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The maternal effects of dietary restriction on Dnmt expression and reproduction in two clones of *Daphnia pulex*

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The inheritance of epigenetic marks induced by environmental variation in a previous generation is broadly accepted as a mediator of phenotypic plasticity. Transgenerational effects linking maternal experiences to changes in morphology, gene expression, and life history of successive generations are known across many taxa. While the number of studies linking epigenetic variation to ecological maternal effects is increasing rapidly, few if any attempts have been made to investigate molecular mechanisms governing epigenetic functions in the context of ecologically relevant maternal effects. *Daphnia* make an ideal model for investigating molecular epigenetic mechanisms and ecological maternal effects because they will reproduce asexually in the lab. *Daphnia* are also known to have strong maternal effects, involving a variety of traits and environmental variables. Using two clones of *Daphnia pulex*, we investigated the plasticity of life history and DNA methyltransferase (Dnmt) gene expression with respect to food limitation within and across generations. We found strong evidence of genotypic variation of responses of life history and Dnmt expression to low food diets, both within and across generations. In general, effects of offspring diet were larger than either the direct maternal effect or offspring-maternal environment interactions, but the direction of the maternal effect was usually in the opposite direction of the within-generation effect. For both life history and Dnmt expression, we also found that when offspring had low food, effects of the maternal environment were stronger than when offspring had high food.

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INTRODUCTION

A century ago, researchers working on the pulmonate snail, *Limnaea peregra*, observed that the coil pattern of offspring shells was identical to that of the mother regardless of the offspring genotype (Boycott and Diver 1923; Sturtevant 1923). This was early evidence that mothers affect embryonic and post-embryonic development, a phenomenon scientists have documented extensively since (Mousseau and Fox 1998; Wolf and Wade 2009). Subsequent investigations confirm that maternal effects influence cellular organization and determination of the body axis in early development (Nusslein-Volhard et al. 1987; Bernardo 1996), and are vital for the initiation and maturation of organ systems in later development (Ho and Burggren 2010; Champagne 2012). In animals, mothers can alter the phenotypes of their offspring by modifying egg components and composition (Mousseau and Fox 1998; Groothuis and Schwabl 2008; Ho 2008; Dzialowski et al. 2009), through the gestational environment (Chan et al. 2009; Mastorci et al. 2009), or postnatally via maternal resources and behavior (Cameron et al. 2008).

Interest in how information about the maternal environment can be transmitted across generations and ultimately alter offspring phenotypes has led researchers to investigate a collection of mechanisms rooted in epigenetics. Broadly defined, epigenetics includes any heritable changes in gene expression and function that cannot be explained by changes in DNA sequences (Richards 2006; Bird 2007; Trerotola et al. 2015). Altering gene expression and function can be achieved through reasonably well-defined molecular processes that activate, reduce, or shut off the activity of specific genes. Evidence continues to mount that transmission of epigenetic modifications can be stable across generations, including those induced via environmental cues (Chong and Whitelaw 2004; Richards 2006; Trerotola et al. 2015; Herman and Sultan 2016).

Most epigenetic studies focus on three mechanisms. DNA methylation and histone modification remodel chromatin, while the other is a small-RNA-mediated regulatory process (Berger 2007; Gibney and Nolan 2010). Maternal diet can modify chromatin structure, change histone modification patterns, and increase recruitment of transcription factors during embryonic development (Aagaard-Tillery et al. 2008) and alter epigenome regulation by modulating histone deacetylases (Suter et al. 2012). Regardless of the mechanism studied, genetic recombination presents a potential confounding effect that complicates studies of maternal environmental effects. Study systems that involve asexual reproduction remove that complication.

Current literature linking molecular mechanisms of epigenetics with ecology are limited. Lamka et al. (2022) found that out of 206 studies connecting ecology to epigenetics, chordates and angiosperms accounted for 38 and 41% of all species studied with a heavy bias toward model systems. Most areas of investigation were limited to within-generation studies and responses to environmental changes (Lamka et al. 2022). Few studies assayed transgenerational effects, whether paternal or maternal in origin.

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Nearly all the studies incorporated differential methylation, but none addressed the molecular mechanisms governing methylation. Here, we aim to investigate molecular epigenetic mechanisms themselves in the context of ecologically relevant maternal effects using the model system *Daphnia*.

Daphnia are ecologically important freshwater microcrustaceans that have been well-studied in the context of ecology, evolution, and genomics (Colbourne et al. 1997; Stollewerk 2010). *Daphnia* are cyclic parthenogens, producing diploid parthenogenetic or haploid sexual eggs in response to environmental cues such as photoperiod, temperature, food abundance, and crowding (Zaffagnini 1987; Kleiven et al. 1992). The ability to respond to environmental cues and the parthenogenetic reproductive cycle make *Daphnia* ideal candidates for studying environmentally induced, transgenerational epigenetic effects because variation within clones is unambiguously due to plasticity (Walsh et al. 2014).

Daphnia have strong maternal effects in which diet and age can alter disease resistance and life-history traits (Jian et al. 2013; Clark et al. 2017; Garbutt and Little 2016; Goos et al. 2018) for multiple generations. Exposure to predator cues can induce morphological defenses in future generations (Agrawal et al. 1999; Walsh et al. 2014, 2015) and likewise alter life-history traits and behavior. In addition, abiotic environmental stressors like salinity (Jeremias et al. 2018), heavy elemental metal contamination (Vandegehuchte et al. 2009a, b), and radiation exposure (Trijau et al. 2018) induce transgenerational epigenetic effects.

Evidence of both epigenetic machinery and the ability to modify and transmit stable epigenetic alterations has been documented in *Daphnia* (Vandegehuchte and Janssen 2011; Vandegehuchte et al., 2009a, b; Kvist et al. 2018; Jeremias et al. 2018; Trijau et al. 2018; Nguyen et al. 2021). *Daphnia* possess three homologs of cytosine-5-methyltransferases (DNA methyltransferases or Dnmts), enzymes that catalyze the addition of methyl groups to cytosines. These enzymes are capable of copying established methyl marks during DNA replication (Dnmt1) or adding new marks in a de novo fashion (Dnmt3). No function for Dnmt2 has yet been established in DNA methylation (Bewick et al. 2017).

Diet-induced maternal effects are well known in *Daphnia* (Goos et al. 2018; Frost et al. 2010; Garbutt and Little 2016; Clark et al. 2017), with potential links to epigenetic alterations including miRNA packaging (Hearn et al. 2018) and changes in the methylation across the genome (Hearn et al. 2019). Older studies in *Daphnia* have shown that vitamin B_{12} is linked to reproductive performance (Keating 1985), while more recent work has shown that increased B_{12} leads to global hypermethylation of the *Daphnia* genome as well as increasing juvenile-specific growth rate (Kusari et al. 2017). It is not currently known how the expression of any of the three Dnmts responds to environmental cues and whether their expression is subject to maternal influences. Changes in Dnmt expression do not always equate to altered global methylation levels. Dorts et al. (2016) observed significant increases in zebrafish Dnmt3 expression in response to stress but no changes in methylation percentage. Dnmts use methyl groups supplied by the one-carbon metabolic pathway (Anderson et al. 2012) that generates the methyl-donor S-adenosylmethionine (SAM). This pathway is made up by the folate and methionine pathways (Ducker and Rabinowitz 2016), making SAM dependent upon both nutrient and vitamin intake. Reduction in nutritional or vitamin content directly impacts the SAM pathway's ability to produce methyl groups for Dnmt activity independently of Dnmt gene expression.

Our study explores the effects of food quantity on life history and Dnmt expression in *Daphnia pulex*. Specifically, we aimed (1) to determine if expression values of the de novo methyltransferase (Dnmt3), and subsequent maintenance transferase (Dnmt1), would respond to a reduction in food quantity within and across generations and (2) to identify genotypic differences in life history within and across generations potentially linked with changes in gene expression. To accomplish these goals, we manipulated food levels over two generations to test for dietary maternal effects on reproduction and Dnmt expression in two clones of *D. pulex*. We also included Dnmt2 since little is known about it in any organism. We measured life-history traits for both generations including age at maturation, time between clutch release, reproduction type, and clutch size followed by gene expression analysis. Our hypotheses are (1) that dietary restriction would negatively affect life history in the maternal generation, G_0, and increase Dnmt gene expression, and (2) that offspring, G_1, from low-food mothers would perform better than offspring from high-food mothers when challenged with dietary restriction and Dnmt gene expression would be lower in offspring from low food mothers.

MATERIALS AND METHODS

The experiment used two *Daphnia pulex* clones, Morg-5 and Tro-3, to establish experimental populations. Unlike most clones of *D. pulex*, Morg-5 and Tro-3 are obligate parthenogens. Thus, like typical *Daphnia*, they produce broods of parthenogenetic subitaneous eggs that begin development once deposited into the brood chamber, but unlike typical *Daphnia* they also produce resting eggs parthenogenetically. Resting eggs are enclosed in a modified carapace known as an ephippium (Hebert 1981; Innes et al. 2000).

Ephippia of each clone were removed from storage at −80 °C and placed in tissue culture plates containing a small amount of filtered lakewater. To mimic natural conditions for hatching, plates were stored for 1 week in darkness at 4 °C, then moved to a 20 °C incubator at a high light intensity on a 12:12 (light:dark) photoperiod. Seven neonates from Tro-3 hatched after 8 days five neonates hatched from Morg-5 after 13 days. Individuals were transferred into 150 ml beakers with 100 ml of filtered lakewater and kept under standard conditions detailed below. Once all individuals reached reproductive maturity, one exephippial individual per clone was randomly selected to be the progenitor for the experimental setup.

Standard culture conditions and pre-experiment acclimation

All animals were maintained in filtered lakewater (to 1 µm) from Lake Murray in Lexington County, SC, USA. Each individual was placed into a 150 ml Pyrex beaker with 100 ml of filtered lakewater and kept in climate-controlled chambers under a 12:12 (light:dark) photoperiod at 20 °C. Surface entrapment was prevented through dusting cetyl alcohol on the water surface (Desmarais 1997). Animals were fed a quantitative diet of 20,000 cells/ml of vitamin-fortified *Ankistrodesmus falcatus* (Goulden and Horning 1980) daily to allow ad libitum filter feeding. Individuals were transferred every other day into a new beaker of fresh lakewater. Three acclimation generations of 25 individuals were passed before producing experimental individuals. All experimental animals were taken from the third clutch of the third acclimation generation within 8–12 h of release from the brood chamber. Overall, our aim was to minimize variation due to non-experimental maternal effects.

Maternal effects assay

The experimental maternal (G_0) and offspring (G_1) generations for both clones were established by randomly assigning an equal number of neonates from an individual to either a high or low-food environment. High food constituted a daily diet of 20,000 cells/ml of *A. falcatus*, low food was 5000 cells/ml. Animals were transferred every other day into new beakers with 100 ml of fresh filtered lakewater and checked every 8–12 h for maturation starting on the fifth day of the respective assay. Individuals were considered mature when eggs were deposited into the brood pouch. Day and number of neonates released were recorded for all individuals in both generations as well as the type of reproductive output (parthenogenic egg, resting egg, abortive event/no data). Animals were kept individually in beakers for the duration of the experiment. The experimental design and total number of animals used in each generation can be seen in Fig. 1.

Gene expression

Gene-specific primers for qPCR were designed from the genome sequence of *D. pulex* (Colbourne et al. 2011). Primer sequences and validation details are given in the Supplementary Information. *Daphnia* from each treatment were collected after releasing their third clutch and stored in RNAlater (Invitrogen) for 24 h at 4 °C. Eggs were removed from the brood chamber

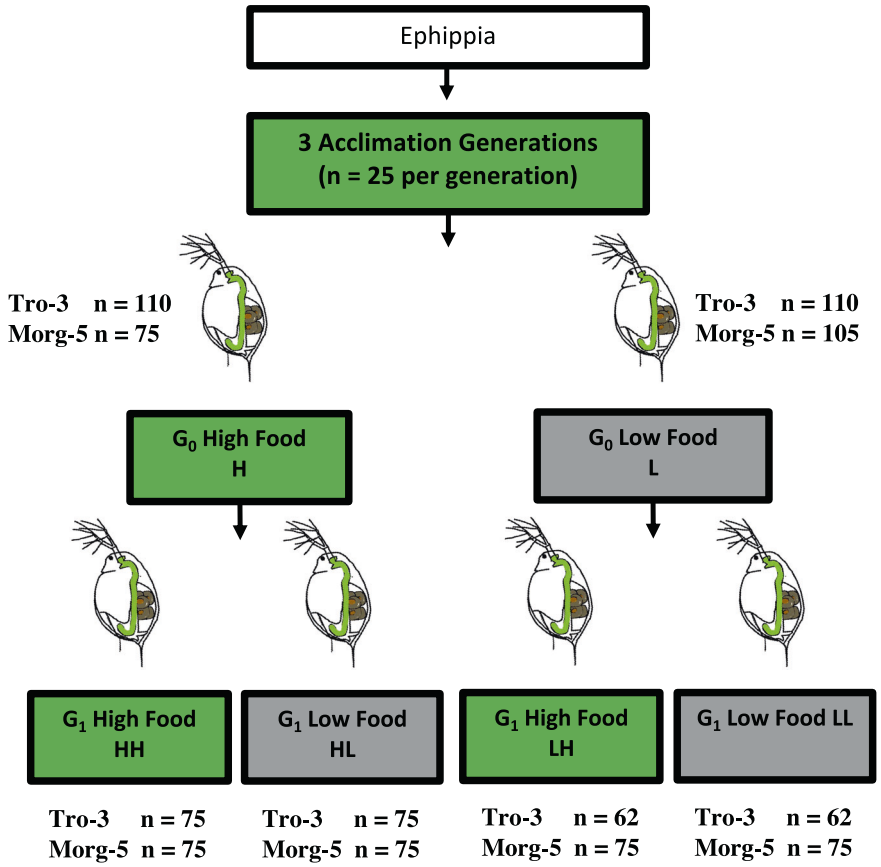

Fig. 1 Experimental design setup. Three acclimating generations under constant conditions were used following hatching Tro-3 and Morg-5 from ephippia. G_0 represents the maternal generation for the main study and G_1 the offspring produced. Animals under high food (H) were fed 20,000 cells/ml of *Ankistrodesmus falcatus* daily, low food (L) were fed 5000 cells/ml. For G_1, the first letter denotes the maternal food level, the second the offspring food level. Numbers, *n*, of animals used for each clone in each treatment are listed.

of each animal. For total RNA extractions, we pooled cohorts of 10 *Daphnia* per treatment into a single biological replicate. Each treatment had a total of five biological replicates yielding a total of 60 samples (2 clones * 6 treatments * 5 biological replicates = 60 samples). All samples were stored at −80 °C until extraction. Total RNA was extracted from homogenized tissue using a trizol/purezol-chloroform method (Purezol, Bio-Rad, CA). Purity and concentration were assessed via Nanodrop 2000 and Qubit, respectively. RNA subunit integrity was confirmed through gel electrophoresis. Each replicate was treated for genomic DNA contamination and converted into first-strand cDNA with gDNAse Iscript (Bio-Rad, CA) using random primers according to the manufacturer's protocol. qPCRs were run with three technical replicates using PowerUp SYBR Green Master Mix (Applied Biosystems) in a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad, CA). Thermal cycling conditions were: 2 min at 50 °C and 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Dissociation-curve analysis and gel electrophoresis confirmed correct amplicon size and primer specificity. No-template (NT) and no-reverse-transcriptase (NRT) controls were used to confirm no contamination was present. Relative Dnmt mRNA levels were normalized to β-actin transcript level (Dudycha et al. 2012) using the Pfaffl method, with an efficiency correction calculated from Real-time PCR Miner (Zhao and Fernald 2005).

Statistical analysis

Statistical analyses were conducted using R, version 3.6.2 (R Core Team 2021) and plots were made using ggplot2 (Wickham 2016). All models were fit accordingly using the formulas $y \sim G_0$ or $y \sim G_0 * G_1$ depending on the generation being analyzed. Time to maturation and clutch interval are both considered "time to event" data and were analyzed using Cox proportional hazards regressions with an efron method to handle tied event times more accurately. Clutch-size data were log-transformed to meet the assumptions of normality. Maternal and resource effects on clutch size were analyzed using a type-III ANOVA; effect sizes reported are η^2. Maternal and resource effects on reproductive output type were analyzed using a generalized linear regression following a binomial distribution. Gene expression data were analyzed using a pairwise *t*-test (G_0) or one-way analysis of variance (G_1). A post hoc analysis (Tukey's honest significant difference) was used to test particular differences between treatments in G_1.

RESULTS

Time to maturation and day of clutch release

Restricted food levels significantly delayed time to maturation in the maternal generation for both clones (Table 1 and Fig. 2). The effect size of low food was greater in the Tro-3 clone as indicated by the hazard ratio, HR (Table 1). HR values less than 1 indicate a delay in maturation time, equal to 1 show no difference in maturation, and greater than 1 indicate earlier maturation. G_0 plots denote food level treatments using either an H, high food, or L, low food. Animals in G_1 denote maternal and offspring diet using two letters. The first letter indicates maternal diet and the second offspring; HH, high maternal food and high offspring, HL, high maternal and low offspring, LH, low maternal and high offspring, LL, low maternal and low offspring. The current generation's food level is indicated in Fig. 1.

Effects in the offspring generation differed markedly between the two clones. In Tro-3, the G_1 maturation time was jointly influenced by G_1 food level, resulting in a HR of 0.34, indicating a delay in maturation when G_1 had low food (Table 1 and Fig. 2). Maternal low-food diets and the interaction between G_0 and G_1 food levels significantly reduced the negative effects of offspring low-food diets (Table 1). Maturation was delayed when offspring food level was low only when the maternal food level was high. In

Table 1. The effects of diet on time to maturation.

Clone	Generation	Variable	χ^2	HR	95% CI	*p* value
Tro-3	G_0	Diet	59.92	0.29	0.21–0.40	**0.0001**
	G_1	Offspring diet	30.25	0.34	0.24–0.50	**0.0001**
		Maternal diet	15.86	1.19	0.84–1.68	**0.0001**
		Interaction	6.86	1.93	1.18–3.17	**0.008**
Morg-5	G_0	Diet	9.89	0.60	0.44–0.82	**0.0001**
	G_1	Offspring diet	49.92	0.43	0.31–0.60	**0.0001**
		Maternal diet	3.89	1.43	1.03–1.98	**0.04**
		Interaction	0.32	0.88	0.55–1.38	0.57

Cox Proportional Hazard Regression using an Efron approximation.
HR hazard ratio.
Bold values indicate a significant *p*-value.

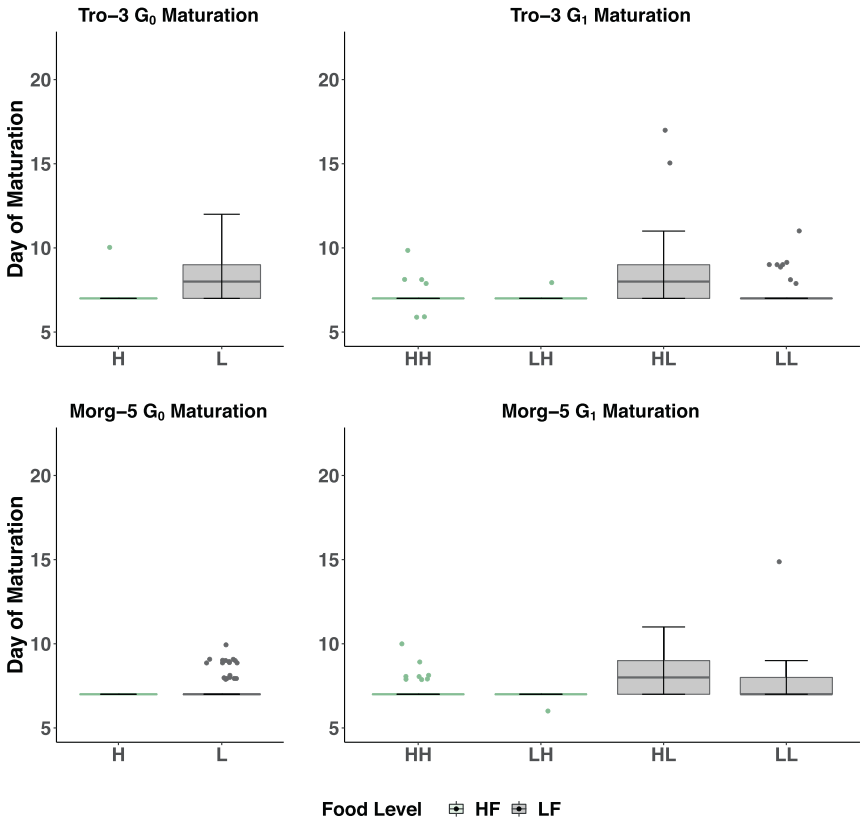

Fig. 2 Box plots of the effects of diet and maternal diet on the age at maturation in the clones Tro-3 and Morg-5, for both generations. Green boxes denote a high-food diet, gray boxes low food in the generation being observed. Data were collected within an 8–12 h window starting on day 5. For G_1, the first letter denotes the maternal food level, the second the offspring food level. Color indicates the current generation's food level.

contrast, in Morg-5 there was no interaction between generations, although time to maturation in G_1 was significantly influenced by the main effects of both offspring food level and maternal food level.

In the maternal generation, the day of neonate release for all three clutches was significantly delayed in Tro-3 when food availability was low. The same was true in Morg-5 for only clutches 2 and 3 (Supplementary Table 1 and Supplementary Figs. 1 and 2). The same trend was observed in G_1 and is largely explained by offspring food level, with low food resulting in significant delays. However, offspring from low-food mothers reproduce earlier compared to offspring from high-food mothers. The interaction between G_0 and G_1 diet was significant for all three clutches in Morg-5 but only clutch 1 in Tro-3 (Supplementary Table 1). In addition to effects on means, we also observed effects of maternal diet on variability of reproductive timing in Tro-3 (Supplementary Fig. 1), with offspring from low-food mothers releasing clutches in a relatively narrow 8–12 h window.

Clutch size

Low food significantly reduced clutch size for the first three clutches of G_0 both Tro-3 and Morg-5 (Supplementary Table 2 and Supplementary Figs. 3 and 4). There was no significant interaction between maternal and offspring diet on clutch size for either clone in the first three clutches, and in most cases, there was no main effect of G_0 resource level on G_1 clutch sizes. The one

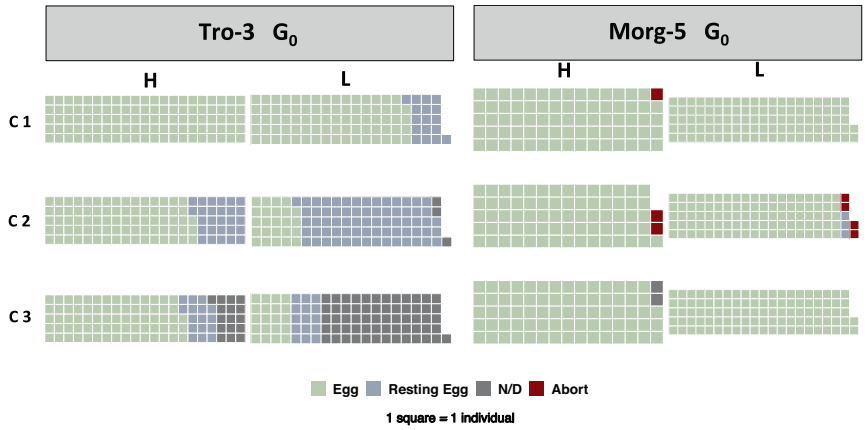

Fig. 3 Waffle plot representation of diet effect on reproduction type in G_0 where each square represents one individual. Green squares represent subitaneous egg production, purple squares denote resting egg production, gray squares no reproduction, and red represent individual who produced a clutch of subitaneous eggs but resulted in an abortive event.

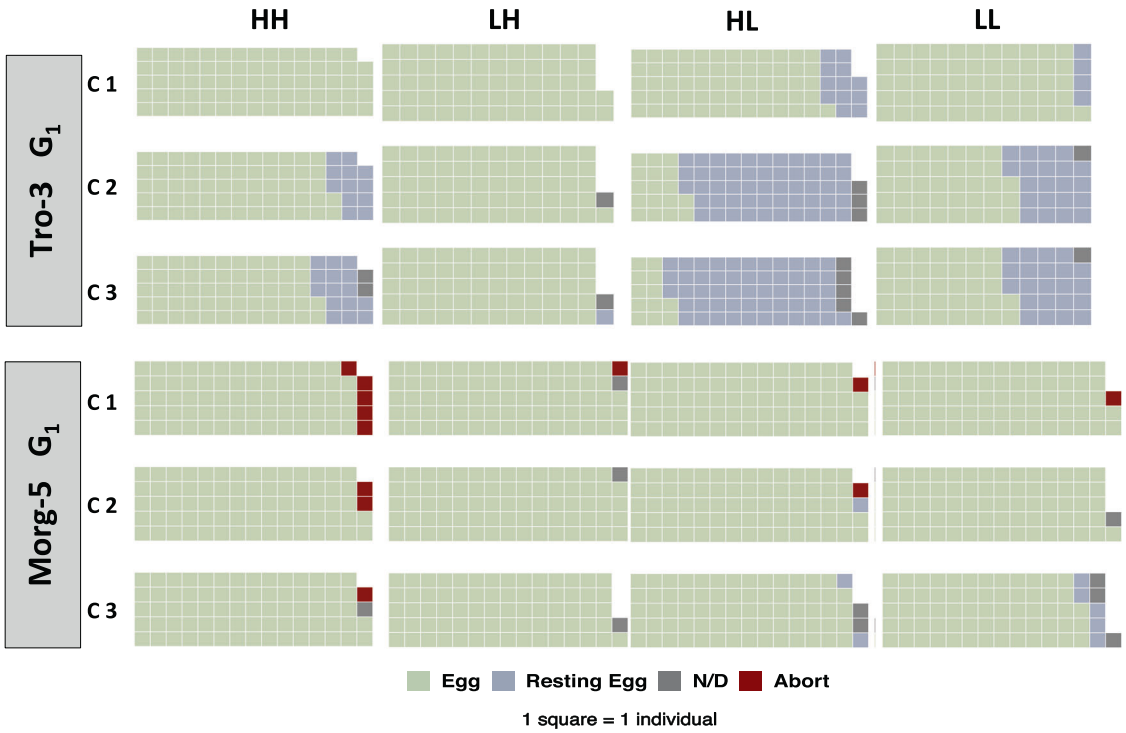

Fig. 4 Waffle plot representation of the effect of diet on reproduction type in G_1. Each square represents one individual. Green squares represent subitaneous egg production, purple squares denote resting egg production, gray squares no reproduction, and red represent individual who produced a clutch of parthenogenic eggs but resulted in an abortive event. For G_1, the first letter denotes the maternal food level, the second the offspring food level. Clutch number is indicated by C and the following number. Plots are grouped by offspring food level.

exception was in clutch 2 of Tro-3, which were larger when their mothers had low food (Supplementary Table 2 and Supplementary Figs. 3 and 4). Clutch size in G_1 was significantly reduced by low food in both clones.

Reproductive output type

Diet did not affect the type of reproduction in the maternal generation of Morg-5 (Supplementary Table 3 and Fig. 3), but resting egg production in the 2nd and 3rd clutches of Tro-3 was significantly increased by low food (Supplementary Table 4 and Fig. 3). Lack of variation in the high-food treatment of Tro-3, where no replicates produced resting eggs, rendered the statistical error terms unintelligible. However, low food did result in the production of resting eggs, suggesting there is a diet effect for clutch 1.

There was no significant interaction between G_0 and G_1 diet on the type of reproductive output (egg, resting egg, or no output) in G_1 for either Tro-3 or Morg-5. There was no main effect of diet, from either G_0 or G_1, on reproductive output type in G_1 for the first three clutches of the Morg-5 clone (Supplementary Table 5 and Fig. 4).

Unlike Morg-5, Tro-3 showed substantial treatment effects on reproductive type. Tro-3 G_1 from high-food mothers, HH and HL showed the same effect as G_0: low food resulted in increased

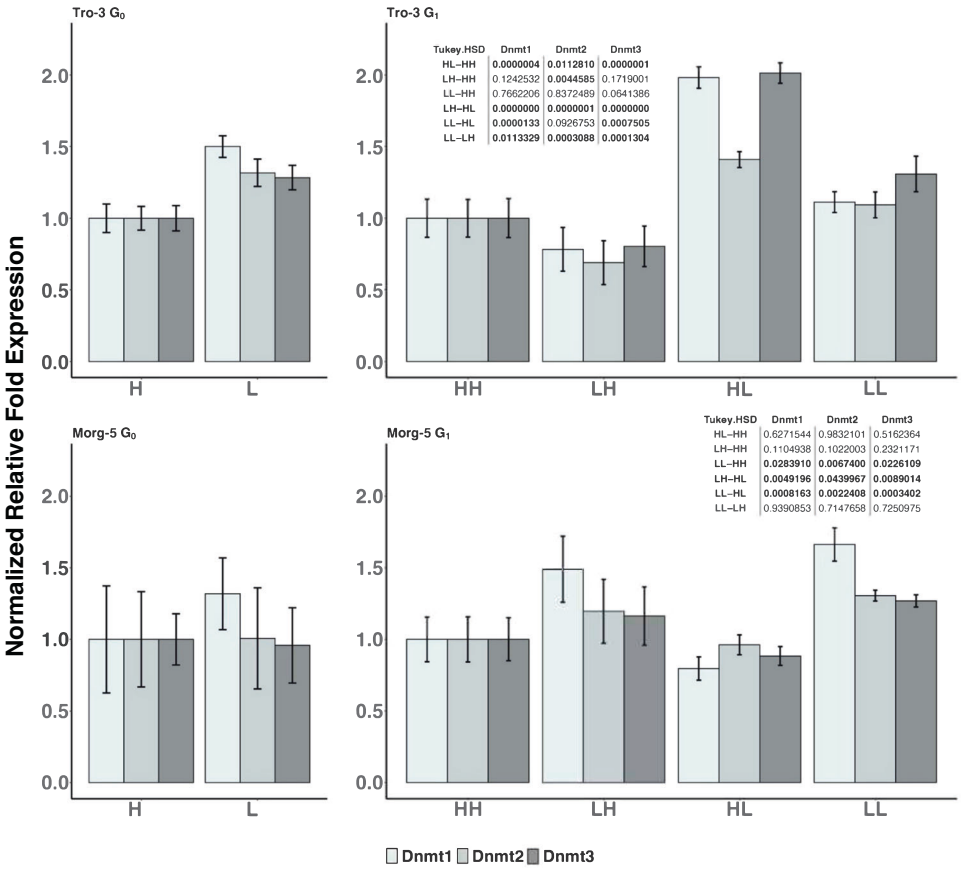

Fig. 5 Normalized relative fold expression and standard error for DNA methyltransferases 1, 2, and 3 in the *D. pulex clones* Tro-3 and Morg-5. For G_1, the first letter denotes the maternal food level, the second the offspring food level; post hoc Tukey HSD results are displayed for both clones in the G_1 generation.

resting egg production. In contrast, resting egg production was significantly reduced in the 2nd and 3rd clutches of G_1 if the G_0 diet was low food (Supplementary Table 6). Low-food G_1 diets reduced the log-odds of producing subitaneous eggs by 2.995 and 2.836 for clutches 2 and 3, respectively, while low-food G_0 diets increased the log-odds of producing subitaneous eggs in G_1 by 2.469 and 2.191. The Tro-3 G_1 clutch 1 statistical model suffers from the same lack of variation in which replicates did not produce resting eggs within a treatment.

Gene expression

Dnmt expression was scaled to high-food treatments (H in G_0 or HH in G_1), rendering the expression value of 1. Expression of all three Dnmt genes trended in the same direction for both clones, with low food resulting in increased expression. Maternal gene expression in Tro-3 was significantly upregulated in low food for Dnmt1 ($t_{14} = 3.49$, $p = 0.0036$), Dnmt2 ($t_{14} = 2.57$, $p = 0.022$), and Dnmt3 ($t_{14} = 2.45$, $p = 0.028$). No significant differences of maternal gene expression were observed in Morg-5 (Fig. 5). Comparatively, there was greater variability in expression for all three Dnmt genes in G_0 of Morg-5 than of Tro-3.

Maternal food level affected Dnmt expression in opposite directions in offspring of the two clones. In Morg-5, significant increases in offspring gene expression were observed for all three DNA methyltransferases when maternal food was low (Dnmt1: $F_{3,\ 56} = 7.371$, $p = 0.0003$; Dnmt2: $F_{3,\ 56} = 6.613$, $p = 0.000655$; Dnmt3 $F_{3,\ 56} = 7.532$, $p = 0.000255$). The effect was most notable for Dnmt1. In contrast, in Tro-3 offspring in the HL treatment had higher Dnmt expression than the other environments (Dnmt1: $F_{3,\ 56} = 25.82$, $p = 1.28 \times 10^{-10}$; Dnmt2: $F_{3,\ 56} = 15.65$, $p = 1.64 \times 10^{-7}$; Dnmt3: $F_{3,\ 56} = 28.23$, $p = 2.98 \times 10^{-11}$) (Fig. 5). In addition, in G_1 of Tro-3 expression of Dnmts in LH was reduced relative to HH (post hoc Tukey tests shown in Fig. 5). There was also a marginally significant elevation of Dnmt3 in LL relative to HH ($p < 0.064$; Fig. 5).

DISCUSSION

We found evidence for effects of maternal food availability on offspring life history and Dnmt expression. While such effects are reasonably well known for life history, to our knowledge we present the first data quantifying plasticity of Dnmt expression in response to food availability within and across generations. Significant differences in Dnmt gene expression were observed for all three genes in both clones within and across generations, although the details differed among genes and between clones. One key pattern we found was that when offspring experience poor resource environments, effects of the maternal environment are generally stronger for both life history and Dnmt expression. A second key pattern was that while effect sizes (η^2, β/log-odds) of the offspring diet were larger than either maternal or interaction term effect sizes, the direction of the maternal effect was almost always in the opposite direction. Furthermore, our data provide strong evidence of genotypic variation of responses to low-food diets—both within and across generations—for both life history and Dnmt expression.

Ecological research on maternal effects has a long history at the phenotypic scale, but is largely segregated from developments in molecular biology that may shed light on molecular mechanisms that enable maternal effects. We sought to bring these fields closer together. However, our goal for this study was not to use Dnmt expression to directly explain the specific life-history effects

we observed; it is not possible to predict specific mechanistic causes in the absence of detailed knowledge of the genetic architecture of these complex traits. Instead, we used life-history traits to illustrate broad patterns and genetic variation in maternal effects, and asked whether similar patterns and types of variation are seen in a crucial component of epigenetic regulation. In addition, our data on Dnmt expression shows that broad mechanisms with the potential to modulate maternal effects are themselves subject to maternal effects.

Diet effects on life-history traits

Maternal effects of diet on life history have been studied for multiple species of *Daphnia* (e.g., Glazier 1992; Gliwicz and Guisande 1992; Boersma 1995, 1997; Urabe and Sterner 2001; Pieters and Liess 2006; Hearn et al. 2018; Gillis and Walsh 2019), and our results confirm general expectations. Reduction in food quantity and quality results in delayed maturation, decreased clutch size, increased time between clutches and potentially the production of resting eggs. Both clones responded to food level but overall, maternal life history was more sensitive to diet in Tro-3 than in Morg-5. For example, maturation was delayed in both clones, but the effect was almost double in size for Tro-3. Both clones also saw a significant reduction in clutch size for all three clutches, but again diet had a larger effect on Tro-3.

We observed a significant shift from subitaneous to resting egg production in clutch 2 in response to low-food diets in Tro-3. The majority of Tro-3 G_0 clutch 3 then shifted to not reproducing at all, further illustrating the strength of diet-induced effects in this clone. Morg-5 did not shift its type of reproductive output, indicating a clear genotypic difference between the two *D. pulex* clones and how they respond to an environmental challenge. Maternal and grandmaternal food environments are well known to influence the production of resting eggs (Alekseev and Lampert 2001; LaMontagne and McCauley 2001); however, all G_0 animals we used were taken from the third acclimation generation of animals that were grown under constant high-food conditions. Given this, we believe that the differences observed in the G_0 are due to treatment rather than a maternal artifact from the acclimation generations.

Studies investigating diet-induced effects across generations show that maternal effects are more prominent when the offspring food environment is poor and that the strength of maternal effects is more pronounced in early developmental stages (Brett 1993; Hearn et al. 2018). *Daphnia* eggs from low-resource mothers are typically greater in volume and dry mass with larger reserves of carbon, nitrogen, protein, and lipids (Boersma 1997; Guisande and Gliwicz 1992). While we did not investigate the composition of eggs produced, our results from Tro-3 offspring show a clear maternal effect. Low-food offspring from low-food mothers (LL) have life-history traits that resembles that of their high-food siblings. Time to maturation, time between clutch release, and reproductive output type all resemble that of maternal individuals receiving a high-food diet, suggesting that mothers of this clone are able to inoculate their offspring against the challenges of a low-food environment.

Offspring food level dictated the direction of the effects on life-history traits for both clones studied. However, despite often having smaller effect sizes, the maternal effects observed in Tro-3 G_1 were always opposite in direction to the within-generation effect. In the case of reproductive output type, the effect size of offspring food level on offspring output type increased over time to the point of almost equaling the effect size of maternal diet, but it was in the opposite direction. These findings may suggest the presence of an adaptative maternal effect in Tro-3.

Diet effects on Dnmt expression

We proposed two hypotheses for Dnmt expression in response to diet and generation: (1) Dnmt expression would be higher in a low-food environment than in a high-food environment, and (2) offspring from mothers grown in low-food environments would have lower Dnmt expression than their counterparts from mothers grown in high-food environments. We observed significant increases in gene expression for all three Dnmts in low food compared to high food in the maternal generation of Tro-3 but not in Morg-5 (Fig. 5), partially confirming our first hypothesis. There was greater variance in expression between biological replicates in Morg-5 for each methyltransferase, so it is possible that this clone is simply less tightly regulated. Other investigations of epigenetics have also found conflicting results among genotypes. Hearn et al. (2019) found significant genome-wide methylation differentiation in response to dietary restriction for one clone of *D. magna*, yet a second study using *D. magna* from the same location found no effect (Hearn et al. 2021).

Data from Tro-3 supported our second hypothesis, that offspring from mothers reared in low food environments would have lower Dnmt expression. Tro-3 offspring from low-food mothers, both LH and LL, had a significantly lower expression for all three methyltransferase genes compared to diet-restricted offspring from high-food mothers, HL, but not HH. LH mean Dnmt gene expression values were lower than the reference, HH, and significantly different from LL, with Dnmt3 expression with a 50% greater relative fold difference in LL.

In contrast, data from Morg-5 seems to refute our second hypothesis. In this clone, offspring from low-food mothers had *higher* Dnmt expression than offspring from high-food mothers, although the differences were statistically significant only when offspring experienced low food.

Past work has shown that within-generation environmental stimuli alter DNA methylation across the genome or at select genes, including in *Daphnia* (Vandegehuchte and Janssen 2011, 2014; Head 2014). Data from Kusari et al. (2017) highlighted the importance of diet on the one-carbon metabolic pathway (OMC) in *Daphnia* (OMC is responsible for supplying methyl groups used for DNA methylation). Decreased diet quality resulted in hypomethylation across the genome and reduced fitness, suggesting that epigenetic modifications are directly influenced by environmental changes.

The do novo methyltransferase, Dnmt3 has already been shown to respond to dietary restriction treatments in *Daphnia* and has been linked to a tradeoff between reproduction and growth (Nguyen et al. 2020, 2021). In our study, we observed increased expression of Dnmt3 in response to low food in both Tro-3 generations, except for the offspring from low-food mothers, LH and LL. The change in expression may play a vital role in the life-history changes overserved. LH animals produced, on average, more offspring in clutches 2 and 3 than HH and reduced the variation in day of clutch release. LL animals produced significantly fewer resting eggs compared to their HL counterparts and the day of neonate release was comparable to that of the HH control.

Daphnia change offspring phenotype and life history in response to dietary shifts with clear genotypic variation (Glazier 1992; Mkee and Ebert 1996; Gabsi et al. 2014; Tessier and Consolatti 1989, 1991; Gliwicz and Guisande 1992; Burns 1995; LaMontagne and McCauley 2001; Li and Jiang 2014). Differences in how species achieve the alteration are becoming clearer, e.g., by modulating gene expression (Hearn et al. 2018), embryo provisioning (Gabsi et al. 2014, references within), and DNA methylation (Asselman et al. 2015; Hearn et al. 2019, 2021) but mechanisms behind the changes remain elusive. Our study helps close the gap between the phenotypic changes and the molecular mechanisms by showing changes in gene expression of crucial parts of epigenetic machinery responsible for maintaining genome stability and responding to the environment. It adds to the complexity, however, by showing that the operation of epigenetic mechanisms differs between genotypes. Future studies

investigating maternal effects in *Daphnia* should include multiple genotypes and also factor in changes in methylation across the genome as proximate mechanisms for understanding how maternal experiences shape offspring life history.

DATA AVAILABILITY

Data can be accessed at Dryad: https://doi.org/10.5061/dryad.1c59zw3zz.

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AUTHOR CONTRIBUTIONS

TCA and JLD conceived the ideas and designed the experiments and led the writing of the manuscript. TCA and JA collected the data. TCA analyzed the data. All authors contributed critically to the drafts and gave final approval for publication.

COMPETING INTERESTS

The authors declare no competing interests.

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