Embryogenesis plasticity and the transmission of maternal effects in *Daphnia pulex*

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Abstract
Understanding how genetic, nongenetic, and environmental cues are integrated during development may be critical in understanding if, and how, organisms will respond to rapid environmental change. Normally, only post-embryonic studies are possible. But in this study, we developed a real-time, high-throughput confocal microscope assay that allowed us to link *Daphnia* embryogenesis to offspring life history variation at the individual level. Our assay identified eight clear developmental phenotypes linked by seven developmental stages, the duration of which were correlated with the expression of specific offspring life history traits. *Daphnia* embryogenesis varied not only between clones reared in the same environment, but also within a single clone when mothers were of different ages or reared in different food environments. Our results support the hypothesis that *Daphnia* embryogenesis is plastic and can be altered by changes in maternal state or maternal environment. As well as furthering our understanding of the mechanisms underpinning parental effects, our assay may also have an industrial application if it can be used as a rapid ecotoxicological prescreen for testing the effect that pollutant doses have on offspring life histories traditionally assayed with a 21-day *Daphnia* reproduction test.

1 | INTRODUCTION

One of the fundamental assumptions underpinning the Modern Synthesis is that inheritance is “hard,” meaning that the environment cannot directly influence the process (Bonduriansky, 2012). However, other inheritance mechanisms operate alongside Mendelian genetic inheritance that may be environmentally sensitive. Nongenetic inheritance refers to the transmission of any aspect of the parental phenotype or environment that influences the gene expression and development of the offspring (Bonduriansky, 2012; Bonduriansky & Day, 2009; Danchin et al., 2011) and includes mechanisms such as epigenetic inheritance and genomic imprinting, the transmission of substances via the gametes, or from parents to offspring (e.g., hormones, nutrients, parasites, antibodies), or the transmission of behavior or culture through learning (see Bonduriansky & Day, 2009, 2018; Danchin et al., 2011; Jablonka & Lamb, 2005 for reviews). Such transmission may not only reflect the parents’ current environment or state, but also the genetic variation associated with how parents respond to their environment (i.e., indirect genetic effects; Wolf, 2003; Wolf, Brodie, Cheverud, Moore, & Wade, 1998).
The incorporation of nongenetic mechanisms into evolutionary thinking is changing our assumptions about how populations respond to rapid environmental change (Bonduriansky 2012; Hallsson, Chenoweth, & Bonduriansky, 2012), and driving an incentive to understand how the integration of genetic, nongenetic, and environmental cues during development generate persistent phenotypic variation that we observe in offspring and later descendants (Bonduriansky & Day, 2018; Day & Bonduriansky, 2011; Leimar & McNamara, 2015). From an empirical perspective, parthenogenetic organisms are useful for studying the integration of developmental cues, because genetic and nongenetic effects can easily be separated, and large numbers of genetically identical individuals can be reared across different environments and over multiple generations (Harney, Paterson, & Plaistow, 2017; Harris, Bartlett, & Lloyd, 2012; Plaistow, Shirley, Collin, Cornell, & Harney, 2015). As a result, there is already a large body of work in Daphnia demonstrating that parental effects influence the life histories of offspring in various ways including effects on offspring size (Glazier, 1992; Harney et al., 2017), offspring size and age at maturity (Harney et al., 2017), inducible predator defenses (Tollrian, 1995), strain-specific immunity (Little, O’Connor, Colegrave, Watt, & Read, 2003), mode of reproduction (LaMontagne & McCauley, 2001), the development of resistance to heavy metals (Bosuyt & Janssen, 2003) and pesticides (Brausch & Smith, 2009), and the onset and rate of senescence (Plaistow et al., 2015). However, the mechanisms linking parental environment and/or state to offspring phenotypic variation remain unknown. One reason for this is that genetic, nongenetic, and environmental cues are integrated during development, yet a majority of Daphnia studies start after offspring are born as neonates.

After leaving the maternal brood pouch, neonates still have to grow and mature, and genetic, nongenetic, and environmental cues all contribute to this process (Harney et al., 2017). However, a mechanistic understanding of the clone-specific integration of developmental cues (Harney et al., 2017) requires the ability to link changes in parental environments or states to changes in egg development patterns and the effect that this then has on offspring phenotypic variation. To date, studies of Daphnia embryogenesis have focussed on the staging of development (reviewed in Mittmann, Ungerer, Klann, Stollewerk, & Wolff, 2014; Toyota et al., 2016) and ecotoxicity testing (Sobral et al., 2001). The methods that have been developed so far are destructive, preventing the study of individual-level variation and links between embryogenesis and subsequent life history variation. The objectives of this study were, therefore, twofold. First, we aimed to carry out a nondestructive, real-time, in vitro assay of Daphnia egg development to understand how developmental differences translate into subsequent life history trait variation. Second, we tested the hypothesis that maternal food and maternal age effects generate egg developmental plasticity which can explain some of the life history trait variations we see in the offspring.

## 2 METHODS

### 2.1 Experimental animals

*Daphnia pulex* clones used in this experiment (LL14, LL18, and LL32) were isolated from a single population in Wales (53°14′45″N, 4°08′12″W) in 2012 (approx. 200 generations). Since isolation they were maintained as laboratory stock cultures in a controlled temperature incubator at 21 ± 1°C on a 14:10 light:dark cycle in hard artificial pond water (ASTM; OECD, 1998) enriched with standard organic extract (Baird, Barber, Bradley, Calow, & Soares, 1989). The clone lines are frequently bottle-necked (restated from a single individual) to prevent the possibility of mutation-derived genetic variation existing in each clone line.

### 2.2 Experimental design

Three neonates from the stock clone lines were each placed in their own individual jar containing artificial pond water (ASTM; OECD, 1998) enriched with standard organic extract (Baird et al., 1989). They were fed 200,000 cells/ml of batch-cultured *Chlorella vulgaris* (high food) daily and the media was changed 3 days a week (Monday, Wednesday, and Friday). Neonates from the first two clutches were discarded. Once individuals produced their third clutch, three offspring from the total produced were randomly selected and used to set up the next generation. On reaching their second generation, offspring from all three mothers were mixed and randomly allocated to two food treatments. To maximize the chances of catching mothers depositing their third and fifth clutches into the brood pouch, 20 individuals were fed high food daily (200,000 cells/ml), and 30 were fed low food (40,000 cells/ml) for each clone. On reaching their third clutch, eggs at the correct stage (see below) were removed for the development and life history assay. This was repeated for the fifth clutch for mothers not sampled at the third clutch stage (see Figure 1).

### 2.3 Egg development assay

The egg development assay was carried out in a Corning clear flat-bottomed 96-well plate. To prevent
the eggs from moving out of the field of view during the imaging, a Rondelle Spacer Bead (Clear Czech Pressed Glass, 6 × 2 mm; www.beadaholique.com) with a 0.9-mm hole was placed into each well on the plate. For the assay, the eggs were placed in this hole where they were able to develop and swim freely once they had hatched from the vitelline membrane. The plates were prepared before eggs were removed from the mother. First, the beads were placed in the wells, each well was then filled with medium from which the mother had been living, and any air bubbles present were removed using a tungsten wire.

Mothers were checked hourly after releasing their second clutch neonates until the clutch three eggs were visible in the brood pouch. Using preliminary experiments, we determined that eggs were at the correct stage for removal (Stage 1; see Figure 2a) approximately 7 hours after the eggs were first seen in the brood pouch. At this stage, eggs were yellow/green in color, with a visible central fat drop. The cells had also begun to pull away from the membrane leaving a transparent gap between the cells and the membrane of the egg (Figure 2a). Eggs taken any earlier than this stage often did not develop.

Once the eggs had reached Stage 1, mothers were placed on a slide and photographed to get a measure of body size (see Section 2.5 for details). To remove the eggs, the mother was first placed onto a slide and the liquid was removed to restrict movement. Using a tungsten wire, the individual was held in place to further restrict her movement. Using a second wire, the dorsal side of her carapace was carefully torn to create a small hole from which the eggs could be extracted. Once all eggs had been removed, the mother was removed from the slide to prevent any movement causing damage to the eggs and the number of eggs in the clutch was counted. A few drops of ASTM were added to the slide using a Pasteur pipette and a 100-µl pipette was used to individually transfer each egg into the center of a bead placed in each well of a flat-bottomed 96-well plate.

Time-lapse images of each individual’s development were taken on a Zeiss LSM 710 Confocal Microscope at ×10 magnification using a 488-nm laser at a transmission rate of 1.2. All individuals were imaged in nine different z planes to ensure that even if the embryos moved, they remained in focus over the course of development. Images were taken using an automated system, with an interval of 28 min until all individuals in the plate had developed.

**FIGURE 1** Experimental design. For each clone, different food treatments were set up by randomly allocating individuals to a high- or low-food environment. A developmental assay and life history assay were carried out on individuals from the third and fifth clutch, enabling us to compare maternal environment and maternal age effects on the development and life history of each individual [Color figure can be viewed at wileyonlinelibrary.com]
reached developmental phenotype 8 (see Figure 3h). Once the eggs had hatched from the vitelline membrane and were swimming freely around the well, the time lapse was stopped. At this stage, individuals would usually still be within the mother’s brood pouch. They have not yet developed their gut, tail spine, or second antennae and the maternal yolk is still visible (see Figure 2b). The beads were removed but the individuals remained in the wells until they had developed to the neonate stage (see Figure 2c); the stage at which they are normally released from the mother’s brood pouch. During this time, they were kept under standard rearing conditions.

**FIGURE 2** (a) Stage 1 of *Daphnia pulex* egg development. At this stage, eggs are yellow/green in color, have a visible fat drop, and a transparent gap between the membrane and the cells. (b) Stage 5 of *D. pulex* development. Individual has hatched from vitelline membrane. Maternal yolk (My) is still visible, gut is not formed, and tail spine (Ts) is not yet extended. (c) Stage 6 of *D. pulex* development (neonate). Individual has a fully formed gut (G), carapace and the Ts and second antennae (An) are extended. [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 3** Egg development phenotypes. (a) The individual has just hatched from the external chorion (Ec). (b) Clear cephalic invaginations (Cl₁ and Cl₂) can be seen, and symmetry changes from radial to bilateral. (c) Individuals head shape begins to form (H), the secondary antennae form (An), and there is an obvious posterior invagination (Pi). (d) The posterior end briefly protrudes (Pp₁) enlarging the vitelline membrane. (e) Posterior end protrudes further (Pp₂), filling the vitelline membrane, and thoracic segments (Ts) are clearly visible. (f) Eye spots are first seen (Es₁). (g) Eye spots become more prominent (Es₂) and posterior end becomes rounded (Pr) as carapace extends. (h) Individual hatches from vitelline membrane (Vm). [Color figure can be viewed at wileyonlinelibrary.com]
conditions (21 ± 1°C on a 14:10 light:dark photoperiod). All surviving neonates were then used for life history assays.

The time-lapse images collected from each individual egg were processed and analyzed using Zeiss AIM imaging software (Carl Zeiss Canada Ltd.). Time-lapse videos were cropped to ensure that the start point was the same for all individuals (phenotype 1 = time zero; see Figure 3a). The point at which the individual hatched from the vitelline membrane (Figure 3h) was set as the end point. An image of the egg at the start of the time lapse was used to get an accurate measure of egg length. All images for each individual were then combined to create a time-lapse video of the individual's development.

2.4 Embryo phenotyping

We identified eight different developmental phenotypes that describe the development of *D. pulex* using staging information from preliminary experiments and previous papers describing *Daphnia* development using destructive assays (e.g., Mittmann et al., 2014). The preliminary experiments were carried out on many individuals, using five different clones from two different populations, to ensure that the phenotypes observed could be consistently recognized in the different specimens. In addition, the phenotypes had to be identifiable from different planes of view, as some eggs developed in a dorsal/ventral view, while others developed giving a lateral view. The duration of time between each of the eight developmental phenotypes was calculated and used as a seven-trait multivariate phenotype to compare the development of individuals in different treatments (see Figure 3).

2.5 Life history assay

Neonates collected from the egg development assay were kept in isolated jars, containing 150 ml of hard artificial pond water (ASTM; OECD, 1998) enriched with a standard organic extract (Baird et al., 1989) which were replaced three times a week. All individuals were fed on a high food diet (200,000 cells/ml of batch-cultured *C. vulgaris*) daily. Individuals were first photographed as neonates and subsequently every time they molted. Photographs were taken using a Canon EOS 600D digital camera attached to a Leica M60 optical microscope. Body size was measured as the distance from the top of the individual's head, to the base of the tail spine using Image J (Rasband, 1997). Maturation was recorded as the time when eggs were first seen in the brood pouch. For each subsequent clutch of eggs, neonates were counted upon release and five were photographed to calculate the average neonate size for each clutch. This was carried out until each individual reached their third clutch. Each individual, therefore, had a measure of six life history traits: mean neonate size produced in each clutch (mm), pre- and post-maturation growth rate (mm/day), size at maturity (mm), age at maturity (days), and fecundity for their first three clutches laid.

2.6 Statistical analysis

A canonical correlation analysis using Pillai's trace statistic and corresponding *F* tests was used to test for a significant association between multivariate developmental phenotypes (seven developmental stage durations) and multivariate life history phenotypes (six traits). Standardized canonical coefficients were then used to evaluate the relative importance of variables in the model and interpret the relationship between developmental traits and life history traits. Clonal variation in development and life history was then tested for using a permutational multivariate analysis of variance (perMANOVA) with the seven durations of the developmental stages and the six life history traits as response variables and clone as a fixed factor with three levels (LL32, LL14, LL18). For the maternal effects experiment, the effect of maternal environment and maternal age on development and life history was tested using a perMANOVA with the same variables as the test for clonal differences but with maternal age fitted as a fixed factor with two levels (third clutch, fifth clutch) and maternal food fitted as a fixed factor with two levels (high food, low food). Differences in treatments and clones were visualized using the ordiplot function in the R vegan package to plot the centroids for each treatment group surrounded by 95% confidence interval ellipses. All analyses were carried out in R, version 3.2.0, using the CCorA, prcomp, and adonis functions from the R vegan package (Oksanen et al., 2018).

3 RESULTS

3.1 Staging of *D. pulex* embryonic development

The first clearly identifiable phenotype in the live assay was hatching from the external chorion (Figure 3a), equivalent to Stage 3A in Gulbrandsen and Johnsen (1990). This was used as a standardized time zero for each assay. Our second phenotype was characterized as the first point at which cephalic invaginations could be
seen, and the symmetry changed from radial to bilateral symmetry (Figure 3b). Phenotype 3 involved the formation of the secondary antennae and a posterior invagination. The head of the individual was also clear at this stage (Figure 3c). The posterior end then expanded for a short time, extending the vitelline membrane (Figure 3d). Phenotype 5 was characterized by the growth of the individual into the vitelline membrane, with an obvious protrusion of the posterior end and clear thoracic segments (Figure 3e). Phenotype 6 marks the formation of the eye (Figure 3f). Phenotype 7 involved the rounding of the posterior end and the eye becoming more prominent (Figure 3g). Phenotype 8 (Figure 3h) occurs when individuals hatch from the vitelline membrane and marks the end of the time lapse because at this point individuals were able to swim freely and moved out of the imaging field of view.

### 3.2 | Linking egg development phenotypes to life history traits

Missed developmental windows, mortality, and restricted access to the confocal microscope meant we were only able to collect full developmental and life history data from 10 LL14 clone individuals, 23 clone LL18 individuals, and 76 clone LL32 individuals. Consequently, we could only conduct a full analysis of maternal food and age effects for clone LL32 (see below). The pooled data set for all clones revealed a significant association between multivariate developmental phenotypes and multivariate life history phenotypes (Pillai’s trace statistic = 0.825; df1 = 49; df2 = 700; approx. F = 1.908; p ≤ .001) but only the first two of the seven canonical dimensions was statistically significant (see Table 1). Canonical loadings for the first two dimensions across both developmental and life history traits are displayed in Figure 4. Dimension 1 had a canonical correlation of 0.56 between developmental and life history traits. Individuals with longer developmental stage 5 and 6 durations have shorter Stage 7 durations which is correlated with reduced pre-reproductive growth rates but higher post-reproductive growth rates and later maturation (see Figure 4). The second dimension had a canonical correlation of 0.49. Individuals that hatched from larger eggs had longer developmental stage 8, 4, and 3 durations but shorter developmental stage 2 and 5 durations and matured later and larger and produced larger offspring (see Figure 4). Figure 4d shows a phenotypic correlation matrix detailing the strength of correlations between all traits measured.

### 3.3 | Clonal variation in offspring development and life history phenotypes

Multivariate developmental and life history phenotypes were clonally variable (F2,107 = 3.4726; p = .008; Figure 5). The differences between clones observed in PC1 resemble the second dimension of the canonical correlation analysis involving developmental stages 5 and 3 and their association with egg size and age at maturity. The differences observed in PC2 resembled the first dimension of the canonical correlation involving a relationship between developmental stages 5 and 6 and the effect they have on rates of pre-maturation growth. Differences between clones were still apparent when only the developmental traits were included in the analysis (F2,107 = 3.4732; p = .01; see Figure S1), confirming that different clones have different patterns of egg development despite being in the same environment.

### 3.4 | Maternal food environment and maternal age effects on offspring

The data set for clone LL32 individuals only also revealed a significant association between multivariate developmental phenotypes and multivariate life history phenotypes. The pooled data set for all clones revealed a significant association between multivariate developmental phenotypes and multivariate life history phenotypes (Pillai’s trace statistic = 0.825; df1 = 49; df2 = 700; approx. F = 1.908; p ≤ .001) but only the first two of the seven canonical dimensions was statistically significant (see Table 1). Canonical loadings for the first two dimensions across both developmental and life history traits are displayed in Figure 4. Dimension 1 had a canonical correlation of 0.56 between developmental and life history traits. Individuals with longer developmental stage 5 and 6 durations have shorter Stage 7 durations which is correlated with reduced pre-reproductive growth rates but higher post-reproductive growth rates and later maturation (see Figure 4). The second dimension had a canonical correlation of 0.49. Individuals that hatched from larger eggs had longer developmental stage 8, 4, and 3 durations but shorter developmental stage 2 and 5 durations and matured later and larger and produced larger offspring (see Figure 4). Figure 4d shows a phenotypic correlation matrix detailing the strength of correlations between all traits measured.

### Table 1 | Tests of canonical dimensions for the pooled three clone data set

| Dimension | Canonical correlation | Approximate F | df1 | df2 | p     |
|-----------|----------------------|---------------|-----|-----|-------|
| 1         | 0.55813468           | 1.90833894    | 49  | 700 | <.001 |
| 2         | 0.49309812           | 1.56969631    | 36  | 714 | .019  |
| 3         | 0.43332008           | 1.16931299    | 25  | 728 | .259  |
| 4         | 0.22290478           | 0.55285937    | 16  | 742 | .918  |
| 5         | 0.14763094           | 0.39522035    | 9   | 756 | .938  |
| 6         | 0.10215734           | 0.30258715    | 4   | 770 | .876  |
| 7         | 0.02344738           | 0.06158015    | 1   | 784 | .804  |

Note: Boldface values highlight statistically significant values.
phenotypes (Pillai’s trace statistic = 1.003; df1 = 49; df2 = 469; approx. F = 1.601; p = .008) but only the first two of the seven canonical dimensions was statistically significant (see Table 2). Canonical loadings for the first two dimensions across both developmental and life history traits are presented in Table 3. Dimension 1 had a canonical correlation of 0.55 between developmental and life history traits. Individuals with short developmental stage 3 durations had longer stage 2 and 8 durations, hatched from bigger eggs, matured at smaller sizes, and had fewer offspring in each clutch (see Table 3). The second dimension had a canonical correlation of 0.51. Individuals with longer stage 5 and 6 developmental durations had shorter stage 7 durations, slower pre-maturation growth, later maturation, more post-maturation growth and larger offspring (see Table 3). Figure S2 shows a phenotypic correlation matrix for the subsetted clone LL32 only data revealing the strength of correlations between all traits measured.

Maternal food had a significant effect on the multivariate development and life history phenotypes of offspring (F1,74 = 3.5616; p = .012; Figure 6a–d). Offspring from mothers reared in a high food environment came from a smaller egg, had longer developmental stage 3 durations, took longer to mature at larger sizes, and produced larger offspring (Figure 6a,c). They also had longer developmental stage 4 and 7 durations and a shorter developmental stage 5 (Figure 6b). Maternal age also had a significant effect on the multivariate development and life history phenotypes (F1,74 = 3.7728; p = .003; Figures 6a,b and 6e,f). Offspring from the fifth clutch hatched from larger eggs resulting in them having reduced stage 3 and stage 6 developmental durations, faster pre-reproductive growth, earlier maturation at a smaller size, and smaller offspring (Figure 6a,e). Maternal food environment and maternal age effects were still apparent when only the developmental traits were included in the analysis (maternal food: F1,74 = 3.3427; p = .015; see Figure S2 and maternal age: F1,74 = 3.8283; p = .007; see Figure S3).

4 | DISCUSSION

The mechanisms responsible for linking parental environment and/or state to offspring phenotypic variation often remain unknown. One reason for this is that genetic, nongenetic, and environmental cues are integrated.
during embryogenesis, yet parental effect studies are often post-embryonic. We developed a high-throughput confocal microscope assay that allowed us to link *Daphnia* embryogenesis to offspring life history variation at the individual level. We found that patterns of embryogenesis varied not only between clones reared in the same environment, but also within a single clone when mothers were of different ages, or experienced different food environments. The duration of particular developmental stages was also correlated with the expression of specific offspring life history traits, raising the possibility that our *Daphnia* embryogenesis assay might also be useful for predicting the later life consequences of exposure to adverse environments.

There is a growing consensus that development has to be integrated into evolutionary biology (Abouheif et al., 2014; FIGURE 5 Principal component analysis of offspring development and life history phenotypes across clones. Contributions to principal component space are shown in (a) a biplot of PC1 (19.8% of data variation) versus PC2 (15% of data variation), (b) a vector plot showing PC1 and PC2 trait loadings, (c) a biplot of PC2 (15%) versus PC3 (12.9%), (d) a vector plot showing PC2 and PC3 trait loadings. Ellipses indicate 95% confidence intervals around centroids for clones LL32 (blue), LL14 (red), and LL18 (orange) [Color figure can be viewed at wileyonlinelibrary.com]

| Dimension | Canonical correlation | Approximate F | df1 | df2 | p   |
|-----------|----------------------|---------------|-----|-----|-----|
| 1         | 0.5487226            | 1.6014131     | 49  | 469 | .008|
| 2         | 0.5143757            | 1.4959946     | 36  | 483 | .035|
| 3         | 0.4403585            | 1.3257768     | 25  | 497 | .136|
| 4         | 0.4116050            | 1.1520929     | 16  | 511 | .303|
| 5         | 0.2224216            | 0.6258345     | 9   | 525 | .775|
| 6         | 0.1399774            | 0.4797104     | 4   | 539 | .751|
| 7         | 0.0723736            | 0.4141070     | 1   | 553 | .520|

*Note: Boldface values highlight statistically significant values.*
Theory focusing on the integration of genetic, nongenetic, and environmental cues is developing rapidly (McNamara, Dall, Hammerstein, & Leimar, 2016), but empirical studies of cue integration are lagging behind because they require study systems in which it is possible to link changes in parental environments or states to changes in egg development patterns and the effect that this has on offspring phenotypic variation. Embryological assays are conducted in a diverse range of model organisms to understand developmental processes (Barresi & Gilbert, 2020), or as a tool for replacing whole-organism acute toxicity tests (Braunbeck et al., 2015). However, they are often destructive, or terminated at the end of development, for ethical or practical reasons (Barresi & Gilbert, 2020), making it impossible to understand how different patterns of embryogenesis are translated into offspring phenotype differences. Moreover, in sexual organisms, offspring are genetically and nongenetically different from their parents (and their siblings) making it difficult to tease genetic, nongenetic, and environmental cues apart. Clonal organisms, such as Daphnia, provide a great opportunity to study cue integration (Harney et al., 2017) but Daphnia embryogenesis assays are also normally destructive (reviewed in Mittmann et al., 2014; Toyota et al., 2016).

Giardini, Yan, and Heyland (2015) used a non-destructive Daphnia magna embryogenesis assay to demonstrate that mothers provision their offspring with calcium in low calcium environments, but they did not link embryogenesis to life history trait variation at the individual level as we have done here. Having a non-destructive, real-time, in vitro assay of Daphnia egg development is important for a number of reasons. First, because it allows us to study cue integration in different situations. Nongenetic effects, such as maternal effects, are often modeled as a static coefficient linking parental phenotype to offspring phenotype and/or fitness (Hoyle & Ezard, 2012; Kirkpatrick & Lande, 1989). Studies are already demonstrating that responses to different cues vary with genotype (Hallsson et al., 2012; Harney et al., 2017; Plaistow et al., 2015; Walsh, Cooley, Biles, & Munch, 2014), parental state (Lind, Berg, Alavioon, & Maklakov, 2015; Plaistow et al., 2015), and/or environmental context (Czesak & Fox, 2003; Plaistow, Lapsley, & Benton, 2006). We can only understand how these effects arise if we can study the mechanisms responsible for them directly. Quantifying the duration of seven different developmental stages occurring between Stages 3A and 6 of Gulbrandsen and Johnsen (1990) destructive assay will help if it allows us to target critical developmental windows with omics technologies more precisely.

Second, our results demonstrate that embryogenesis is plastic, sensitive to parental cues, and correlated with offspring life history variation. The relationship between embryogenesis and offspring life histories varied slightly between samples and different methods of ordination, but generally revealed a consistent association between developmental stages 5, 6, and 7 and a trade-off between pre- and post-maturation growth, and an association between egg length, developmental stages 2–5 and 8 and 7 and a trade-off between age and size at maturity, fecundity, and the size of offspring produced. A similar pattern of trait association explained clonal differences in offspring development and life history phenotypes, and maternal age and maternal food effects in clone LL32. We did not get enough data to test for clonal variation in maternal age and maternal food effects on offspring development and life history phenotypes in this study, but we have previously demonstrated clonally variable maternal food effects on post-embryonic developmental phenotypes (Harney et al., 2017), and maternal age effects on post-embryonic developmental phenotypes (Plaistow et al., 2015). Consequently, we hypothesize that differences in embryogenesis plasticity in response to parental cues may be important for explaining variation to the extent that nongenetic cues are transmitted across generations.

It is not surprising that the mechanism underpinning maternal age and maternal food effects are similar given that Daphnia are indeterminate growers, meaning that older mothers are also normally larger mothers. Consequently, it can be difficult to separate the effects of

### TABLE 3

Canonical loadings for the first two dimensions of clone LL32 only data set

| Variable               | Dimension 1 | Dimension 2 |
|------------------------|-------------|-------------|
| Developmental          |             |             |
| d2                     | 0.556       | 0.198       |
| d3                     | −0.552      | 0.021       |
| d4                     | 0.342       | 0.055       |
| d5                     | 0.038       | 0.470       |
| d6                     | −0.109      | 0.636       |
| d7                     | 0.290       | −0.627      |
| d8                     | 0.560       | 0.013       |

| Life history           |             |             |
|------------------------|-------------|-------------|
| Egg length             | 0.708       | 0.034       |
| Age at maturity        | −0.274      | 0.449       |
| Size at maturity       | −0.580      | −0.095      |
| Fecundity              | −0.681      | 0.038       |
| Pre-maturation growth rate | −0.101    | −0.386      |
| Post-maturation growth rate | −0.008    | 0.432       |
| Mean offspring size    | −0.083      | 0.767       |
maternal age from effects attributed to maternal size in *Daphnia* (Plaistow et al., 2015). Further studies will be required to determine if embryogenesis is also plastic in other species. In many systems, it may not be feasible to link parental effects all the way through to offspring life history variation as we have done here. However, fish embryological assays have been developed for ecotoxicity purposes (Braunbeck et al., 2015), and existing embryological assays for nematodes, insects, amphibians, and mammals (Barresi & Gilbert, 2020) might conceivably also be adapted to study parental effects on embryogenesis.

Third, our assay permits us to study *Daphnia* maternal effects in a controlled environment. In *D. magna*, the clonetype-specific integration of genetic, nongenetic, and environmental cues was explained by differences in the expression of post-embryonic developmental traits in different environments (Harney et al., 2017). However, the adaptive significance of the nongenetic cues observed in Harney et al.’s (2017) study (maternal effects) was difficult to interpret because the environmental conditions experienced by the offspring interacted with maternal environmental cues. Our assay allows us to separate maternal provisioning effects from other maternal and early life effects such as the state of the brood pouch environment (Bartosiewicz, Jabłoński, Kozłowski, & Maszczyk, 2015), or the extent that mothers oxygenate their eggs (Seidl, Pirow, & Paul, 2002).

Finally, our assay could have an industrial application because *Daphnia* is a model system for ecotoxicological studies used to monitor environmental pollution all over the world (Shaw, 2008). The current OECD standard test—the *Daphnia magna* reproduction test—requires a test duration of 21 days which is both time consuming and costly. If the in vitro embryogenesis assay that we have developed here can predict variation in later adult life history traits, it could potentially inform about the

**Figure 6** Principal component analysis of maternal food, and maternal age, effects on offspring development and life history phenotypes in clone LL32. Contributions to principal component space are shown in (a) a vector plot showing PC1 (19%) and PC2 (17.9%) trait loadings, (b) a vector plot showing PC2 (17.9%) and PC3 (13.1%) trait loadings, (c) a biplot of PC1 (19% of data variation) versus PC2 (17.9% of data variation), (d) a biplot of PC2 (17.9% of data variation) versus PC3 (13.1% of data variation), (e) a biplot of PC1 (19% of data variation) versus PC2 (17.9% of data variation), (f) a biplot of PC2 (17.9% of data variation) versus PC3 (13.1% of data variation). Ellipses indicate 95% confidence intervals around centroids; (c, d) maternal food environment: high food (red) and low food (blue), (e, f) maternal age: third clutch (red) and fifth clutch (blue) [Color figure can be viewed at wileyonlinelibrary.com]
toxicity effects on *Daphnia* on a large number of individuals in a much shorter time frame and conceivably be used as a prescreen to define doses to be used in more standard OECD tests in the same way that fish embryology tests are beginning to be used (Braunbeck et al., 2015). Encouragingly, we found that the duration of certain developmental stages was consistently associated with offspring life history variation and was, in some cases, better correlated than traits that are often used to encapsulate maternal effects such as differences in egg size (Guinnee, Gardner, Howard, West, & Little, 2007; Guinnee, West, & Little, 2004; Lampert, 1993). For example, the duration of developmental stages 5, 6, and 7 was associated with a trade-off between pre- and post-maturation growth that was not predicted by egg size.

Pinpointing the precise developmental stages affected by parental effects will help us to understand the mechanisms responsible for parental effects on offspring. However, we still also need to understand how changes in the maternal environment or maternal state are transmitted to the egg phenotype. Otherwise, the data sets supporting this article will be uploaded to DRYAD if it is accepted.

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