induced hatching in all host populations. Exposure to water-borne cues revealed population-specific responses. We found significant additive genetic variation for hatching time, and genetic variation in trait plasticity. In conclusion, hatching is induced in response to infection and can be affected by water-borne cues of infection, but populations and families differ in their reaction to the latter.

Keywords: Additive genetic variation, fish embryo, induced hatching, niche shift, phenotypic plasticity, reaction norm, salmonid
Introduction

The timing of life-history transitions and niche shifts is expected to be influenced by the risk-benefit ratio before and after the change (Werner and Gilliam 1984; Werner 1986; Rowe and Ludwig 1991). As such risk-benefit ratios often vary according to environmental conditions, some degree of phenotypic plasticity is expected (Gomez-Mestre et al. 2008; Reed et al. 2011). Hatching represents such a life-history transition and niche shift, and plasticity in this trait has been observed in a number of taxa (Warkentin 2007; Warkentin 2011). Induced hatching is often favored in response to egg-specific stressors (e.g. predators, pathogens) (Warkentin et al. 2001; Wedekind 2002; Moreira and Barata 2005; Vonesh 2005; Gomez-Mestre et al. 2006; Touchon et al. 2006), and conversely, delayed hatching is preferred when there is increased risk of larval mortality (Sih and Moore 1993; Moore et al. 1996; Martin 1999; Laurila et al. 2002; Schalk et al. 2002).

Notably, the physical presence of a threat is not always required to induce a plastic response in hatching time. Cues from both predators (Moore et al. 1996; Smith and Fortune 2009; Miner et al. 2010) and pathogens (Kiesecker et al. 1999; Wedekind 2002) have been observed to alter hatching time. These cues can be quite specific, allowing animals to distinguish between predators and non-predators, and even predator diets (Chivers et al. 2001; Kusch and Chivers 2004). Induced responses can equally be elicited from injured or infected conspecifics (Kiesecker et al. 1999; Chivers et al. 2001; Wedekind 2002; Miner et al. 2010).

The ability to respond to a threat before physical contact is not only practical for slower hatching species (e.g. that require proteolytic cleavage of the egg membrane; Anderson and Brown 2009), but is particularly well suited for pathogen challenge as it decreases infection risk.

The ability for such inducible defenses to evolve in a population resides in the persistence of genetic variation in trait plasticity (reaction norms) (Pigliucci 2001); however, the genetic organization of a trait can change according to environmental condition, and not necessarily in a consistent direction. For example, genetic variation has been shown repeatedly to increase from favorable to unfavorable conditions (Hoffmann and Merilä 1999; Agrawal et al. 2002; Relyea 2005; Kraft et al. 2006), while other studies reported either a decrease (Merilä 1997; Laugen et al. 2005) or no change in heritable variation with increasingly stressing conditions (Merilä and Fry 1998; Pakkasmaa et al. 2003; Merilä et al. 2004). Taken together, these studies suggest that the expression of genetic variation in a trait is not only trait dependent but can also be reliant on the stressor. To add an additional level of complexity, the amount of genetic variation in a trait can also vary among populations, due to genetic drift and/or local adaptation (Einum and Fleming 2000; Gomez-Mestre and Warkentin 2007). Indeed, in the case of salmonids, life histories are typically finely tuned to local environmental conditions (Crozier et al. 2008). Consequently, differences in micro-ecological conditions often lead to population-specific reaction norms in early life history traits.

While much work has been conducted to characterize patterns of genetic variation in plastic traits at more advanced developmental stages, few have done so during the egg stage (Gomez-Mestre et al. 2008; Jensen et al. 2008). To our knowledge, no study to date has assessed genetic variation for induced hatching in response to water borne cues of infection.

Here, we performed full-factorial in vitro fertilizations within five populations of brown trout (Salmo trutta), and infected resulting embryos with the opportunistic fish pathogen, Pseudomonas fluorescens (Austin and Austin 2007). As in Wedekind (2002), water-borne cues from acutely stressed embryos were then taken and, after sterile filtration, applied to conspecifics. By virtue of our experimental design, we were able to observe whether (i) brown trout embryos responded to water-borne cues, (ii) determine if populations differed in their response, (iii) estimate the amount of additive genetic variation for induced or delayed hatching, (iv) and assess whether these patterns differed by population.
Material and Methods

Fertilization protocol and embryo rearing

Adult male and female brown trout were caught by electro-fishing from the river network between the cities of Bern and Thun; Switzerland, i.e. the river Aare (from 7°34’16.67”E/ 46°49’09.58”N to 7°26’46.78”E / 46°56’39.00”N) and four of its tributaries (Kiese: 7°37’11.29” E / 46°50’55.85” N; Gürbe: 7°30’4.19” / 46°52’59.37” ; Giesse: 7°32’44.00” / 46°53’05.33”; Amletenbach: 7°34’04.73” / 46°47’05.95”) during the breeding season in October to November 2009. Pairwise comparisons between these populations not only showed significant genetic differentiation but also important morphological differences (Stelkens et al. 2012). After capture, adults were kept at the cantonal hatchery until the start of the experiment. Four females and six males from each population were haphazardly chosen, anaesthetized (Tricaine mesylate MS-222), measured (total length), and stripped of their gametes. These gametes were used for in vitro fertilizations (following methods described in Jacob et al. 2007). Crossings were performed within populations in a full-factorial breeding design, i.e. for each population all possible crosses of four females and six males (North Carolina II; Lynch and Walsh 1998) yielding in total 120 sib-groups (24 per population).

Water used for fertilizations and thereafter was chemically standardized (OECD 1992), aerated, and cooled to 6.5°C before use.

After water hardening, eggs were transported to a climate chamber (6.5°C) where they were washed (as described in von Siebenthal et al. 2009). Eggs were then distributed singly to 24-well plates (Falcon, Becton Dickinson; 2ml water per well) in a block-wise design, such that a set of five plates contained all types of siblings. They were examined 54 days post-fertilization on a light table (Hama professional, LP 555) and with a stereo zoom microscope (Olympus SZX9) to assess fertilization success.

Isolation of Pseudomonas fluorescens, identification, and infection protocol

To facilitate the collection of a pathogenic strain of the gram-negative bacterium P. fluorescens, brown trout eggs from various origins were left in Petri dishes and incubated until signs of infections could be observed. A plate was randomly selected (eggs originated from Amletenbach), and 100µl of water was streaked onto a non-selective tryptic soy agar media (TSA) (Sigma Aldrich) plate and incubated at 22°C for 48 hours. Non-selective media is preferred for single colony selection as it allows for identification of other bacterial contaminants (Holt et al. 1994). A fluorescent colony was selected and restreaked onto a TSA plate, which was allowed to incubate for 24 hours. This procedure was repeated twice to ensure colony purity (Sambali and Mehrotra 2009). For identification, DNA was isolated from the colony using the GenElute™ Bacterial Genomic DNA Kit, according to the manufacturer’s instructions (Sigma-Aldrich). A PCR was performed using a P. fluorescens-specific primer set: 16SPSEfluF (5’-TGCATTCAAAAAGCTGACTG-3’) and 16SPSER (5’AATCACCCGTTGGTAACCGG-3’) (Sparpellini 2004). P. fluorescens strain ATCC 17400 was used as the positive control. All PCR reactions were performed in a total volume of 50 µl, and contained: ~50-100ng bacterial genomic DNA, 5 µl of 10X PCR buffer, 200 µM of each dNTP, 2 mM of MgCl2, 0.5 µM of each primer, and 0.5 U of Taq polymerase (Qiagen). The thermal profile was modified from Sparpellini et al. (2004): 2 min at 94°C; 5 cycles of 94°C for 45s, 55°C for 1 min, 72°C for 2 min; 35 cycles of 92°C for 45s, 60°C for 45s, 72°C for 2 min; final extension of 72°C for 2 min; and final cooling at 12°C. Following the PCR, the amplified products were purified with the Wizard® SV Gel and PCR Clean-Up System (Invitrogen) and sequenced in the forward and reverse directions with 16SPSEfluF/16SPSER on an ABI Prism 3100 genetic analyzer (Applied Biosystems).

To prepare the bacterial inoculum, 200 ml of tryptic soy broth (TSB) was inoculated with a single colony of P. fluorescens. The flask was then placed on a shaker (200rpm) for 36
hours at 22°C, until reaching exponential growth phase. The contents were transferred to 50 ml conicals and centrifuged for 15 minutes at 4000 rpm. Pellets were washed twice with sterile standardized water and pooled. Bacterial cells were counted with a Helber hemocytometer using a phase contrast microscope (400x). The suspension was diluted such that an inoculation with 100 µl/well would result in a concentration of 6.0 x 10^8 cells/ml.

Eight of the twenty replicates per sibgroup were treated with P. fluorescens 54 days post-fertilization. The remaining 12 replicates received only standardized water. Nine days later, 100µl of water were taken from each treated well, pooled, and sterile filtered (0.2µm) to produce the water-borne cue (WBC). Eight of the 12 non-infected embryos per sibgroup were then exposed to 100µl of WBC. The remaining four embryos served as controls and were sham-treated with sterile-filtered standardized water. Following treatment, embryos were monitored daily and mortality and hatching date were recorded.

Statistical analyses
Analyses were performed with Jmp7.0 (JMP 1989-2007) and R (R Development Core Team 2006) using the lme4 package for linear and logistic mixed effect model analyses (Bates and Sarkar 2007). Eggs that were not eyed on day 54 post fertilization were assumed to be non-fertilized and hence excluded from all further analyses. Survival was analyzed as a binomial response variable in generalized linear mixed models (GLMM), and hatching time as a continuous variable in linear mixed models (LMM). Treatment was considered as fixed effect, and population, dam, sire, treatment x population, treatment x dam, and treatment x sire interactions as random effects. Starting with a reference model that included treatment, population, dam, and sire, we added or removed one effect at a time and compared the reference and the changed model with likelihood ratio tests (LRT) using restricted maximum likelihood (REML) (Zuur et al. 2009). Sire x dam interactions were not included in the models to avoid low sample sizes in some experimental cells.

Because infection with P. fluorescens and inoculation with WBC were performed at two different time points during embryo development, and reactions to stressors may be conditional to developmental stage (Johnson et al. 2001; Schotthoefer et al. 2003; Solllid et al. 2003; E. S. Clark et al., unpubl. data), infected embryos were not directly compared to WBC-exposed embryos. Instead, both treatments were compared to the controls only. Moreover, because embryo survival was low in the infection treatment, survival but not hatching date was analyzed in the corresponding mixed effect models. Analogously, because survival turned out to be close to 100% in the WBC treatment (see Results), hatching date but not survival was analyzed in the corresponding mixed effect models.

Variance components for hatching time were extracted from mixed models using REML. With a full-factorial breeding design, assuming that epistasis is negligible, additive genetic variance (VA) is estimated as four times the sire variance (VSIRE) (Lynch and Walsh 1998). Maternal environmental effects (VM), i.e. the part of dam variance (VDAM) not explained by additive genetic effects, was calculated by subtracting VSIRE from VDAM. Residual variance (VRES) includes environmental variance as well as ½ VA and ¼ dominance variance (VD) (Kearsey and Pooni 1996). Narrow sense heritability was estimated as VA divided by total variance, i.e VDAM, VSIRE and VRES, as in Lynch and Walsh (1998). Standard errors of variance components and heritabilities were calculated using a bootstrap procedure, taking the standard deviation of 1000 estimates produced by reshuffled hatching dates separately per population and treatment in R. To facilitate comparisons with other studies, we also report the coefficients of additive genetic variation (CVA) (Houle 1992). Variance components and heritability were not estimated for the binominal response variable survival, because mortalities turned out to be either very low (controls, WBC) or very high (infected) rendering heritability estimates from count data estimates spurious. Genetic correlations for
hatching time across two environments were estimated using Pearson's product moment correlations for sire sib-group means (Roff 1997; Relyea 2005; Gomez-Mestre et al. 2008).

**Results**

**Embryo survival**

Exposure to *P. fluorescens* significantly decreased mean survival until hatching from 98.8 ± 0.5% (SE) in the control to 11.6 ± 1.1% in infected eggs (Table 1). Populations did not differ significantly in average embryo viability or susceptibility to infection (Table 1). While sire x treatment or dam x treatment interactions were not significant (Table 1), there were significant overall dam and sire effects in offspring viability (Table 1). Survival in the WBC treatment was on average 97.3% ± 1.0% (SE) and not significantly different from the controls (GLMM $z = -1.58$, $p = 0.12$).

**Treatment and population effects on hatching time**

Embryos treated with *P. fluorescens* hatched significantly earlier than controls (comparing the hatching times of the first hatching per sibgroup: median difference = 60 hours, Wilcoxon signed rank test, $V = 561.5$, $p = 0.006$; mean hatching time per sibgroup: median difference = 57 hours, $V = 587$, $p = 0.002$; Fig. 1). This median difference in the timing of first hatching per sibgroup corresponded to a median hatching acceleration of 18% since exposure to *P. fluorescens* and did not significantly among the populations (Kruskal-Wallis $\chi^2 = 4.7$, d.f. = 4, $p = 0.32$). Reaction to WBC treatment varied among populations (Table 1; Fig. 1). Treatment with WBC induced hatching in the Gürbe population, delayed hatching in Giesse and Amletenbach, and appeared to have no significant overall effect in Kiese and Aare (Fig. 1).

We found significant overall dam and sire effects on hatching time (the main effects in Table 1). We also found significant genetic variation for hatching plasticity (the sire x treatment and dam x treatment interaction in Table 1). In the control, sire effects accounted for a significant part of the variance in two populations, i.e. Aare ($\chi^2 = 7.85$, $p = 0.005$) and Gürbe ($\chi^2 = 17.53$, $p < 0.0001$), but were not significant in Kiese ($\chi^2 = 0.08$, $p = 0.78$), Giesse ($\chi^2 = 0.07$, $p = 0.80$) or Amletenbach ($\chi^2 = 0.00$, $p = 1.00$) (Table 2). However in the WBC treatment, sire effects were significant in four out of five populations, i.e. Aare ($\chi^2 = 13.97$, $p = 0.0002$), Gürbe ($\chi^2 = 19.30$, $p < 0.0001$), Giesse ($\chi^2 = 56.66$, $p < 0.0001$) and Amletenbach ($\chi^2 = 13.93$, $p = 0.0002$), but not Kiese ($\chi^2 = 0.56$, $p = 0.45$) (Table 2).

**Cross-environmental trait correlations**

As populations differed significantly in hatching response to treatments (Table 1), cross-environment genetic correlations between control and WBC were calculated separately for each population. For the Kiese and Giesse, there is no correlation in hatching time among the two environments ($r = -0.01$, $p = 0.98$; and $r = 0.66$, $p = 0.15$ respectively). However, hatching time was positively correlated among the two environments in Aare ($r = 0.82$, $p = 0.04$), Gürbe ($r = 0.89$, $p = 0.02$) and Amletenbach ($r = 0.86$, $p = 0.03$).

**Discussion**

In whitefish (*Coregonus* sp.), i.e. in another salmonid genus, exposure to *P. fluorescens* has been shown to cause significant embryonic mortality (Wedekind et al. 2004; von Siebenthal et al. 2009), as well as to induce hatching (Wedekind 2002). Brown trout embryos differ in many respects from whitefish embryos, including mean size, various characteristics of their typical habitat, and the behavior of freshly hatched alevins (Wedekind and Müller 2005). We nevertheless found that treatment with *P. fluorescens* increased mortality and also prompted precocious hatching. Sensitivity to *P. fluorescens* and the host’s life-history response therefore appears to extend to brown trout embryos. This allowed us to compare populations...
that live in different habitats (but still within the same catchment area) and for which Stelkens et al. (2012) found significant morphological and genetic differentiation despite the rather small geographic scale. Some of the previously observed morphological differentiation within this catchment area could even be linked to habitat characteristics (Stelkens et al. 2012), consistent with local adaptation.

Although experimental infection with *P. fluorescens* induced mortality and precocious hatching in all study populations, we found that the populations varied significantly in their hatching response to water-borne cues from infected embryos, i.e. we found significant differences in the reaction norms. Such variation at the level of the population could potentially indicate differential selection pressures (Laurila et al. 2002; Jensen et al. 2008). As the microbial community composition can differ vastly between streams (Evans and Neff 2009), it is possible that our five study populations would typically be exposed to different pathogen communities. However, differences between populations could also result, for example, from genetic drift. We further found significant dam and sire by treatment interactions, i.e. additive genetic variation for hatching plasticity. Such variability in reaction norms could indicate a potential for adaptation in response to environmental changes (Hutchings 2011). However, as dams and sires were nested in population, family effects cannot fully be disentangled from potential population differences.

The study populations not only differed in their reaction to water-borne cues but also in the genetic organization of hatching time. While our overall analysis showed significant amounts of additive genetic and maternal environment variation in hatching time across treatments, closer examination within populations revealed more variable patterns. Certain populations seemed to have little genetic variation for hatching time under benign conditions. This finding would meet traditional expectations for life-history traits, which anticipate reduced genetic variation as a consequence of directional selection (Mousseau and Roff 1987) or stabilizing selection (Fisher 1930). However, significant additive genetic variance for the trait was found in some other populations, conforming with studies which have shown considerable heritable variation in fitness-related traits (Houle 1992; Merilä and Sheldon 1999).

The water-borne cues we used likely consisted of a complex mixture of chemicals of both pathogen and embryo origin. On the one hand, these chemicals could mainly convey an imminent danger to embryos, as it did in Kiesecker et al. (1999) and Wedekind (2002). On the other hand, they may also relay other kinds of information that may be important in other than the host-parasite context, e.g. they could reveal that conspecifics were in the environment. The presence or absence of conspecifics has been shown to induce or delay hatching time in other species (Miner et al. 2010). It therefore remains unclear what aspect of a potential multiple signal the embryos reacted to in our experiment. Additionally, it remains to be seen how the difference in the timing of exposure to pathogen versus the water-borne cues may have impacted the behavior response to treatment. Nevertheless, the population differences we observed suggests that hatching time in brown trout is not only dependent on environmental factors, but that these environmental factors are differently weighted by embryos of different origins.

Despite significant overall dam and sire effects on embryo mortality, we found no significant population x treatment effects on mortality, indicating a more or less uniform susceptibility to infection. We further found no sire or dam by treatment interactions, indicating a lack of genetic variability in survival reaction norms. Lack of variation in reaction norms at both the family and population level could have important implications on the species’ ability to cope with infectious disease—a stressor which is anticipated to increase in occurrence due to anthropogenic activities (Dewitt et al. 1998; Harvell 1999; Dobson and Foufopoulos 2001). However, significant gene by environment interactions on larval survival
could be found in other salmonids, e.g. in the Chinook salmon (*Oncorhynchus tshawytscha*) (Evans et al. 2010) or in whitefish (*Coregonus* sp.) (von Siebenthal et al. 2009), and even in other populations of brown trout (Wedekind et al. 2008; Jacob et al. 2010). Genetic variability in reaction norms may therefore depend on the kind of stressor, the developmental stage, and the species or population in question.

As with pathogen susceptibility, we found significant additive genetic variation for hatching time. In addition, we found genetic variation in hatching plasticity after exposing embryos to water-borne cues of infection. We further observed that hatching time was correlated across control and WBC treatments in the majority of the populations, indicating that trait expression is mediated by the same loci in different environments (Via 1984). Trait means, therefore, may not be able to evolve independently, which places a constraint on the evolutionary potential of the reaction norm (Pigliucci 2001).

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**Ethical standards**

Permissions for handling embryos were granted by the local authority (i.e. the Fishery Inspectorate of the Bern canton). The manipulations of the adults were part of the yearly hatchery program of the Bern canton. Experimental manipulations on embryos were performed prior to yolk sac absorption. All manipulations comply with the current law of the country in which they were performed (Switzerland). The authors declare that they have no conflict of interest.

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Table 1 Likelihood ratio tests on mixed model logistic regressions on (a) embryo survival, and (b) hatching time. Two treatments are compared each, i.e. control vs. infected and control vs. WBC (see main text). The Akaike information criterion (AIC) indicates the goodness of fit of a model. P-values were obtained from comparisons between the respective model and the reference model (in bold).

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<th>P</th>
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<td>(a) Survival</td>
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Table 2 Within-environment variance components (\(V_A\): additive genetic variance, \(V_M\): maternal effect variance, \(V_{RES}\): residual variance), narrow sense heritability estimate (\(h^2\)) and coefficients of additive genetic variation (\(CV_A\)) for hatching time in the control and the WBC treatment. Numbers in parentheses indicate standard errors; asterisks indicate whether males explain a significant part of the variance (see main text).

<table>
<thead>
<tr>
<th>Population</th>
<th>(V_A)</th>
<th>(V_M)</th>
<th>(V_{RES})</th>
<th>(h^2)</th>
<th>(CV_A)</th>
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<td>80.9 (16.9)</td>
<td>0.05 (0.12)</td>
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<td>Aare</td>
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<td>-2.7 (6.1)</td>
<td>85.4 (24.8)</td>
<td>0.79 (0.14)</td>
<td>2.30 (0.56)</td>
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<td>132.7 (19.2)***</td>
<td>31.8 (6.3)</td>
<td>81.4 (26.0)</td>
<td>0.74 (0.12)</td>
<td>2.60 (0.60)</td>
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<tr>
<td>Giesse</td>
<td>4.5 (13.4)</td>
<td>31.0 (4.5)</td>
<td>82.6 (13.2)</td>
<td>0.04 (0.12)</td>
<td>0.47 (0.49)</td>
</tr>
<tr>
<td>Amletenbach</td>
<td>0.0 (15.7)</td>
<td>38.5 (5.2)</td>
<td>96.6 (17.4)</td>
<td>0.00 (0.12)</td>
<td>0.00 (0.54)</td>
</tr>
<tr>
<td>(b) WBC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiese</td>
<td>4.7 (6.3)</td>
<td>49.1 (2.1)</td>
<td>63.0 (9.8)</td>
<td>0.04 (0.06)</td>
<td>0.49 (0.34)</td>
</tr>
<tr>
<td>Aare</td>
<td>67.2 (6.6)**</td>
<td>-13.1 (2.3)</td>
<td>70.7 (17.8)</td>
<td>0.74 (0.08)</td>
<td>1.81 (0.35)</td>
</tr>
<tr>
<td>Gürbe</td>
<td>63.0 (7.9)**</td>
<td>47.6 (2.6)</td>
<td>78.0 (11.2)</td>
<td>0.40 (0.06)</td>
<td>1.82 (0.39)</td>
</tr>
<tr>
<td>Giesse</td>
<td>107.4 (5.1)**</td>
<td>-2.6 (1.7)</td>
<td>47.3 (10.4)</td>
<td>1.09 (0.06)</td>
<td>2.29 (0.30)</td>
</tr>
<tr>
<td>Amletenbach</td>
<td>28.2 (5.7)**</td>
<td>48.9 (1.9)</td>
<td>45.6 (8.1)</td>
<td>0.26 (0.06)</td>
<td>1.20 (0.33)</td>
</tr>
</tbody>
</table>

*p<0.05; **p<0.01; ***p<0.001
Fig. 1 Cumulative proportion hatched per population (a: Kiese; b: Aare; c: Gürbe; d: Giesse; e: Amletenbach) and treatment (control: round symbols; *P. fluorescens*-treated: squares; WBC-treated: triangles). The arrows indicate the timing of the WBC treatment.