RESEARCH PAPER

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

Running title: Egg development plasticity in *Daphnia*

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ABSTRACT

Understanding how genetic, non-genetic and environmental cues are integrated during development may be critical for 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 between clones reared in the same environment, but also within a single clone when mothers were 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 pre-screen for testing the effect that pollutant doses have on offspring life-histories traditionally assayed with a 21-day *Daphnia* reproduction test.

Key words: parental effects, *Daphnia pulex*, embryogenesis

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. Non-genetic 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 behaviour or culture through learning (see Bonduriansky & Day, 2009, 2018; Danchin et al., 2011; Jablonka & Lamb, 2005 for reviews). Such transmission may reflect the parents' current environment or state, but also genetic variation associated with how parents respond to their environment (i.e. Indirect genetic effects, (IGE's) (Jason B Wolf, 2003; J B Wolf, Brodie III, Cheverud, & Moore, 1998).

The incorporation of non-genetic mechanisms into evolutionary thinking is changing our assumptions about how populations respond to rapid environmental change (Bonduriansky, Crean & Day 2012; Hallsson, Chenoweth & Bonduriansky 2012), and driving an incentive to understand how the integration of genetic, non-genetic 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 non-genetic 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 defences (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 (Bossuyt & 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, non-genetic and environmental cues are integrated during development, yet the 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, non-genetic 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 two-fold. First, we aimed to carry out a non-destructive, 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 variation we see in offspring.

METHODS

(a) 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 have been maintained as laboratory stock cultures in a controlled temperature incubator at 21°C ± 1° on a 14:10 L:D cycle in hard artificial pond water (ASTM; OECD 1998) enriched with standard organic extract (Baird et al. 1989). The clone lines are frequently bottle-necked (re-started from a single individual) to prevent the possibility of mutation derived genetic variation existing in each clone line.

(b) 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^{-1} of batch-cultured *Chlorella vulgaris* (high food) daily and the media changed three days a week (Monday, Wednesday, Friday). Neonates from the first two clutches were discarded. Once individuals produced their 3rd clutch, three offspring from the total produced were randomly selected and used to set up the next generation. On reaching their 2nd generation, offspring from all three mothers were mixed and randomly allocated to two food treatments. In order to maximise the chances of catching mothers depositing their 3rd and 5th clutches into the brood pouch, 20 individuals were fed high food daily (200 000 cells $mL^{-1)}$, and 30 were fed low food (40 000 cells mL^{-1} day^{-1}) for each clone. On reaching their 3rd clutch, eggs at the correct stage (see below) were removed for the

development and life history assay. This was repeated for the 5th clutch for mothers not sampled at the 3rd clutch stage (see Figure 1).

(c) Egg development assay

The egg development assay was carried out in a Corning clear flat-bottomed 96 well plate. In order to prevent the eggs from moving out of the field of view during the imaging, a Rondelle Spacer Bead (clear czech pressed glass, 6mm x 2mm; www.beadaholique.com) with a 0.9mm 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 2nd 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 one, see Figure 2A) approximately seven hours after the eggs were first seen in the brood pouch. At this stage, eggs were yellow/green in colour, 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 one, mothers were placed on a slide and photographed to get a measure of body size (see methodology section(e) for details). To remove the eggs the mother was first placed on to a slide and liquid 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

158 prevent any movement causing damage to the eggs and the number of eggs in the clutch was
159 counted. A few drops of ASTM were added to the slide using a Pasteur pipette and a 100µl
160 pipette was used to individually transfer each egg into the centre of a bead placed in each well
161 of a flat- bottomed 96-well plate.
162 Time lapse images of each individual's development were taken on a Zeiss LSM 710 Confocal
163 Microscope at x10 magnification using a 488nm laser at a transmission of 1.2. All individuals
164 were imaged in nine different z-planes to ensure that even if the embryos moved, they remained
165 in focus over the course of development. Images were taken using an automated system, with
166 an interval of 28 minutes until all individuals in the plate had reached developmental phenotype
167 eight (see Figure. 3.8). Once the eggs had hatched from the vitelline membrane and were
168 swimming freely around the well, the time lapse was stopped. At this stage individuals would
169 usually still be within the mother's brood pouch. They have not yet developed their gut, tail
170 spine or second antennae and the maternal yolk is still visible (see Figure 2B). The beads were
171 removed but the individuals remained in the wells until they had developed to the neonate stage
172 (see Figure 2C); the stage at which they are normally released from the mother's brood pouch.
173 During this time, they were kept under standard rearing conditions (21°C ± 1°C on a 14:10
174 light: dark photoperiod). All surviving neonates were then used for life history assays.
175 The time lapse images collected from each individual egg were processed and analysed using
176 Zeiss AIM imaging software (Carl Zeiss Canada Ltd). Time lapse videos were cropped to
177 ensure the start point was the same for all individuals (phenotype one = time zero; see Figure
178 3.1). The point at which the individual hatched from the vitelline membrane (Figure 3.8) was
179 set as the end point. An image of the egg at the start of the time lapse was used to get an accurate
180 measure of egg length. All images for each individual were then combined to create a time
181 lapse video of the individual's development.
182

183 *(d) Embryo phenotyping*

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

194

195 *(e) Life-history assay*

196 Neonates collected from the egg development assay were kept in isolated jars, containing
197 150ml of hard artificial pond water (ASTM; OECD 1998) enriched with a standard organic
198 extract (Baird et al. 1989) which were replaced three times a week. All individuals were fed on
199 a high food diet (200 000 cells mL^{-1} of batch-cultured *Chlorella vulgaris*) daily. Individuals
200 were first photographed as neonates and subsequently every time they moulted. Photographs
201 were taken using a Canon EOS 600D digital camera attached to a Leica M60 optical
202 microscope. Body size was measured as the distance from the top of the individual's head, to
203 the base of the tail spine using Image J (Rasband 1997). Maturation was recorded as the time
204 when eggs were first seen in the brood pouch. For each subsequent clutch of eggs, neonates
205 were counted upon release and five were photographed to calculate the average neonate size
206 for each clutch. This was carried out until each individual reached their 3rd clutch. Each
207 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^{-1}), size at maturity (mm), age at maturity (days), and fecundity for their first three clutches laid.

(f) Statistics

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 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 (3^{rd} clutch, 5^{th} clutch) and maternal food fitted as a fixed factor with two levels (high food, low food). Differences in treatments and clones were visualised 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. 2015).

RESULTS

(a) *Staging of D. pulex embryonic development*

The first clearly identifiable phenotype in the live assay was hatching from the external chorion (Figure 3.1), equivalent to stage 3A in Gulbrandsen & Johnson (1990). This was used as a

233 standardised time zero for each assay. Our second phenotype was characterised as the first
234 point at which cephalic invaginations could be seen, and the symmetry changed from radial to
235 bilateral symmetry (Figure 3.2). Phenotype three involved the formation of the secondary
236 antennae and a posterior invagination. The head of the individual was also clear at this stage
237 (Figure 3.3). The posterior end then expanded for a short time, extending the vitelline
238 membrane (Figure 3.4). Phenotype five was characterised by the growth of the individual into
239 the vitelline membrane, with an obvious protrusion of the posterior end and clear thoracic
240 segments (Figure 3.5). Phenotype six marks the formation of the eye (Figure 3.6). Phenotype
241 seven involved the rounding of the posterior end and the eye becoming more prominent (Figure
242 3.7). Phenotype eight (Figure 3.8) occurs when individuals hatch from the vitelline membrane
243 and marks the end of the time lapse because at this point individuals were able to swim freely
244 and moved out of the imaging field of view.
245
246 *(b) Linking egg development phenotypes to life history traits*
247 Missed developmental windows, mortality and restricted access to the confocal microscope
248 meant we were only able to collect full developmental and life-history data from 10 LL14 clone
249 individuals, 23 clone LL18 individuals and 76 clone LL32 individuals. Consequently, we could
250 only conduct a full analysis of maternal food and age effects for clone LL32 (see below). The
251 pooled data set for all clones revealed a significant association between multivariate
252 developmental phenotypes and multivariate life history phenotypes (*Pillai's trace statistic* =
253 0.825, df1=49, df2=700, *approx. F* = 1.908, P= <0.001) but only the first two of the seven
254 canonical dimensions was statistically significant (see Table 1). Canonical loadings for the
255 first two dimensions across both developmental and life-history traits are displayed in Figure
256 4. Dimension 1 had a canonical correlation of 0.56 between developmental and life-history
257 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.

(c) Clonal variation in offspring development and life history phenotypes

Multivariate developmental and life history phenotypes were clonally variable ($F_{2,107}$= 3.4726, p=0.008; 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 ($F_{2,107}$=3.4732, p= 0.01, see Figure S1), confirming that different clones have different patterns of egg development despite being in the same environment.

(d) 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 (*Pillai's trace statistic* = 1.003, df1=49, df2=469, *approx. F* = 1.601, P= 0.008) 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 stage 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 stage 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 sub-setted Clone 32 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 ($F_{1,74}$= 3.5616, p= 0.012; Figure 6A,B,C,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 ($F_{1,74}$= 3.7728, p= 0.003; Figure 6A,B,E,F). Offspring from the 5th 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 6 A,E). Maternal food environment and maternal age effects were still apparent when only the developmental traits were included in the analysis (Maternal food: $F_{1,74}$= 3.3427, p= 0.015, see Figure S2; Maternal age: $F_{1,74}$= 3.8283, p= 0.007, see Figure S3).

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, non-genetic 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 between clones reared in the same environment, but also within a single clone when mothers were 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; Gilbert, Bosch, & Ledón-Rettig, 2015; Sommer, 2009; Sultan, 2007). Theory focussing on the integration of genetic, non-genetic 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 then has on offspring phenotypic variation. Embryological assays are conducted in a diversity 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 non-genetically different from their parents (and their siblings) making it difficult to tease genetic, non-genetic 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 *et al.* (2015) used a non-destructive *Daphnia magna* embryogenesis assay to demonstrate that mothers provision their offspring with calcium in low calcium environments, but they didn't 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. Non-genetic effects, such as maternal effects, are often modelled 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, Chenoweth, & Bonduriansky, 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 stage 3A and stage six of Gulbrandsen & Johnson's (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 traits such as 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 didn't 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 in the extent that non-genetic cues are transmitted across generations.

It isn't 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 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 clone-specific integration of genetic, non-genetic 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 non-genetic cues observed in Harney et al.'s (2017) study (maternal effects) were 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 et al. 2015), or the extent that mothers oxygenate their eggs (Seidl, Pirow, & Paul, 2002).

382 Finally, our assay could have an industrial application because *Daphnia* are a model system
383 for ecotoxicological studies used to monitor environmental pollution all over the world (Shaw,
384 2006). The current OECD standard test – the *Daphnia magna* Reproduction test – requires a
385 test duration of 21-days which is both time-consuming and costly. If the *in-vitro* embryogenesis
386 assay we have developed here can predict variation in later adult life-history traits, it could
387 potentially inform about toxicity effects on *Daphnia* on a large number of individuals in a much
388 shorter timeframe and conceivably be used as a pre-screen to define doses to be used in more
389 standard OECD tests in the same way that fish embryology tests are beginning to be used
390 (Braunbeck et al., 2015). Encouragingly, we found that the duration of certain developmental
391 stages were consistently associated with offspring life-history variation and were, in some
392 cases, better correlated than traits that are often used to encapsulate maternal effects such as
393 differences in egg size (Guinnee, Gardner, Howard, West, & Little, 2007; Guinnee, West, &
394 Little, 2004; Lampert, 1993). For example, the duration of developmental stages 5,6 and 7 was
395 associated with a trade-off between pre- and post-maturation growth that was not predicted by
396 egg size.
397 Pinpointing the precise developmental stages affected by parental effects will help us to
398 understand the mechanisms responsible for parental effects on offspring. However, we still
399 also need to understand how changes in maternal environment, or maternal state are transmitted
400 to the egg phenotype. Cross-generational continuity is typically understood to be a function of
401 inherited genes (Dobzhansky, 1970). But, offspring don't just inherit genes from their parents;
402 they also inherit a phenotype in the form of a gamete, a newly divided cell, or a group of cells
403 that is donated by a parent or both parents (West-Eberhard, 2003). This fully functioning
404 'bridging phenotype' (West-Eberhard, 2003) is already adapted to respond to the environment
405 and is responsible for transmitting developmental templates and resources to the offspring
406 which then determine how offspring genes are expressed (Badyaev & Uller, 2009). These

complex phenotypes might include maternally derived RNAs, organelles, ribosomes, proteins, cytoplasmic gradients, hormones and symbionts. Hence there are likely to be many possible ways that parents can manipulate the phenotypes of their offspring (Badyaev & Uller, 2009).

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DATA ACCESSIBILITY

The data sets supporting this article will be uploaded to DRYAD if it is accepted.

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TABLES

Table 1: Tests of Canonical dimensions for the pooled three clone data set

Dimension	Canonical correlation	*Approx. F.*	df1	df2	P
1	0.55813468	1.90833894	49	700	**<0.001**
2	0.49309812	1.56969631	36	714	**0.019**
3	0.43332008	1.16931299	25	728	0.259
4	0.22290478	0.55285937	16	742	0.918
5	0.14763094	0.39522035	9	756	0.938
6	0.10215734	0.30258715	4	770	0.876
7	0.02344738	0.06158015	1	784	0.804

Table 2: Tests of Canonical dimensions for the clone LL32 only data set

Dimension	Canonical correlation	*Approx. F.*	df1	df2	P
1	0.5487226	1.6014131	49	469	**0.008**
2	0.5143757	1.4959946	36	483	**0.035**
3	0.4403585	1.3257768	25	497	0.136
4	0.4116050	1.1520929	16	511	0.303
5	0.2224216	0.6258345	9	525	0.775
6	0.1399774	0.4797104	4	539	0.751
7	0.0723736	0.4141070	1	553	0.520

Table 3: Canonical loadings for the first two dimensions of clone LL32 only data set.

Variable	Dimension	
Developmental	*1*	*2*
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

FIGURE LEGENDS

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 3rd and 5th clutch, enabling us to compare maternal environment and maternal age effects on the development and life history of each individual.

Figure 2a: Stage one of *Daphnia pulex* egg development. At this stage eggs are yellow/green in colour, have a visible fat drop and a transparent gap between the membrane and the cells. **(b)** Stage five 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 six of *D. pulex* development (neonate). Individual has a fully formed gut (G), carapace and the tail spine (Ts) and second antennae (An) are extended.

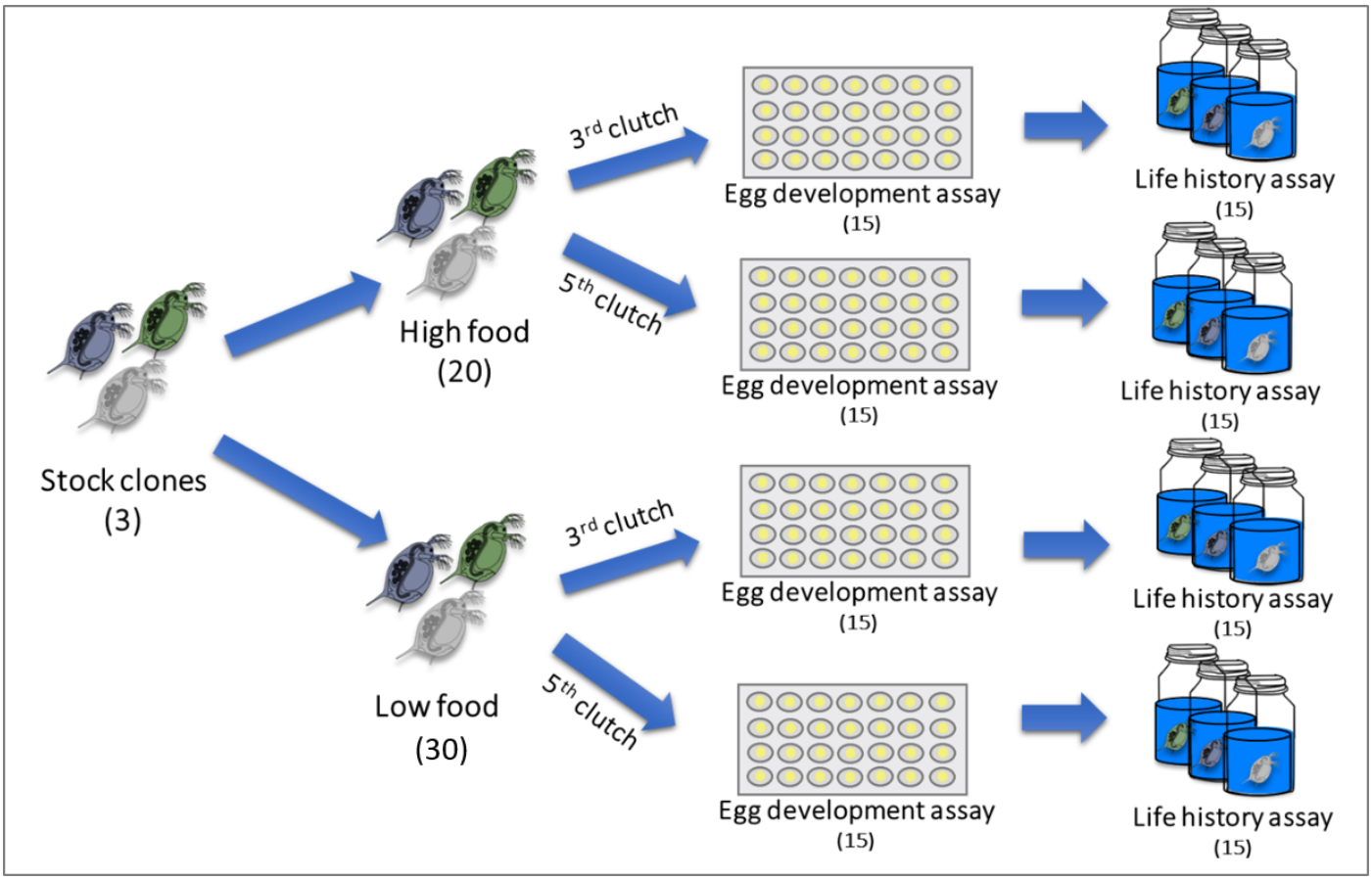
Figure 3: Egg development phenotypes. **(1)** The individual has just hatched from the external chorion (Ec). **(2)** Clear cephalic invaginations (Ci_1 andCi_2) can be seen, and symmetry changes from radial to bilateral. **(3)** Individuals head shape begins to form (H), the secondary antennae form (An) and there is an obvious posterior invagination (Pi). **(4)** The posterior end briefly protrudes (Pp_1) enlarging the vitelline membrane. **(5)** Posterior end protrudes further (Pp_2), filling the vitelline membrane, and thoracic segments (Ts) are clearly visible. **(6)** Eye spots are first seen (Es_1). **(7)** Eye spots become more prominent (Es_2) and posterior end becomes rounded (Pr) as carapace extends. **(8)** Individual hatches from vitelline membrane (Vm).

Figure 4: Vector plots of the canonical loadings for **(a)** seven developmental stage durations and, **(b)** six life-history traits. **(c)** Canonical loadings for all traits. **(d) A phenotypic correlation matrix for all developmental and life-history traits measured.**

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) vs PC2 (15% of data variation), **(b)** a vector plot showing PC1 and PC2 trait loadings, **(c)** a biplot of PC2 (15%) vs PC3 (12.9%), **(d)** a vector plot showing PC2 and PC3 trait loadings. Ellipses indicate 95% confidence intervals around centroids for clone LL32 (Blue), LL14 (Red) and LL18 (Orange).

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) vs PC2 (17.9% of data variation), **(d)** a biplot of PC2 (17.9% of data variation) vs PC3 (13.1% of data variation), **(e)** a biplot of PC1 (19% of data variation) vs PC2 (17.9% of data variation), **(f)** a biplot of PC2 (17.9% of data variation) vs 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: 3rd clutch (Red) and 5th clutch (Blue).


Stock clones
(3)
High food
(20)
Low food
(30)
3rd clutch
5th clutch
3rd clutch
5th clutch
Egg development assay
(15)
Egg development assay
(15)
Egg development assay
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Life history assay
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Life history assay
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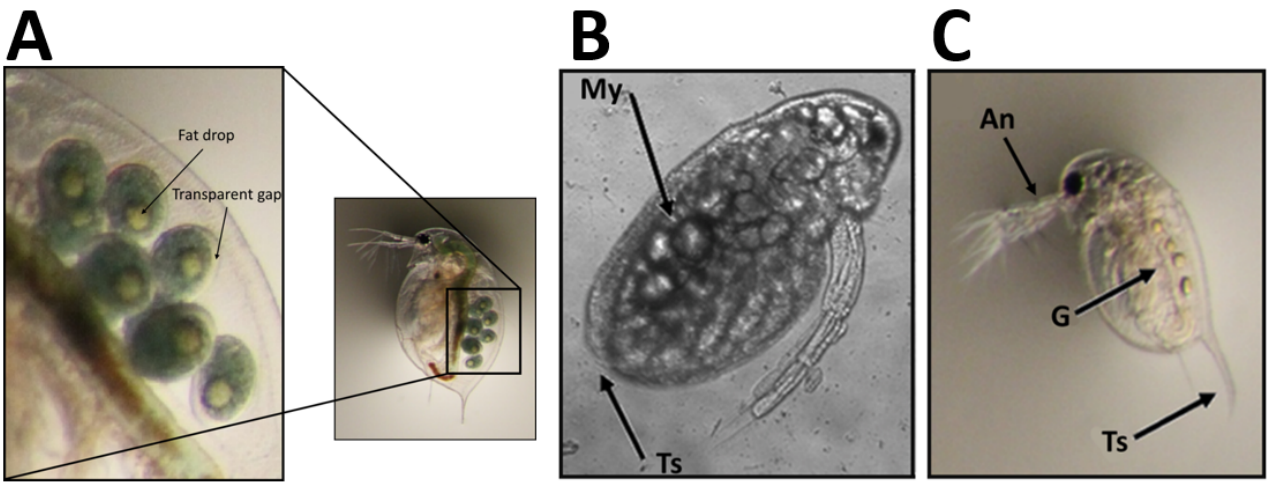
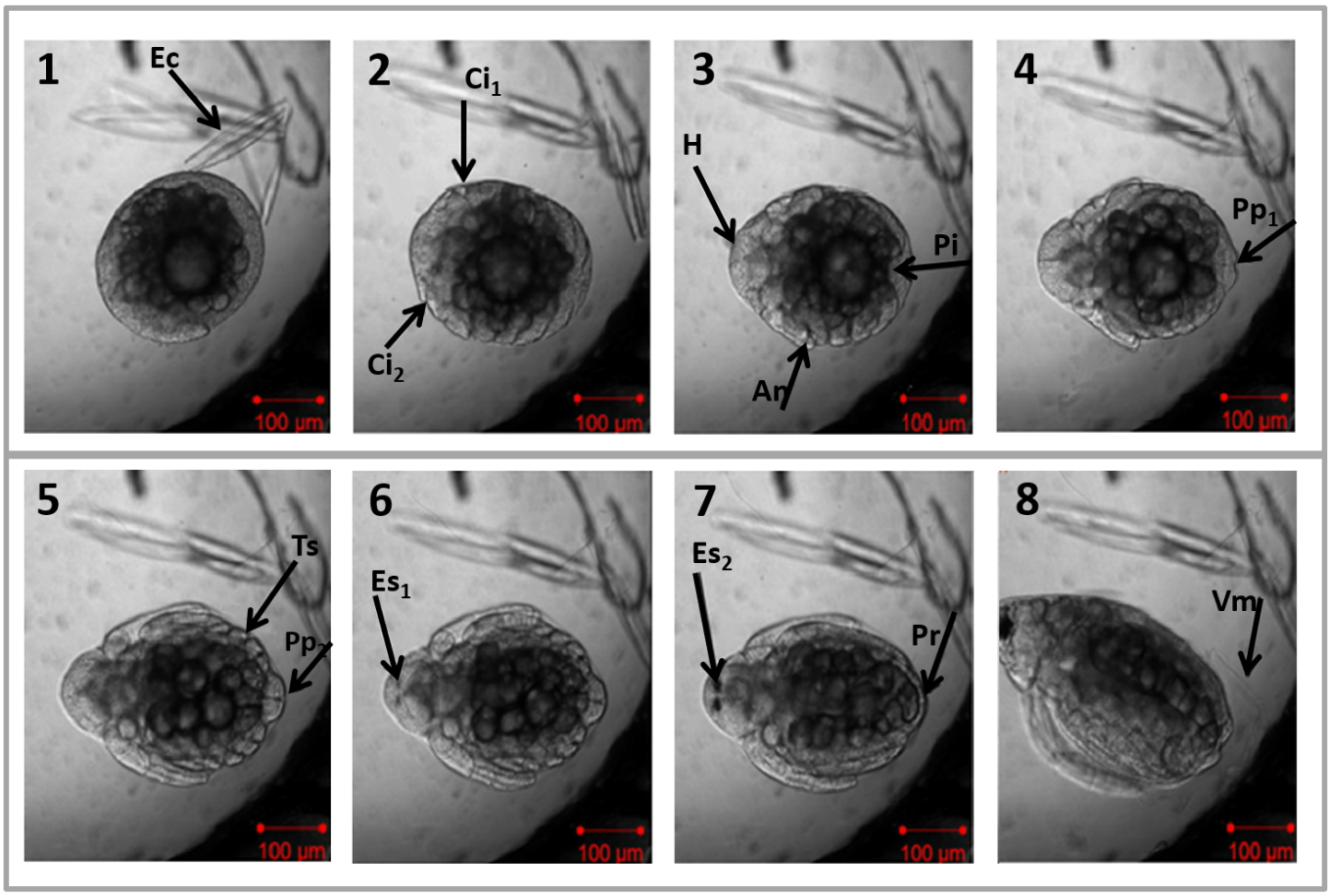

A
Fat drop
Transparent gap
B
My
Ts
C
An
G
Ts


1
Ec
100 µm
2
Ci1
Ci2
100 µm
3
H
Pi
An
100 µm
4
Pp1
100 µm
5
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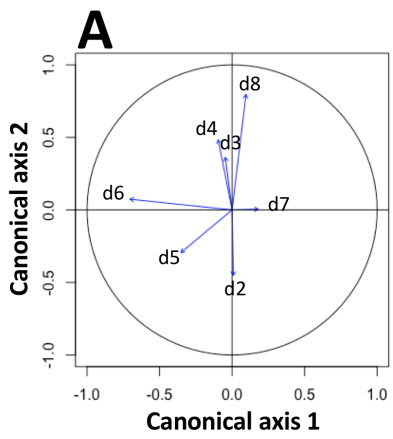
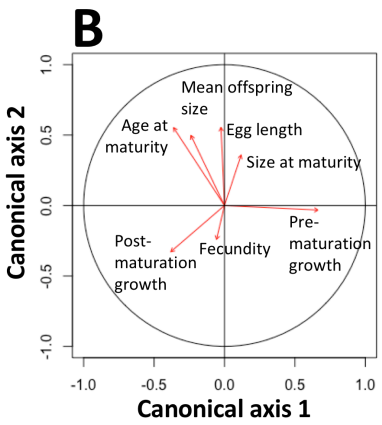
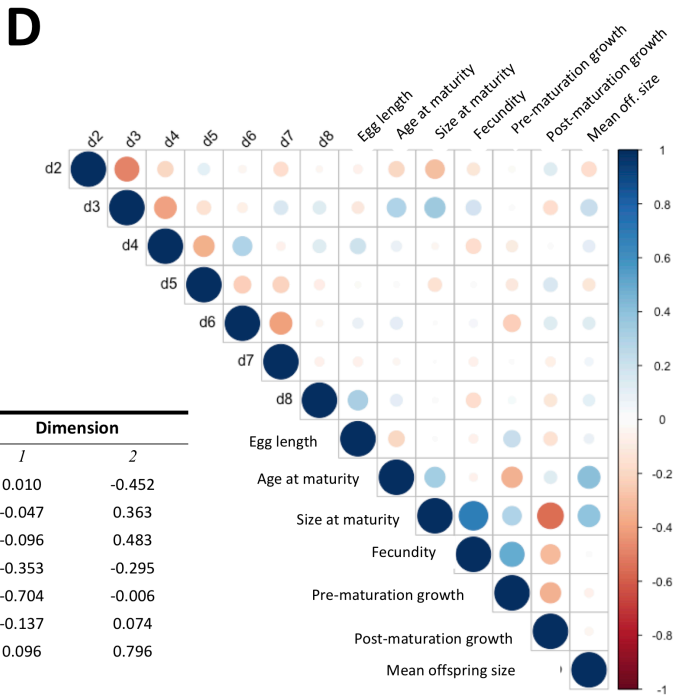
Es1
100 µm
7
Es2
Pr
100 µm
8
Vm
100 µm

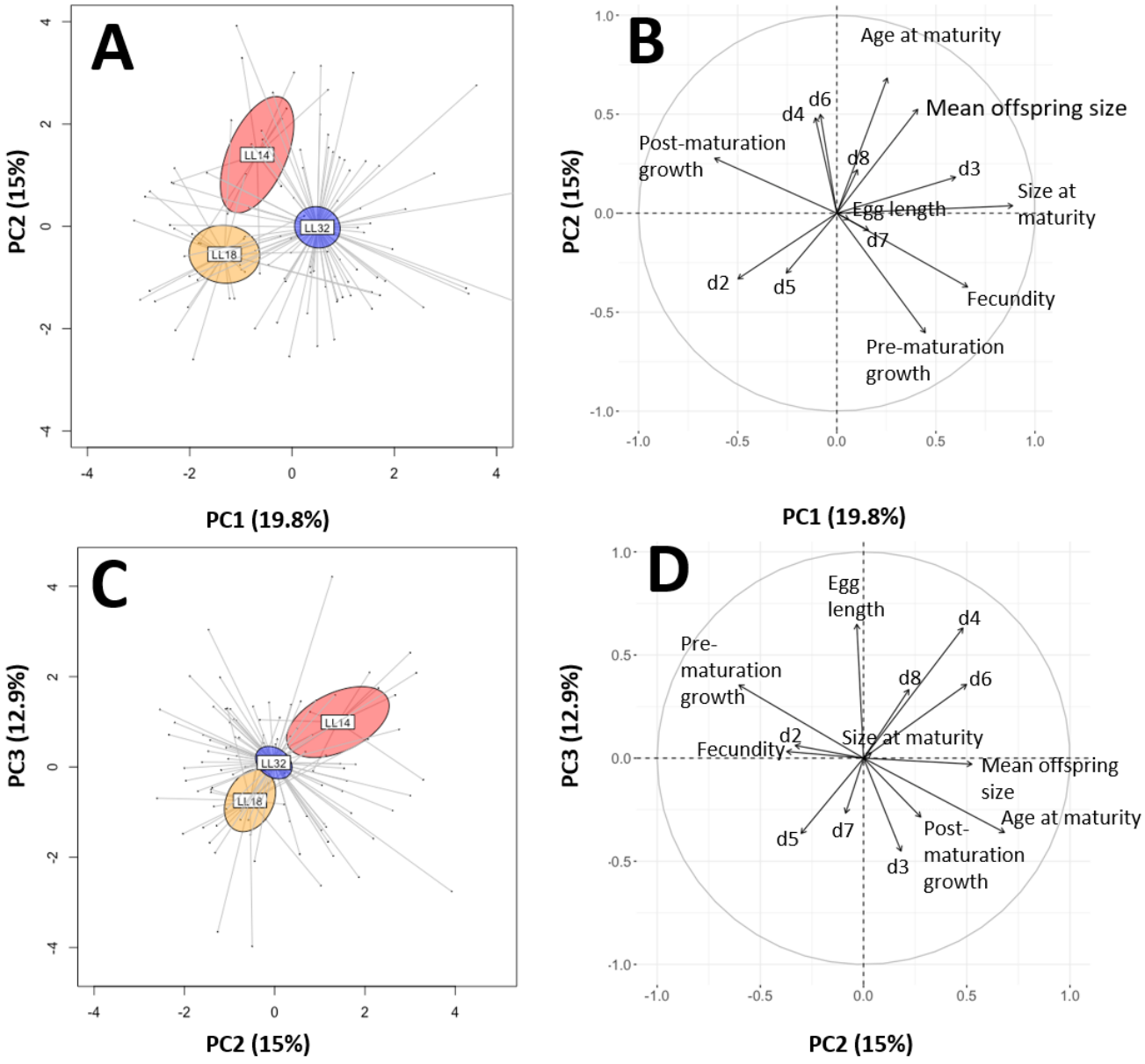


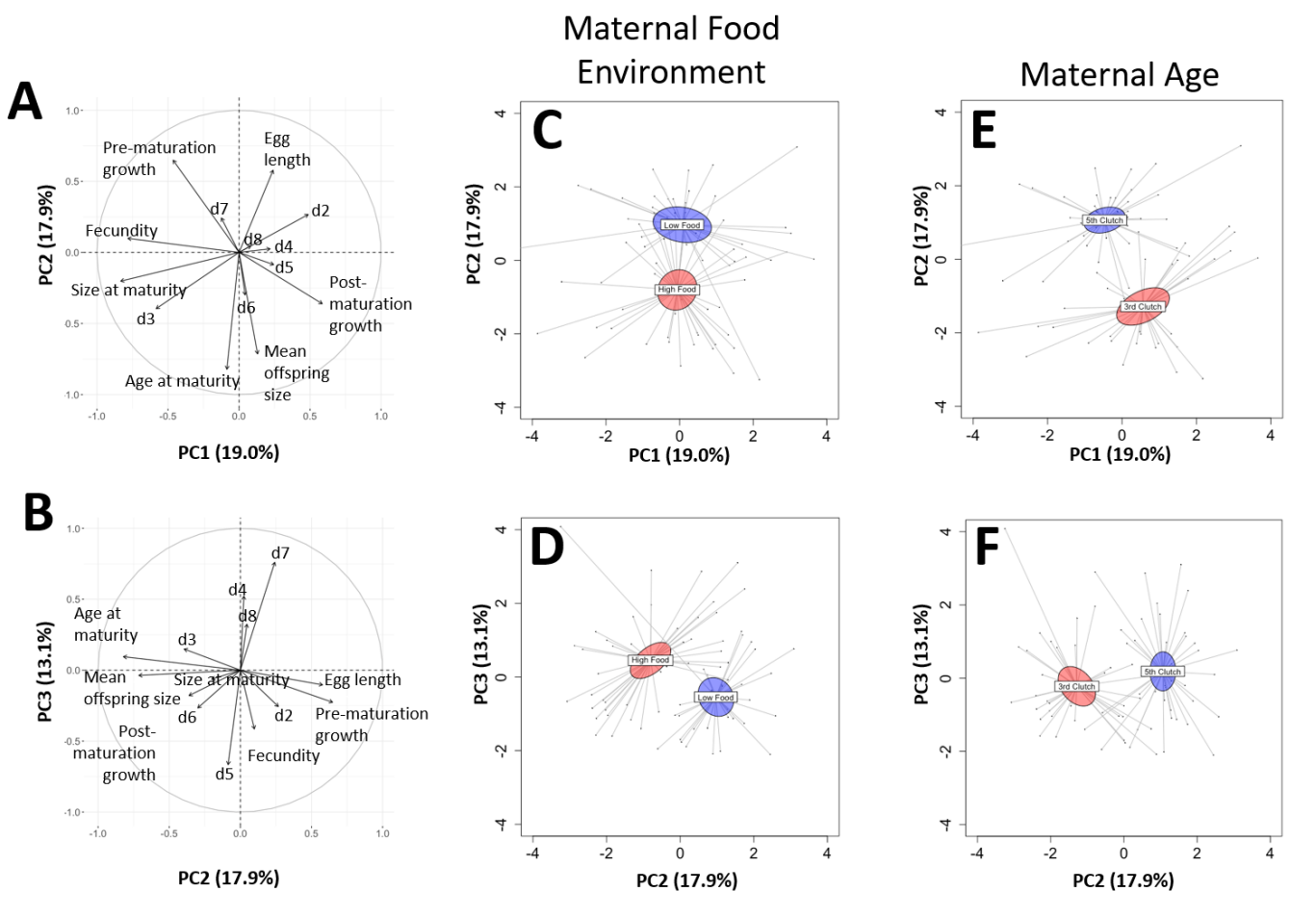


C

Variable	Dimension	
Developmental	*1*	*2*
d2	0.010	-0.452
d3	-0.047	0.363
d4	-0.096	0.483
d5	-0.353	-0.295
d6	-0.704	-0.006
d7	-0.137	0.074
d8	0.096	0.796
Life History		
Egg length	-0.025	0.555
Age at maturity	-0.362	0.553
Size at maturity	0.118	0.358
Fecundity	-0.056	-0.241
Pre-maturation growth rate	0.665	-0.033
Post-maturation growth rate	-0.382	0.005
Mean offspring size	-0.239	0.497




A
LL14
LL32
LL18
PC2 (15%)
PC1 (19.8%)
B
Age at maturity
Mean offspring size
Post-maturation growth
Size at maturity
Egg length
Fecundity
Pre-maturation growth
d2
d3
d4
d5
d6
d7
d8
PC2 (15%)
PC1 (19.8%)
C
LL14
LL32
LL18
PC3 (12.9%)
PC2 (15%)
D
Egg length
Pre-maturation growth
Fecundity
Size at maturity
Mean offspring size
Age at maturity
Post-maturation growth
PC3 (12.9%)
PC2 (15%)


Maternal Food Environment
Maternal Age
A
B
C
D
E
F
Pre-maturation growth
Egg length
d7
d2
Fecundity
d8
d4
d5
Size at maturity
Post-maturation growth
d3
d6
Mean offspring size
Age at maturity
PC1 (19.0%)
PC2 (17.9%)
PC3 (13.1%)
Low Food
High Food
5th Clutch
3rd Clutch