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Paternal identity impacts embryonic development for two species of freshwater fish

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ABSTRACT
Paternal, compared to maternal, contributions were believed to have only a limited influence on embryonic development and larval fitness traits in fishes. Therefore, the perspective of male influence on early life history traits has come under scrutiny. This study was conducted to determine parental effects on the rate of eyed embryos of Ide Leuciscus idus and Northern pike Esox lucius. Five sires and five dams from each species were crossed using a quantitative genetic breeding design and the resulting 25 sib groups of each species were reared to the embryonic eyed stage. We then partition variation in embryonic phenotypic performance to maternal, paternal, and parental interactions using the Restricted Maximum Likelihood (REML) model. Results showed that paternal, maternal, and the paternal × maternal interaction terms were highly significant for both species; clearly demonstrating that certain family combinations were more compatible than others. Paternal effects explained 20.24% of the total variance, which was 2-fold higher than the maternal effects (10.73%) in Ide, while paternal effects explained 18.9% of the total variance, which was 15-fold higher than the maternal effects (1.3%) in Northern pike. Together, these results indicate that male effects are of major importance during embryonic development for these species. Furthermore, this study demonstrates that genetic compatibility between sires and dams plays an important role and needs to be taken into consideration for reproduction of these and likely other economically important fish species.

1. Introduction
Parental effects on early life history (ELH) traits of fish (i.e. eggs to small juveniles) are widely recognized (Heath and Blouw, 1998). The driving force behind each parental contribution is likely to be strongly modified by genotype, environmental effects, as well as the interaction between genes and the environment (Conover, 1998; Neff and Pitcher, 2005; Politis et al., 2014). As a result, understanding factors that influence the viability of offspring during early life is critical for aquaculture as well as fisheries science and management, as survival during these life history stages can vary dramatically and ultimately influence commercial production and year-class strength for any given species (Bromage et al., 1992; Nagler et al., 1999).

Paternal contributions were believed to have only a limited influence on embryonic development and larval fitness traits in fishes as compared to maternal contributions (Keckeis et al., 2000; Marteinsdottir and Begg, 2002). Paternal contributions to progeny are solely the product of nuclear-genetic material from the spermatozoon (Trippel et al., 2000: Marteinsdottir and Begg, 2002). Paternal contributions to progeny are solely the product of nuclear-genetic material from the spermatozoon (Trippel et al., 2000; Rideout et al., 2004). Therefore, the perspective of male influence on ELH traits has come under scrutiny (Trippel, 2003; Rideout et al., 2004). Nevertheless, in the last few decades, a number of studies have demonstrated that paternal genetic
effects contribute to variation in morphology and performance during ELH of both marine and freshwater fishes (Vellestad and Lillehammer, 2000; Rideout et al., 2004; Pitcher and Neff, 2007; Butts and Litvak, 2007a;b; Houde et al., 2015). For instance, paternal effects on ELH traits have been demonstrated for Atlantic salmon Salmo salar (Thorpe and Morgan, 1978; Houde et al., 2015), brown trout Salmo trutta (Vellestad and Lillehammer, 2000), rainbow trout Oncorhynchus mykiss (Rinchard et al., 2003), chinook salmon Oncorhynchus tshawytscha (Pitcher and Neff, 2007), masu salmon Oncorhynchus masou (Yamamoto and Reinhardt, 2003), herring Clupea harengus (Panagiotaki and Geffen, 1992; Evans and Geffen, 1998), European sea bass Dicentrarchus labrax (Saillant et al., 2001), haddock Melanogrammus aeglefinus (Rideout et al., 2004), winter flounder Pseudopleuronectes americanus (Butts and Litvak, 2007a,b), Trinidadian guppy Poecilia reticulata (Reynolds and Gross, 1992), alpine whitefish Coregonus sp. (Wedekind et al., 2001) and Atlantic cod Gadus morhua (Butts et al., 2009; Kroll et al., 2013; Politis et al., 2014).

Maternal contributions to progeny are the product of both nuclear-genetic material and extra-nuclear non-genetic environmentally influenced material (Rideout et al., 2004; Neff and Pitcher, 2005). The underlying cause of maternal effects include (among others) female age, female size, broodstock nutrition (Lavens et al., 1999; Furuta et al., 2000; Morehead et al., 2001), photoperiod manipulation (Watanabe et al., 2001), habitat quality (Perry et al., 1991), time of reproduction in the spawning season (Buckley et al., 2006), and hormonal-induced ovulation (Harmin and Crim, 1992). Together, these factors result in variation in egg quality (Chambers and Leggett, 1996; Chambers, 1997; Marteinsdottir and Steinarsson, 1998; Rideout et al., 2005). Because of these aforementioned processes, maternal contributions are generally considered more important than paternal influences (Trippel, 2003; Rideout et al., 2004), as good quality eggs are considered a prerequisite for the production of viable offspring, especially during the "critical" ELH stages (Trippel et al., 1997).

For this study we use two different species, ide Leuciscus idus L., and Northern pike, Esox lucius. Ide, is a rheophilous cyprinid, widely distributed in Europe, especially in rivers and lakes (Jamróz et al., 2008; Cejko et al., 2010; Ciesla et al., 2014). In recent years, reproduction of ide in captivity has received increasing attention to meet the demand of high quality juveniles for restocking (Wojda, 2004; Krejszeff et al., 2009), sport fishing, aquarium industry (Jamróz et al., 2008; Cejko et al., 2010), toxicity studies (Witeska et al., 2014) and as a bio-monitoring agent (Fenske et al., 2006). On the other hand, Northern pike is an iteroparous, large-bodied and long-living (>10 years) fish species inhabiting the northern hemisphere (Craig, 2008; Forsman et al., 2015). There is interest in fishing this species but it has also been cultivated extensively in Europe and Asia since the middle ages (Steinberg, 1992). Males and females sexually mature at the age of 2–3 and 3–4 years, respectively. Females and males swim through and over the vegetation in water and release eggs and sperm simultaneously at irregular intervals for up to several hours (Froese and Pauly, 2015).

The objective of this study was to determine the effect of genetic compatibility and/or incompatibility during embryonic development for ide and Northern pike. More specifically, we crossed 5 females with 5 males, in a quantitative genetic breeding design and reared the resulting 25 sib groups of each species (in replicate) to the embryonic eyed stage. We then attempt to attribute variation in embryonic phenotypic performance to maternal, paternal, and parental interactions.

2. Materials and methods

2.1. Broodstock husbandry and gamete collection for ide

Wild-caught ide broodstock (age 1–3 years and weight 700 g to 2 kg) were collected from an earthen pond from Zurawia Fish Farm (Central Poland) in the middle of April 2015. In total, 13 males and 12 females were kept separately in a recirculating aquaculture system (1000 dm³ volume) at 10 °C and under a 14 h light/10 h dark photoperiod for two weeks (Kujawa et al., 1999). Mature males were recognized by gentle abdominal pressure and females were selected by assessing the maturation stage with catheterization according to Krejszeff et al. (2009). Males and females were stimulated with Ovopel (Interfish, Hungary) containing mammalian GnRH analogue and metoclopramide as a dopamine antagonist. One ovopel pellet (50–55 mg) contains a mammalian GnRH analogue (v-ala³, Pro⁶Net-mGnRH at dose 25 mg) and dopamine antagonist: metoclopramide (dose 8–10 mg) (Horvath et al., 1997). Prior to administration, Ovopel pellets were homogenized in a mortar and dissolved in 0.9% NaCl solution (10 mL physiological solution for 10 pellets) following methods of Siddique et al. (2016).

For spermiation, each male received a single dose of 1 mL ovopel solution kg⁻¹ body weight, intraperitoneally under the left pectoral fin. Ovulation was induced by an initial injection of 0.2 mL ovopel solution kg⁻¹ body weight and a second injection of 1 mL ovopel solution kg⁻¹ body weight 12 h after the first injection. Ripe gamete donors were anaesthetized in a solution of 2-phenoxyethanol (0.5 mL L⁻¹). Milt was collected from the genital papilla under abdominal pressure avoiding contamination with urine, using a 5 mL plastic syringe. Until the beginning of the experiments stripped milt was stored at 4 °C for 30 min. Females were checked every 2 h between 30 and 36 h after injection. After 36 h of hormonal stimulation, ovulated oocytes were collected by abdominal pressure and stored in 250 mL plastic containers at 4 °C until used for fertilization.

2.2. Broodstock and gamete collection for Northern pike

For Northern pike, ~30 males and 20 females (age 1–3 years and weight 1–3 kg) were caught by gillnet from Kortowskie Lake (Olsztyn, North-East Poland) during the spawning season (at the beginning of April 2015). Without hormonal stimulation, gametes were collected from wild-caught Northern pike broodstock. Males and females were separated by physical observation on abdomen and genital papilla. Male and female fish were stripped in the field near the lake. Naturally ovulating males were stripped first. Milt from 11 males were collected from the genital papilla under abdominal pressure avoiding contamination with urine, using a 5 mL plastic syringe. Among 20 females, we obtained ovulated oocytes from 8 females. Eggs were collected by abdominal pressure and stored in 250 mL plastic containers. Sperm and eggs both were kept in an ice box in order to transfer them to the laboratory and stored at 4 °C until used for fertilization.

2.3. Experimental design for ide

The fish used in this experiment were randomly chosen and crossed using a full factorial (North Carolina II) breeding design (5 sires × 5 dams) with 3 replicate crosses per family combination. Freshwater (5 mL, 15 °C, pH 7.2), as an activating medium, was first placed in 75 glass petri dishes. Eggs from each female were fertilized by the sperm from each male with 3 replicates. Eggs (~100; 0.15 g) were gently added to each petri dish so that they

could be spread throughout the dish. Then, 10 µL of milt were immediately added to the eggs using a micropipette. After insemination, petri dishes were shaken by hand for 20 s to facilitate fertilization.

Following fertilization, all the petri dishes containing eggs were kept in a 300 L incubation tank at 15.6 °C with continuous water flow for 5 days. After 5 days of incubation, all the petri dishes were gently removed from the incubation tank and digital images were taken under a stereoscopic microscope (SteREO Discovery.V20, Zeiss) at 4.7× mag to count the number of eyed embryos. The percentage of eyed embryos ($E_r$) was then calculated for each treatment from the total number of eggs ($E_t$) placed in the petri dish minus dead eggs ($E_d$) by the following equation:

$$E_r = \frac{[(E_t - E_d)/E_t] \times 100}{1}$$

### 2.4. Experimental design for Northern pike

As above, the fish used in this experiment were randomly chosen and crossed using a full factorial (North Carolina II) breeding design (5 sires × 5 dams) with 3 replicate crosses per family combination. Freshwater (10 mL, 10 °C, pH 7.2), as an activating medium, was first placed in 75 glass petri dishes. Eggs (~140; 1 g) were gently added to petri dishes and spread throughout the dish with a plastic spoon. Then, 10 µL of milt was immediately added to the eggs using a micropipette. After insemination, petri dishes were shaken by hand for 20 s to facilitate fertilization.

Following fertilization, all petri dishes containing eggs were kept in a 1000 L incubation tank at 10 °C with continuous water flow for 5 days. After 5 days of incubation, all the petri dishes were gently removed from the incubation tank and digital images were taken under a stereoscopic microscope (SteREO Discovery.V20, Zeiss) at 2.5× mag to count the number of eyed embryos. The percentage of eyed embryos ($E_r$) was then calculated for each treatment, as above.

### 2.5. Sperm analysis

Sperm analysis was done with the use of milt collected from 5 males of each species. Sperm quality was estimated on the basis of sperm motility parameters analyzed with a computer assisted sperm analysis (CASA) system (Sperm Class Analyzer v. 4.0.0. by Microptic S.L., Barcelona, Spain). The concentration of sperm was determined using the spectrophotometric method (Ciereszko and Dabrowski, 1993). To measure motility, a milt sample (1 µL) was activated in freshwater (1 mL) and then placed on a Leja slide. Motility parameters of sperm were recorded ~8 s after activation of motility. Analysis was done separately for each male in triplicate. For Northern pike we measured sperm density and percent motility, while for Ide we measured sperm density, percent motility, as well as curvilinear velocity, straight line velocity, and average path velocity.

### 2.6. Statistical analyses

Data were then analyzed using fullfact (Houde and Pitcher, 2016), a software program for analyzing full factorial breeding design data using mixed-effects models to extract the variance explained by random and fixed effects and provide their significance. In particular, the software integrates nonnormal error structures for estimating variance components for nonnormal data types.

### 3. Results

#### 3.1. Ide experiment

The mean (±SEM) sperm density of the five males was $10.8 ± 1.6 \times 10^9$ cells mL$^{-1}$, ranging from $6.4$ to $14.9 \times 10^9$ cells mL$^{-1}$ for Ide. The mean (±SEM) percent motility of the five males was $40.8 ± 7.2\%$. The mean (±SEM) values of curvilinear velocity, straight line velocity, and average path velocity of the sperm from all the males were $29.2 ± 2.4$ (range: $21.6–35.3$), $20.2 ± 2.4$ (range: $13.0–25.5$), and $24.0 ± 2.6$ (range: $16.0–32.0$) µm s$^{-1}$, respectively.

Eyed embryo rate ranged from $8.69 ± 3.02\%$ to $91.15 ± 2.24\%$ for the 25 family crosses (Fig 1A). The full-factorial model showed that the paternal, maternal, and paternal × maternal interaction term were highly significant (p < 0.0001) (Fig. 1B); this significant interaction term confirms that certain family combinations were.

#### Table 1

<table>
<thead>
<tr>
<th>Source of variation: ide</th>
<th>VC ± SD</th>
<th>%Total variance</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td>0.55 ± 0.74</td>
<td>10.73</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Paternal</td>
<td>1.04 ± 1.02</td>
<td>20.24</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Maternal × Paternal</td>
<td>0.25 ± 0.50</td>
<td>4.83</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>3.29</td>
<td>64.19</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Eyed embryo rate for Ide, Leuciscus idus. (A) Rate of eyed embryo for 25 family crosses; (B) interaction between sire and dam at the eyed embryo stage.
4. Discussion

Reproductive success relies on offspring quality, which is primarily influenced by intrinsic interactions between parental genes (Neff and Pitcher, 2005; Politis et al., 2014). In the present study, we prohibited any sort of natural mate choice, i.e. reproduction involving secondary sexual characteristics and complex combinations of behaviors within and between sexes (Trippel and Morgan, 1994; Hutchings et al., 1999), by experimentally creating controlled crosses between randomly selected individuals. Here, we show that sires and dams, independently as well as interactively, had a significant impact on the rate of eyed embryos in two separate fish species. Paternal × maternal interactions explained 4.83% and 8.3% of the total variance of Ide and Northern pike, respectively. Paternal effects were ~2-fold and ~15-fold higher than the maternal effects in Ide and Northern pike respectively. Thus, paternal identity was the main driver influencing early embryonic survival to the eye stage. Furthermore, eyed embryo rate ranged from 8.69% to 91.15% for Ide and 7.87% to 89.78% for Northern pike, clearly indicating the genetic compatibility of some and incompatibility of other crosses.

Paternal effects on offspring characteristics are presumed to solely be related to the genetic (haploid) contribution, because no nutritional or energy reserves for metabolism and development of the embryo are provided by the male parent (Green, 2008). Paternal effects on early life history of teleost fishes, have in recent years gained scientific attention (Panagiotaki and Geffen, 1992; Rideout et al., 2004; Butts and Litvak, 2007b). More specifically, paternal history traits via spawning site selection, fecundity, egg quality, egg size, and provisioning of yolk (Bengtson et al., 1987; Green, 2008). Females contribute in contrast to males, not only 50% of the nuclear genomic DNA, but also non genetic components for instance in form of a yolk and carotenoid supply (Rideout et al., 2004; Butts and Litvak, 2007b). Additionally, the influence of paternal was found to explain 56% and 44% of the observed variation in hatching success of Atlantic and Baltic cod, Gadus morhua, respectively (Dahlke et al., 2016). In our study, paternal influences were the predominant force influencing embryonic development for Ide and Northern pike. The results of this and the above mentioned studies validate the fact that paternal has a pronounced influence on pivotal life history traits in fish, especially for development of offspring during the critical early embryonic stages.

Maternal factors have, no doubt, a major influence on early life history traits via spawning site selection, fecundity, egg quality, egg size, and provisioning of yolk (Bengtson et al., 1987; Green, 2008). Females contribute in contrast to males, not only 50% of the nuclear genomic DNA, but also non genetic components for instance in form of a yolk and carotenoid supply (Rideout et al., 2004). Maternal factors influence initial offspring quality and size primarily by influencing egg quality and size (Bengtson et al., 1987; Chambers and Leggett, 1996; Marteinsdottir and Steinarsdottir, 1998). Generally, larger and older females normally produce relatively large offspring, which according to the “bigger is better hypothesis” may confer advantages to offspring during ELI (Chambers and Leggett, 1996; Trippel et al., 1997). Differences
in the nongenetic maternal contributions might superimpose potential paternal effects, thus, a common though inadequate assumption in fish research is that most of the variation in ELH of fishes is due to maternity rather than paternity (Chambers and Leggett, 1996). Although paternal effects were in our study greater than the maternal effects for these two species, nuclear and extra nuclear maternal influences should not be disregarded. Here we provide evidence that the optimal parental combination and their genetic compatibility increased offspring quality during embryonic development. The “genetic incompatibility hypothesis” states that offspring quality depends on the intragenomic conflicts between maternal and paternal genes (Neff and Pitcher, 2005). We observed that an egg batch of a particular female develops more successfully when sired by a compatible male, but develops poorly when sired by a less compatible (or incompatible) different male. Similar results have previously been reported from full factorial experiments with several different crossings of Alpine whitefish, Coregonus sp. (Wedekind et al., 2001) as well as Atlantic and Baltic cod (Politis et al., 2014; Dahlyke et al., 2016). Common in nature and in culture, the process of polyandry, where one female reproduces with several males, enabling genetic diversity and richness, can through mate competition lead to the right mate choice and thus to higher reproductive success (Hutchings et al., 1999; Bekkevold et al., 2002). Our study clearly shows that some parental combinations are more compatible than others, resulting in higher quality offspring and thus confirms that the choice of mate does matter in order to ensure high reproductive outcome and success.

In conclusion, we demonstrate that paternally induced genetic variation during embryonic development represents a relevant resource for adaptive responses in fish. This study validates the fact that paternity and maternity act independently as well as interactively to influence ELH stages of fish. The generally accepted maternal effects were overshadowed by paternally mediated differences during embryonic development. Paternity was the main factor influencing offspring quality and coincided with a vital morphogenetic process during early life: the eyed embryo stage. Eyed embryo rate differed significantly among the different parental combinations, clearly indicating the genetic compatibility of some and incompatibility of other crosses. Thus, we provided evidence that the right female’s choice of mate can result into increased offspring quality and therefore improve the chances of successful natural recruitment and/or assisted reproduction.

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