DNA Methylation Profiles Suggest Intergenerational Transfer of Maternal Effects

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Abstract

The view of maternal effects (nongenetic maternal environmental influence on offspring phenotype) has changed from one of distracting complications in evolutionary genetics to an important evolutionary mechanism for improving offspring fitness. Recent studies have shown that maternal effects act as an adaptive mechanism to prepare offspring for stressful environments. Although research into the magnitude of maternal effects is abundant, the molecular mechanisms of maternal influences on offspring phenotypic variation are not fully understood. Despite recent work identifying DNA methylation as a potential mechanism of nongenetic inheritance, currently proposed links between DNA methylation and parental effects are indirect and primarily involve genomic imprinting. We combined a factorial breeding design and gene-targeted sequencing methods to assess inheritance of methylation during early life stages at 14 genes involved in growth, development, metabolism, stress response, and immune function of Chinook salmon (*Oncorhynchus tshawytscha*). We found little evidence for additive or nonadditive genetic effects acting on methylation levels during early development; however, we detected significant maternal effects. Consistent with conventional maternal effect data, maternal effects on methylation declined through development and were replaced with nonadditive effects when offspring began exogenous feeding. We mapped methylation at individual CpG sites across the selected candidate genes to test for variation in site-specific methylation profiles and found significant maternal effects at selected CpG sites that also declined with development stage. While intergenerational inheritance of methylated DNA is controversial, we show that CpG-specific methylation may function as an underlying molecular mechanism for maternal effects, with important implications for offspring fitness.

Key words: **evolutionary genetics, epigenetics, maternal effects, genetic architecture, DNA methylation, intergenerational effects.**

Article

Introduction

Maternal effects have been shown to affect offspring and maternal fitness (Galloway and Etterson 2007; Aykanat, Bryden, et al. 2012; Perez et al. 2017; Fan et al. 2019) and can contribute to patterns of local adaptation (Wolf and Wade 2016). Traditionally, maternal effects were thought to be driven primarily by gamete size and maternal loading of gametes with hormones, proteins, mRNA, and energy stores (Nodine and Bartel 2012; Perez et al. 2017) although other mechanisms have been identified (Heath et al. 1999; Aykanat, Heath, et al. 2012; Nodine and Bartel 2012; Videvall et al. 2016; Falica et al. 2017). Maternal effects can affect offspring gene expression patterns (Aykanat, Heath, et al. 2012; Nodine and Bartel 2012; Videvall et al. 2016), and for these effects to be adaptive, they must be targeted to specific genes, though the mechanisms for intergenerational control of early life gene expression remain unclear. Previous research has identified maternal effects driven by epigenetic mechanisms, including transmission of small RNAs, histone modifications and parent-specific genetic imprinting to offspring (Feng et al. 2010).

Genetic imprinting, the monoallelic expression of one parent's genes in offspring, has been extensively studied in mammals and DNA methylation shown to be a contributing mechanism (Inoue et al. 2017). However, methylation is often reset at fertilization in animals such as fish, thus the mechanisms behind intergenerational inheritance of methylation are unclear (Perez and Lehner 2019). In zebrafish, DNA methylation is reset almost immediately after fertilization (Mhanni and McGowan 2004) and subsequent de novo methylation occurs (Mhanni and McGowan 2004; MacKay et al. 2007), after which sperm DNA becomes hypermethylated compared with oocyte DNA in newly fertilized embryos (Mhanni and McGowan 2004; Jiang et al. 2013). Paternal methylation patterns are retained through early development, but maternal methylation patterns are lost by the midblastula stage and altered to resemble paternal methylation patterns (Jiang et al. 2013; Potok et al. 2013). Although overall changes in early developmental methylation landscapes suggest that methylation may serve as a conduit for parental effects in fish (Perez and Lehner 2019), gene-specific

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methylation changes still occur at developmentally critical loci (Fang et al. 2013). As development progresses, fluxes in methylation levels occur (Mhanni and McGowan 2004) before stabilizing to the same levels as adult somatic tissue around the time of gastrulation (Fang et al. 2013).

Regardless of the pattern of loss of maternal methylation signatures during early development, maternal effects on offspring methylation have been reported. Since methylation landscapes differ considerably between early embryogenesis and hatching in zebrafish (McGaughey et al. 2014), DNA methylation remains a possible mechanism for the propagation of maternal effects despite genomic imprinting and resetting of methylation, reported in previous research. DNA methylation is sensitive to environmental changes, such as developmental differences (Anastasiadi et al. 2017), and interspecies variation in global methylation based on temperature (Varriale and Bernardi 2006), altered gene methylation due to seawater acclimation in brown trout (Morán et al. 2013), and hatchery-induced methylation changes in Coho salmon (Le Luyer et al. 2017). Since methylation is affected by developmental stage and environment, it is possible that maternal effects are propagated through methylation in response to the maternal environment, with offspring gaining autonomy over methylation later in development. Previous research has shown that maternal food deprivation resulted in altered offspring gene expression and increased mortality in zebrafish (Fan et al. 2019). Exposure of female zebrafish to BPA resulted in transgenerational effects on offspring gene expression and promoter methylation up to the F3 generation (Santangeli et al. 2019). Similar effects were reported on offspring promoter DNA methylation levels up to the F3 generation when adult zebrafish were subjected to ionizing radiation (Kamstra et al. 2018). Strong family effects on DNA methylation have been reported in stickleback, which suggests a role for DNA methylation in generating interindividual variation (Metzger and Schulte 2018). It is possible that variation in DNA methylation among families reflects intergenerational epigenetic inheritance or maternal effects (Metzger and Schulte 2018), thus epigenetic mechanisms other than imprinting are likely responsible for maternal effects on gene expression later in development. However, it is unclear whether DNA methylation is also responsible for intergenerational fine-tuning of offspring gene expression levels. For methylation to be a viable mechanism for the transmission of maternal effects, it must be targeted to specific noncanalized genes reflecting the mother's environmental experiences and genotype, but not affect genes with highly canalized expression. In contrast, random intergenerational epigenetic inheritance would align with the antiquated view of maternal effects as physiological side effects (e.g., Mousseau and Fox 1998).

Chinook salmon (*Oncorhynchus tshawytscha*) are an ideal species for the study of maternal effects as they show high levels of individual variation in fitness-related and life history traits (Fraser et al. 2011) as well as robust maternal effects (Heath et al. 1999; Aykanat, Bryden, et al. 2012; Aykanat, Heath, et al. 2012; Falica et al. 2017), including strong maternal effects on early life gene transcription patterns (Wellband et al. 2018). Chinook salmon have a semelparous life history where a single, terminal reproductive event (Heath et al. 1999) results in strong selection to maximize the adaptive value of maternal effects through their downstream effects on offspring fitness. Furthermore, Chinook salmon are externally fertilized and receive no parental care, allowing for sophisticated breeding designs but avoiding confounding effects of parental care or behavioral variation.

To quantify the role of DNA methylation in the propagation of maternal effects, we created replicated full-factorial (6X6 North Carolina II design) Chinook salmon crosses and estimated genetic variance components for DNA methylation levels at 14 gene loci. We selected genes involved in growth, developmental control, metabolism, stress response, and immune function (supplementary table S1, Supplementary Material online). We used massively parallel ("Next Generation") bisulfite sequencing in a gene-targeted DNA methylation assay for offspring from the replicated 6×6 crosses over a total of 76 CpG sites at three early developmental stages (864 offspring in total): eyed egg (embryo), alevin (larval), and fry (postexogenous feeding). We hypothesized that if maternal effects are adaptively affecting offspring DNA methylation profiles, they would be gene-specific (Venney et al. 2016) and targeted to specific CpG sites within genes, as random-acting maternal effects during this highly regulated developmental period would be expected to be maladaptive. We further predicted that maternal influences on offspring methylation patterns should decline through development, consistent with phenotypic observations of maternal effects in salmon (Heath et al. 1999; Falica and Higgs 2013; Falica et al. 2017) and in other taxa (Mousseau et al. 2009) as the offspring gains control over their genome and phenome. Maternal effects are thought to decline during development due to a parent-offspring conflict between the mother, who predicts the offspring's environment based on her experience, and the offspring, which seeks to maximize its own fitness based on its actual environmental experience (Heath et al. 1999; Crespi and Semeniuk 2004; Falica and Higgs 2013). Despite the resetting of DNA methylation, maternal effects are successfully passed to offspring and persist until offspring gain autonomy over their own development and function. A molecular mechanism (such as DNA methylation) for maternal effects would be consistent with observed strong maternal effects across taxa, coupled with the growing realization that maternal effects likely evolved as an intergenerational signaling process that facilitates rapid adaptation to variable environments.

Results

Average read depth across all CpG sites was 106 sequences after all quality trimming (supplementary table S2, Supplementary Material online). Linear mixed models (LMM) were used to test for maternal effects on DNA methylation 1) across all assayed loci combined, 2) at each locus, 3) at individual CpG sites across all loci combined, and 4) at individual CpG sites at each locus.

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Overall DNA Methylation

We first combined all CpG site methylation data across the 14 candidate genes to test for dam, sire, dam × sire interaction and locus effects on percent methylation levels averaged across all CpG sites for each gene. Replicate (incubation tray cell) and breeding cross were included as variables, but replicate was not significant and therefore removed from the final model. We found evidence for dam effects acting on DNA methylation across all gene loci at the eyed egg ($P < 0.05$) and the alevin ($P < 0.0001$), but not at the fry stage. Sire (additive) and dam × sire interaction (nonadditive) effects on methylation were not significant, but nonadditive interaction effects were significant at the fry stage ($P < 0.05$). We also found very strong locus effects ($P < 0.0001$) at all developmental stages, indicating substantial variation in methylation levels among the candidate genes, as expected.

Locus-Specific Methylation

Next, we tested for dam, sire, and interaction effects at each locus independently to test for gene-specific effects. At the individual gene level, we found transient gene-specific dam effects at the eyed egg and alevin stages after Bonferroni correction which subsided by the fry stage (fig. 1, supplementary table S2, Supplementary Material online). We found significant dam effects at *GTIIBS* and *hsc71* at the eyed egg stage, and *hsc71*, *GH1*, *metA*, and *ITPA* at the alevin stage. We detected significant maternal effects (dam minus sire variance) by generating 95% confidence intervals using the fullfact (Houde and Pitcher 2016) package in R (R Development Core Team 2016).

Maternal effects on methylation levels at individual gene loci were significant (i.e., confidence intervals excluding zero) for three genes (*metA*, *hsp70a*, *hnrL*) in the eyed egg stage, six genes (*GH1*, *hsp90*, *hsc71*, *itpa*, *BDNF*, *hnrL*) in the alevin stage, and six genes (*GTIIBS*, *pit1*, *metA*, *IL8R*, *hsc71*, *hsp70a*) in the fry stage (fig. 2). At the eyed egg stage, we also found significant sire effects on two genes, *GTIIBS* and *hsc71* ($P < 0.001$ and $P < 0.05$, respectively, after Bonferroni correction) as well as nonadditive genetic effects on *FSHb* methylation ($P < 0.01$, after Bonferroni correction).

CpG-Specific Methylation

Finally, we tested for CpG-specific maternal effects. We found a strong dam × CpG site effect across all candidate loci combined ($P < 0.001$ for the eyed egg and alevin stages, but not at the fry stage) with, as expected, a strong locus effect ($P < 0.001$ for all developmental stages). At the individual locus level, three genes showed a significant dam × CpG interaction: *CK-1* at the eyed egg stage, *ITPA* at the alevin stage, and *GTIIBS* at the eyed egg and alevin stage (fig. 3). While statistically nonsignificant, *hsp70a* methylation at the alevin stage differed based on which cross the mothers were from (fig. 3). No significant dam × CpG site effects were found at the fry stage.

Discussion

Maternal effects can dramatically contribute to variation in offspring phenotype, performance, and fitness at early life stages (Galloway and Etterson 2007), and result in evolutionary change at the population level (Aykanat, Bryden, et al. 2012; Wolf and Wade 2016); thus, maternal effects are an important consideration in evolutionary biology. However, due to the resetting of methylation signatures across the genome during early development (Mhanni and McGowan 2004; Perez and Lehner 2019), it remains unclear whether methylation serves as a mechanism for the propagation of maternal effects across generations. Across all loci, we observed strong maternal effects on overall DNA methylation that subsided by the fry stage in Chinook salmon. Despite the reported loss of maternal methylation signatures early in development (Jiang et al. 2013; Potok et al. 2013), we found that maternal effects persist and influence DNA methylation patterns early in life. The widely reported pattern of declining maternal effects associated with offspring control over their genome matches our results, specifically, negligible maternal effects on methylation levels by the exogenous feeding fry stage (Heath et al. 1999; Falica and Higgs 2013; Falica et al. 2017). We observed strong locus effects on DNA methylation at all stages. Since normal development requires strict regulation of gene expression at critical developmental loci (Zeitlinger and Stark 2010), maternal effects are likely to act to "fine-tune" expression of less canalized genes and leave the expression of highly regulated and developmentally controlled genes unaffected. While the mechanism behind the transmission of maternal effects after the loss of maternal methylation patterns during development remains unclear (Perez and Lehner 2019), our results are consistent with previous findings of intergenerational epigenetic inheritance in fish (Kamstra et al. 2018; Santangeli et al. 2019). Thus, maternal effects on DNA methylation occur in the eyed egg and alevin stages of Chinook salmon, but vary among loci, consistent with the hypothesis that maternal effects must target specific loci to be adaptive.

Our results indicate maternal effects on offspring DNA methylation in early development are gene-specific. We found transient gene-specific dam effects at the eyed egg and alevin stages after Bonferroni correction (fig. 1), consistent with previous research on maternal effects in Chinook salmon (Heath et al. 1999; Falica and Higgs 2013; Falica et al. 2017), and a broad array of other taxa (Mousseau et al. 2009). We observed maternal effects on *GTIIBS* (endocrine function and sex differentiation, Patsoula et al. 2003) and *hsc71* (aids in protein folding, Massicotte et al. 2006) at the eyed egg stage, and *hsc71*, *GH1* (larval body size, Li et al. 2007), *metA* (influenced by maternal contaminant exposure, Wu et al. 2008), and *ITPA* (control of cell replication, Abolhassani et al. 2010) at the alevin stage. These genes are associated with phenotypic effects related to previously documented maternal effects, including effects on offspring size (Janssen et al. 1988; Heath et al. 1999; Falica et al. 2017) and resistance to contaminants (Wu et al. 2008). Conversely, constitutively expressed and developmentally critical genes did not show significant dam effects. Genes such as *Tf*, which is constitutively expressed (Stafford and Belosevic 2003), *BDNF* which is involved in neural function and development (Conner et al. 1997), and *pit1* which is involved in regulating growth

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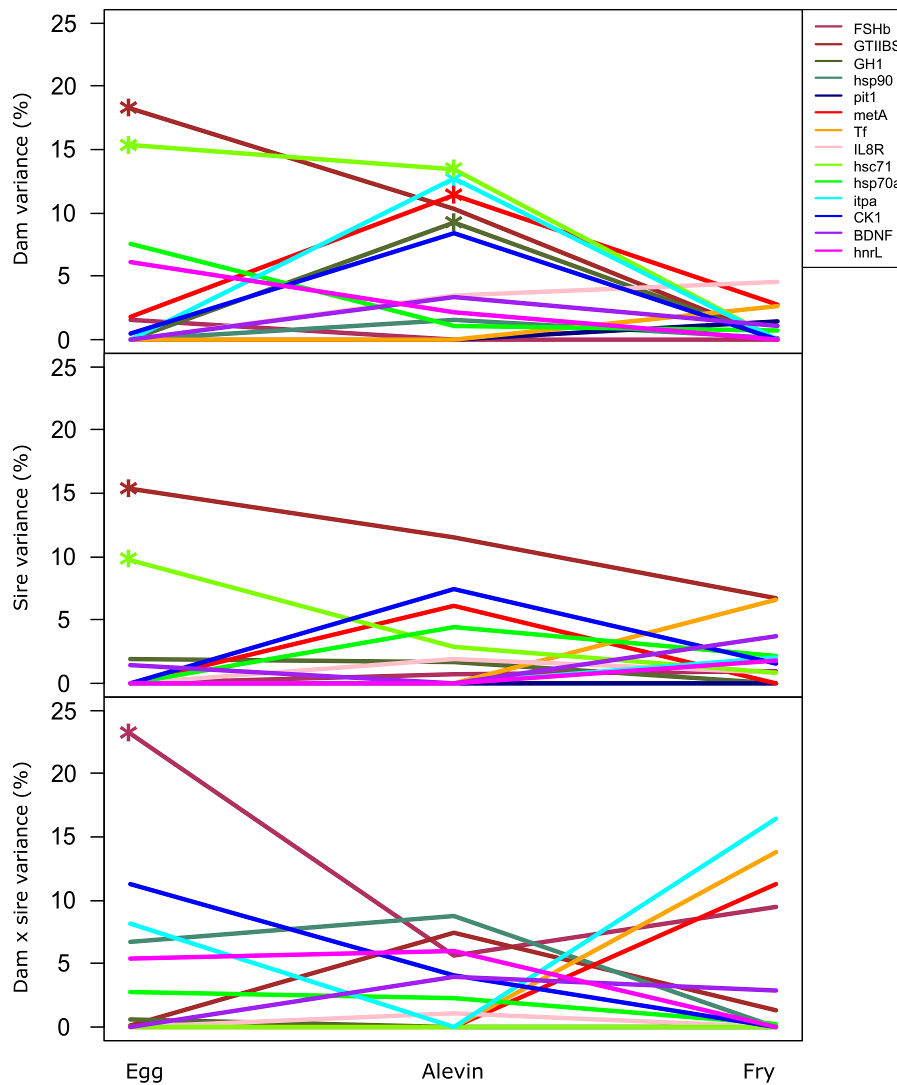

Fig. 1. Mean dam, sire, and dam × sire effect variance component for mean gene-specific DNA methylation rates at 14 selected gene loci across three developmental stages in Chinook salmon offspring. Significant effects of the variance component on percent methylation are denoted by an asterisk.

hormone and other growth-related genes (Yamada et al. 1993) did not show significant effects on methylation at any stage, as expected for developmentally and metabolically critical genes. Previous studies have shown that parental exposure to stressful stimuli results in locus-specific methylation changes in offspring (Kamstra et al. 2018; Santangeli et al. 2019), thus our results support the occurrence of a targeted mechanism for the propagation of maternal effects, though the mechanism remains unclear. Maternal effects are associated with phenotypic and physiological variation which could prove to be adaptive (or maladaptive) depending on the correlation between maternal and offspring environments (Mousseau and Fox 1998), consistent with the theory of the evolution of adaptive maternal-offspring signaling (Sheriff and Love 2013). Our results thus strongly support the hypothesis that methylation serves as a mechanistic mediator for maternal effects (Love et al. 2013).

The discovery of maternal effects influencing offspring DNA methylation at specific loci prompted the question of whether maternal effects act on mean methylation levels across candidate gene loci, or whether maternal effects affect methylation status at specific CpG sites within genes. Since methylation can have variable effects on gene expression depending on which CpG sites are methylated (Lillycrop et al. 2008), CpG site-specific methylation provides an additional level of specificity (and complexity) to the transmission of DNA methylation-based maternal signals. Strong dam × CpG effects across the combined candidate loci at the eyed egg and alevin stage are indicative of broad-scale targeted maternal effects acting on methylation at specific CpG sites. At the individual locus level, dam × CpG interaction effects were detected at *CK-1* (immune response, Lally et al. 2003) and *GTIIBS* at the eyed egg stage, and *GTIIBS* and *ITPA* at the alevin stage (fig. 3). These results support the hypothesis that mothers influence offspring DNA methylation in early development not only at specific genes, but also at specific CpG sites, consistent with a targeted mechanism for maternal effects. The individual genes with significant

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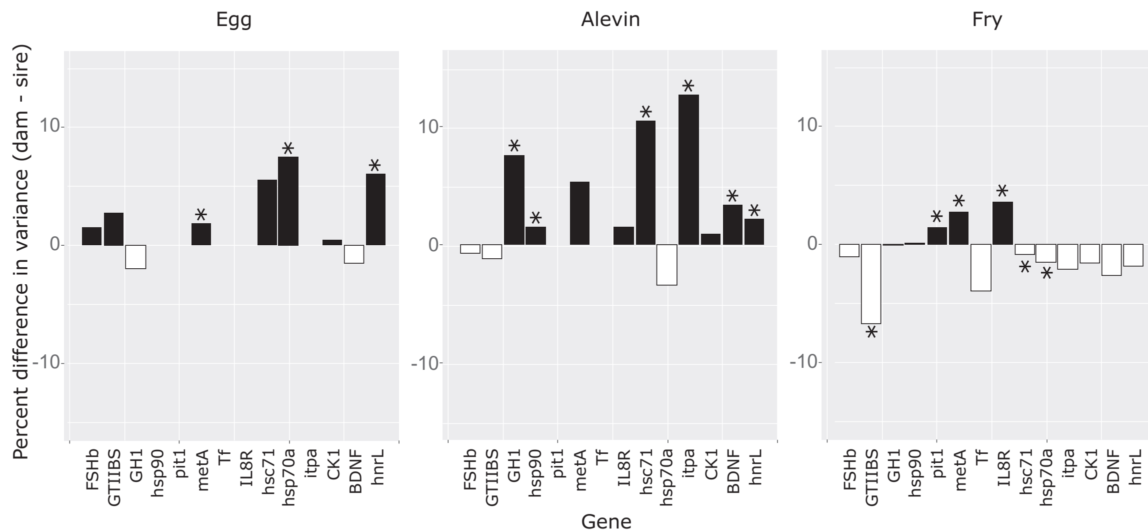

Fig. 2. Percent difference in dam versus sire variance components (maternal effects) for mean DNA methylation at 14 gene loci across three developmental stages in Chinook salmon. Black bars indicate a greater dam component of variance and white bars indicate a greater sire variance component. Results show that the dam component of variance is generally greater than the sire component of variance (black bars) early in development (indicative of maternal effects) but the sire component of variance is generally larger after the onset of endogenous feeding (white bars). Significant maternal effects determined using 95% confidence intervals (see Methods) are denoted by an asterisk and gene descriptions are provided in Table S1.

dam × CpG interaction terms are logical targets for adaptive maternal effects due to their noncanalized expression and role in response to environmental challenges. While the dam component of the methylation profile varies though development, it is lost at the fry (exogenous feeding) stage, as expected for transient maternal effects that are overridden by offspring methylation control as the offspring responds to its environment.

The erosion of maternal effects through early development is well documented (Heath et al. 1999; Falica and Higgs 2013; Falica et al. 2017), but the proximate mechanism of this reduction has not been explored. The loss of maternal control over offspring gene-specific methylation could be due to the degradation of maternally derived proteins regulating DNA methylation (Inoue et al. 2017). At the fry stage, the sire component of variance generally explained more of the variance in DNA methylation than the dam component (fig. 2). This could be due to a delayed paternal effect, as seen in previous studies that have reported increased sire effects later in development in Chinook salmon (Falica and Higgs 2013) and paternal effects in other species (Jensen et al. 2014), or due to a negative maternal effect (Janssen et al. 1988; Heath et al. 1999). However, nonadditive (dam × sire) effects on methylation became significant at the fry stage, suggesting increasing endogenous epistatic and/or dominance effects (Aykanat, Heath, et al. 2012; Wellband et al. 2018) as the offspring genome gains control of methylation and demethylation processes. Our results suggest that intergenerational effects on DNA methylation occur at specific life stages after methylation reset, but before the offspring gains autonomy over their genome. Regardless, our results support DNA methylation as a potential novel mechanism for transient intergenerational maternal effects, which can have important consequences for offspring fitness.

Our results support the idea that CpG-specific DNA methylation has a role in mechanistically propagating maternal effects during early development, which may influence offspring growth and physiology through gene-specific methylation changes. However, further research is required to determine the mechanisms involved in transmitting maternal signals to modify methylation patterns. Our results are unexpected based on the loss of maternal methylation signals early in embryonic development in fish and subsequent adoption of methylation landscapes similar to sperm (Potok et al. 2013; Jiang et al. 2013; Perez and Lehner 2019). A process other than methylation resetting is likely responsible since we detected maternal effects on offspring DNA methylation in life stages after the expected loss of maternal methylation patterns. It is possible that our results are due to our exploration of later developmental stages. At the eyed egg stage, the earliest developmental stage we studied, the developing embryo is in the midst of organogenesis and well past gastrulation (Velsen 1980). While methylation is reset around the time of gastrulation (Mhanni and McGowan 2004), maternal effects on methylation have been reported in developmental stages undergoing organogenesis in zebrafish and are targeted to specific regions of the genome (Fan et al. 2019; Santangeli et al. 2019), consistent with our results. While Chinook salmon development is primarily affected by temperature and time since fertilization (Beacham and Murray 1990), it is possible that some variation in observed methylation is due to differences in developmental rate. However, Chinook salmon tend to show high synchrony in developmental rate when raised in a common, controlled environment, thus we find this unlikely. Although previous studies have identified phenotypic effects of intergenerational epigenetic inheritance (Fan et al. 2019), future research should relate changes in gene-specific and CpG-specific DNA methylation profiles with

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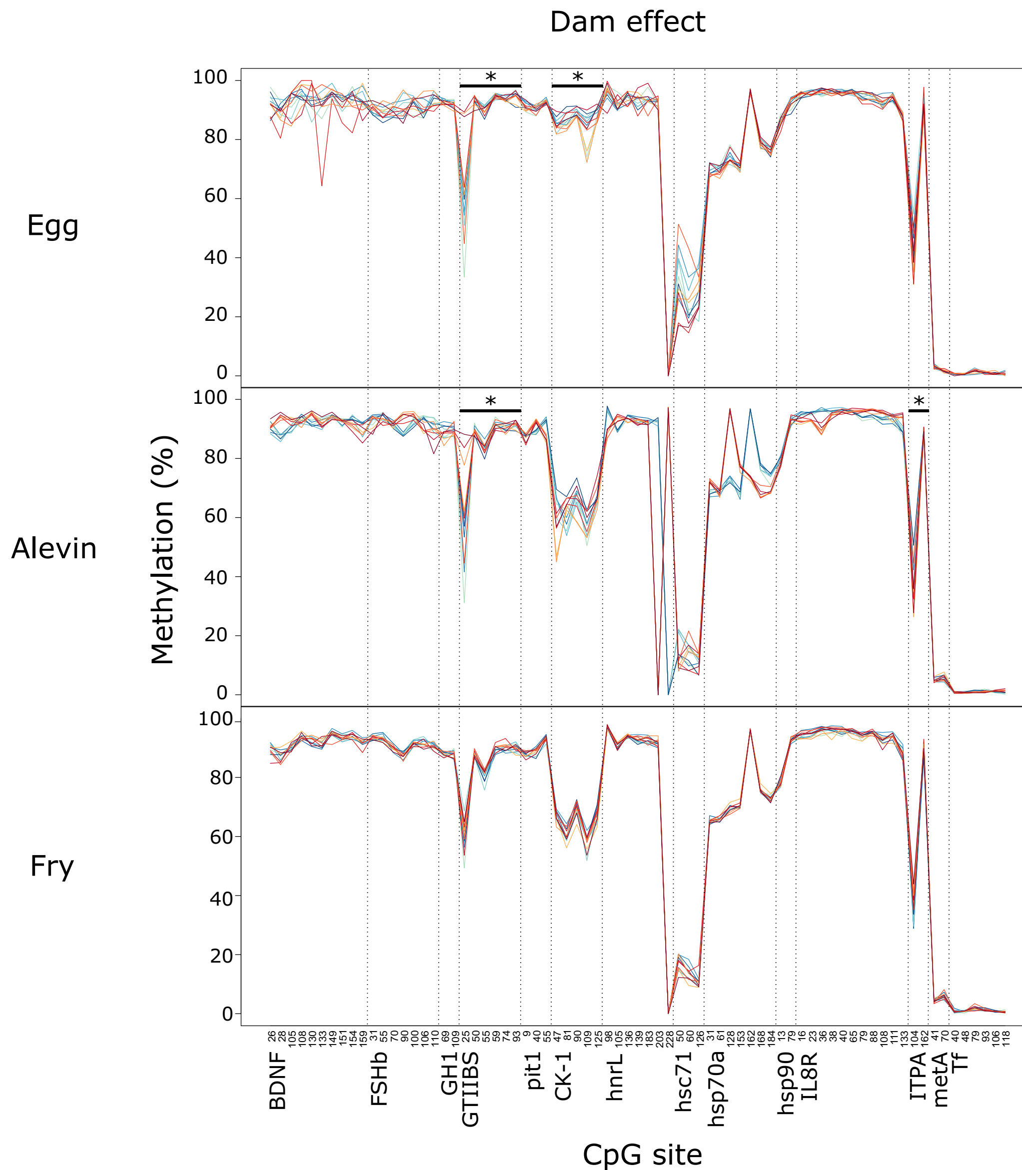

Fig. 3. Maternal DNA methylation profiles for individual CpG methylation sites at 14 gene loci for three developmental stages in Chinook salmon offspring. Individual line graphs show dam-specific effects on CpG-specific DNA methylation rates (%) with the 12 dams used in the crosses shown in different colors. Blue lines represent dams from cross 1 and red lines represent dams from cross 2. Horizontal lines with asterisks denote significant dam × CpG effects on methylation. High levels of dam effects are present when the profiles diverge.

well-documented phenotypic maternal effects, such as those observed in Chinook salmon (Heath et al. 1999; Aykanat, Bryden, et al. 2012; Aykanat, Heath, et al. 2012). Our data further support DNA methylation as a highly targeted mechanism in the underlying genetic architecture of intergenerational effects. Since methylation controls individual variation in gene expression, it has the potential to generate physiological and phenotypic variation upon which selection could act and, ultimately, fine-tune gene expression through maternal inputs to optimize offspring fitness. At present, it is unclear if maternal effects on DNA methylation in early life are indicative of a true mechanism for the transfer of maternal effects, or if they are a downstream consequence of changes in transcription, as reported in other studies (Pacis et al. 2019). Our results highlight the need for future studies on the effects of intergenerational DNA methylation transfer on offspring phenotype and fitness, and their timing with respect to changes in transcription.

The study of DNA methylation in an evolutionary context is in its infancy, with most published studies focused on medical or physiological applications. However, previous research has proposed DNA methylation as a novel adaptive mechanism (Aykanat, Heath, et al. 2012; Venney et al. 2016). In this study, we provide support for targeted DNA methylation as a mechanism for intergenerational signaling in Chinook salmon. Despite loss of maternal methylation patterns shortly after fertilization, strong maternal effects on gene-specific and CpG-specific methylation, suggesting a previously unidentified mechanism allows maternal control over the offspring genome even after loss of parental methylation patterns.

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Parental effects can have far-reaching effects on offspring fitness, resulting in population and evolutionary change (Aykanat, Bryden, et al. 2012; Wolf and Wade 2016). If parentally induced DNA methylation profiles reflect parental environment and experiences, then epigenetic mechanisms may serve as a conduit for parents to affect early-stage offspring phenotype and physiology. Such effects could increase offspring fitness and potentially reinforce local adaptation through maternal effects, a pattern already proposed based on population-level phenotypic divergence (Aykanat, Bryden, et al. 2012).

Materials and Methods

Breeding Design and Sampling

On October 31, 2014, two North Carolina II breeding crosses were set up using Chinook salmon at Yellow Island Aquaculture, Ltd (YIAL), a commercial salmon farm on Quadra Island, BC, Canada. Sexually mature males and females (ages three to five years old) were selected for the breeding experiment, with the first cross created using parents who had been transferred to freshwater tanks and the second cross using parents from saltwater cages. Each of the two crosses were generated by mating six sires with six dams in a factorial design, resulting in 36 families per cross (72 families total). The North Carolina II mating design allows variance to be partitioned to maternal effects by subtracting the sire (additive) component from the dam (additive + maternal) component of variance. Since Chinook salmon die after reproducing, their offspring receive no parental care, and thus any maternal effects are due to underlying egg provisioning or molecular maternal signals.

The fertilized eggs were incubated in freshwater vertical stack incubation trays following standard YIAL protocols, with two replicate cells allotted to each half-sib family. Eyed eggs were sampled from each replicate on December 19, 2014 (~300 ATUs, 49 days since fertilization). Alevins were humanely euthanized and sampled on March 2, 2015 (~700 ATUs, 123 days since fertilization). The remaining alevins were transferred to 200 L freshwater tanks where they were reared until the fry stage. For the transfer, the two replicate incubation cells were pooled, and each mixed family was divided between two replicate rearing tanks. On May 6, 2015, fry were collected by dip netting, humanely euthanized and sampled. Whole fish or eyed egg samples from all developmental stages were preserved immediately in a high salt buffer (25 mM sodium citrate, 10 mM EDTA, 5.3 M ammonium sulfate, pH 5.2) for later analysis. Fry were cut open to promote preservation. A total of four fish (two per replicate cell) per full-sibling family were used for each developmental stage.

DNA Extraction and Processing

Embryos from eyed eggs were dissected from the yolk and digested in 1,000 µl of digestion buffer (100 mM NaCl, 50 mM Tris–HCl pH 8.0, 10 mM EDTA, 0.5% SDS) with 10 µl of proteinase K. Alevins were cut in half and both halves digested in 6,000 µl of digestion buffer with 10 µl of proteinase K (20 mg/ml). The fry (who had their livers removed for another experiment) were cut into three pieces, and the three pieces were digested together in 7,000 µl of digestion buffer with 10 µl of proteinase K. While the liver is an important tissue for regulating growth and metabolism, it represented a minute portion of the total body mass of the fish, and thus the loss of DNA from the liver is not expected to affect our results. All samples were digested at 37 °C for 24 h, and 150 µl of the digested product was used for DNA extraction using a high-throughput plate-based extraction protocol (Elphinstone et al. 2003).

Bisulfite Conversion and DNA Methylation Assay

Extracted DNA was quantified using a Quant-IT PicoGreen dsDNA Assay kit (ThermoFisher Scientific P11496) and 500 ng of DNA underwent bisulfite conversion with an EZ-96 DNA Methylation-Lightning kit (Zymo Research D5033) following the manufacturer's protocol. Bisulfite conversion allows for the analysis of sites of methylation: methylated cytosines are unaffected whereas unmethylated cytosines are converted to uracil.

Published bisulfite sequencing primers for 14 highly conserved genes involved in early development, metabolism, and stress response were used for methylation analysis (Venney et al. 2016). Primers were designed for intragenic exon gene regions with little to no sequence variation to minimize the effects on genetic variation on methylation analysis (Venney et al. 2016). Genes were primarily chosen based on their role in early growth and differentiation, protecting the developing fish from environmental stress, and metabolic regulation. Metabolic and developmental gene expression is highly conserved; thus, these loci are unlikely targets for maternal effects on DNA methylation. Genes with less canalized expression, such as stress and immune genes, are likely candidates for maternal effects. An expected 2249 bp were amplified across the 14 genes ranging from 79 to 225 bp per gene (supplementary table S1, Supplementary Material online); estimates of fragment length exclude primer sequences.

A two-stage PCR approach and sequencing protocol (Venney et al. 2016) was used to generate bisulfite sequencing libraries, which were sequenced using an Ion PGM Sequencing 400 kit with an Ion 318 Chip for the Ion Torrent Personal Genome Machine (PGM). The samples were spread across four sequencing runs with an expected 500 reads per gene with a maximum length of 400 bp.

Data Processing

Using the program mothur (Schloss et al. 2009), the sequencing runs were demultiplexed to create one sequence file per individual and primer sequences were trimmed. The program bwa-meth (Pedersen et al. 2014) was used to align the generated sequence data to existing sequence data for the genes of interest. The use of highly conserved genes in our methylation assay, as well as allowing a maximum of two alignment mismatches in bwa-meth, ensured that the aligned sequences represented the targeted genes. Bwa-meth generated a data table with the percent methylation for each CpG site for each gene in each individual.

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Statistical Analysis

Bwa-meth data tables were imported into R (R Development Core Team 2016), which was used for all statistical analyses. Data for all individuals were analyzed, and CpG sites successfully sequenced in <70% of individuals (with <5 reads per gene per individual) were excluded from the analysis to ensure the represented CpG sites were compared across all individuals.

LMM were run in the R package lme4 (Bates et al. 2015). To determine if maternal effects were targeted to specific loci, an LMM was used to test the effects of dam, sire, dam × sire interaction, gene, and the random effects of (6 × 6 factorial) cross and replicate Heath tray cell on gene methylation across all loci for each developmental stage. To determine which genes were driving significant effects, an LMM was run for each gene in each developmental stage to determine whether dam, sire, dam × sire, cross, and replicate significantly affected locus-specific DNA methylation. Replicate did not significantly affect methylation and was removed from the final model. Cross was retained in the final model as it was significant for at least one gene before Bonferroni correction but nonsignificant after correcting for multiple comparisons. A Bonferroni correction was used to correct for multiple comparisons.

Maternal effects were calculated by subtracting dam minus sire variance components taken from the LMM. The sire component of variance represents solely additive variation, whereas the dam component represents additive + maternal genetic variance. Significant maternal effects were identified by generating 95% confidence intervals in the fullfact (Houde and Pitcher 2016) package. Methylation data were used to generate 1,000 iterations of possible data sets, which were used to calculate confidence intervals. Maternal effects were considered significant when the confidence intervals did not overlap zero.

To test for dam and sire effects on CpG-specific methylation across all loci simultaneously, LMMs were used to test for the random effects of CpG site, dam, sire, and all two- and three-way interaction effects. Cross (freshwater or saltwater) was included initially as a fixed effect, but was nonsignificant in all models and excluded from the final analyses. The final model was used to test the effects of each variable on DNA methylation across all genes in each of the three developmental stages. Likelihood ratio tests were used to determine the significance of each variable in the final model and a Bonferroni correction was used to correct for multiple comparisons. To determine if specific genes were driving dam and sire effects on CpG-specific methylation or if the same effects were observed across all genes, an LMM for the effects of CpG site, dam, sire, and all interactions was tested for each gene in each developmental stage. The significance of the dam × CpG site interaction term determined whether there were differential methylation patterns within a gene based on maternal identity. A Benjamini–Hochberg false discovery rate (FDR) correction was used to correct for multiple comparisons.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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