Intergenerational Effects of Early Life Starvation on Life-History, Consumption, and Transcriptomes of a Holometabolous Insect

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ABSTRACT: Intergenerational effects, also known as parental effects in which the offspring phenotype is influenced by the parental phenotype, can occur in response to parental early life food-limitation and adult reproductive environment. However, little is known about how these parental life stage-specific environments interact with each other and with the offspring environment to influence offspring phenotypes, particularly in organisms that realize distinct niches across ontogeny. We examined the effects of parental early life starvation and adult reproductive environment on offspring traits under matching or mismatching offspring early life starvation conditions using the insect *Athalia rosae* (turnip sawfly). The parental early life starvation treatment had context-dependent intergenerational effects on life-history and consumption traits of offspring larvae, partly in interaction with offspring conditions (intragenerational effects) and sex, while there was no significant effect of parental adult reproductive environment. In addition, parental starvation led to differential expression of only few genes, while offspring larval starvation caused a pronounced differential gene expression and modulation of multiple pathways. Our findings reveal that parental starvation leads to intergenerational effects on offspring life-history traits, consumption patterns as well as gene expression, although the effects are less pronounced than those of offspring starvation.

Introduction

Intergenerational effects, more commonly known as parental effects, are defined as causal influences of parental phenotype on offspring phenotype that are likely mediated by non-DNA sequence-based inheritance (Wolf and Wade 2009; Perez and Lehner 2019). Together with transgenerational effects, which occur across several generations, they play a key role in the ecology and evolution of organisms (Badyaev and Uller 2009) and impact the responses of individuals to changing environmental conditions (Sánchez-Tójar et al. 2020). One important environmental factor that can rapidly change during an organism's lifetime and across generations is the food availability. Periods of starvation are commonly encountered by insects in the wild (Jiang et al. 2019) and, when experienced early in life, often have long-lasting effects on an individual's phenotype, affecting life-history, consumption, and behavior (Miyatake 2001; Wang et al. 2016; McCue et al. 2017). Intergenerational effects of starvation events on offspring developmental trajectories have been found to differ depending on the exact starvation regime, sex, and traits that are considered (Saastamoinen et al. 2013; McCue et al. 2017; Paul et al. 2019). Moreover, to fully elucidate the adaptive nature of intergenerational effects, studies should encompass different phases in development during the parental life cycle and how such experience affects individual offspring phenotypes (English and Barreaux 2020).

The early life environment can shape the phenotype of an individual and its offspring via distinct routes (Monaghan 2008; Burton and Metcalfe 2014; Taborsky 2017). Individuals experiencing a benign environment in early life may pass on their superior condition to their offspring (van de Pol et al. 2006), often called silver spoon or carry-over effects (Monaghan 2008), resulting in offspring that are better able to deal with stressful conditions (Franzke and Reinhold 2013). Similarly, negative effects of a poor start in life might also be passed on, resulting in offspring less able to cope with stressful conditions (Naguib et al. 2006). Alternatively, such stressed

individuals may produce offspring that are buffered against stressors, for example, through parental provisioning (Valtonen et al. 2012; Pilakouta et al. 2015; Hibshman et al. 2016). Finally, identical environmental conditions experienced in a certain stage (i.e. a match between parental and offspring environment) may be predicted to be optimal for the offspring (match-mismatch scenario). Evidence for such predictive adaptive effects has been found in several species (Raveh et al. 2016; Le Roy et al. 2017), but is weak in others (Uller et al. 2013). Each of these avenues of intergenerational effects are not mutually exclusive. For example, offspring in mismatched environments may do relatively better if their parents were of high quality, due to silver spoon effects (Engqvist and Reinhold 2016). Fully reciprocal designs are needed to determine the relative importance of the experience in a certain life-stage or generation on such developmental plasticity (Groothuis and Taborsky 2015).

In addition, parental investment in offspring can vary depending on the condition of the parent's mate (Ratikainen and Kokko 2010). Both males and females have been shown to enhance investment in response to perceived increases in mate quality or attractiveness (Cunningham and Russell 2000; Cornwallis and O'Connor 2009). Changes in parental investment based on mate cues may potentially counteract or augment the effects of the parental early life environment on offspring phenotype. These effects may act in concert; for example, poor early life conditions can contribute to poor quality of mating partners and therefore affect mating success and reproductive investment (Hebets et al. 2008; Vega-Trejo et al. 2016). As niches often shift across an individual's lifetime, both parental early and later life environments and their interaction must be considered.

With discrete phases during development, insects, particularly holometabolous insects, present ideal organisms in which to investigate how intra- and intergenerational environmental cues, such as food limitation and mate quality, might

influence individual phenotypes (English and Barreaux 2020). Periods of low resource availability during larval development are known to influence developmental trajectories with knock-on effects on adult size, reproductive success and adult starvation resistance (Boggs and Niitepõld 2016; Wang et al. 2016). Individuals may respond to periods of starvation with increased food uptake (Regalado et al. 2017) as well as compensatory growth (i.e. a faster growth rate) or catch-up growth (i.e. attaining a minimum size by prolonging the larval development) (Hector and Nakagawa 2012). Moreover, starvation leads to substantial shifts in gene expression within individuals (Moskalev et al. 2015; Jiang et al. 2019; Etebari et al. 2020; Farahani et al. 2020). However, little is known how long these effects may persist (McCue et al. 2017) and to which extent parental starvation may affect gene expression of offspring experiencing starvation.

In the present study, we investigated the effects of parental early life starvation and parental reproductive environment on offspring (i.e. intergenerational effects), under matching or mismatching larval starvation conditions, using the turnip sawfly, *Athalia rosae* (Hymenoptera: Tenthredinidae). Adult females oviposit on various species of Brassicaceae, including crop species, on which *A. rosae* can become a pest (Riggert 1939; Sawa et al. 1989). The leaf-consuming larvae can readily experience periods of starvation when their hosts are overexploited (Riggert 1939). The adults are nectar-feeding and in addition collect neo-clerodanoid-like compounds (hereafter called ‘clerodanoids’) from non-Brassicaceae plants, which improve their mating probability (Amano et al. 1999) and affect the social interactions between adults of both sexes (preprint Paul et al. 2021; preprint Paul and Müller 2021). We measured various key life-history traits to assess the combined and interactive effects of parental and offspring larval resource availability (starvation/non-starvation), and parental clerodanoid exposure on the offspring. Moreover, we measured effects of different early life starvation regimes on consumption and gene

expression of offspring. We predicted that 1) there will be stronger intragenerational than intergenerational effects of larval starvation treatment on all parameters measured; 2) matching conditions between parental and offspring starvation treatment are beneficial, while a mismatch leads to a reduced performance; 3) offspring phenotype will also be influenced via parental effects such as silver spoon or parental buffering effects that may augment or dampen potential mismatch effects; 4) clerodanoid exposure will increase parental investment in offspring due to an increased mate attractiveness, leading to positive effects for the offspring.

Materials and Methods

Set-Up of Insect Rearing and Plant Cultivation

Adults of *A. rosae* were collected in May 2019 from meadows adjacent to farmland in Germany at two locations (population A 52°02'48.0"N 8°29'17.7"E, population B: 52°03'54.9"N 8°32'22.2"E). Adults from each population were split between two cages (total of 4 cages) and maintained for one generation at room temperature and 16 h: 8 h light:dark on potted plants of white mustard (*Sinapis alba*) and Chinese cabbage (*Brassica rapa* var. *pekinensis*). F1 adults were used to set up a total of six cages with males and females from different populations in four cages and two cages with virgin females only (Fig. 1). This sawfly species is haplodiploid, i.e. virgin females produce male offspring (Naito and Suzuki 1991). Larvae of the final instar, the eonymphs, were placed into individual cups containing soil for pupation. Emerged adults were kept individually in Petri dishes and provided with a honey:water mixture (1:50). For mating, pairs of non-sib F2 females and males (N = 20 per treatment level) were placed together and allowed to mate at least once. Afterwards, mated as well as virgin females were placed individually into boxes (25 x 15 x 10 cm) with a middle-aged leaf of 6-8 week old cabbage plants supplied with water for oviposition and a honey:water mixture in a climate chamber (20 °C:16 °C, 16 h: 8 h light:dark,

146 70% r.h.). The boxes were checked and topped up with honey water daily and an
147 additional leaf was added if the first leaf showed signs of wilting. Females were
148 removed from the boxes after one week and their offspring used to set up the
149 experimental generations (Fig. 1).

150 Plants of *B. rapa* and *S. alba* were grown from seeds (Kiepenkerl, Bruno Nebelung
151 GmbH, Konken, Germany) in a greenhouse (20 °C, 16 h: 8 h light:dark, 70% r.h.) and
152 a climate chamber (20 °C, 16 h: 8 h light:dark, 70% r.h.). Plants of *Ajuga reptans*
153 used for clerodanoid supply were grown from seeds (RHS Enterprise Ltd, London,
154 UK) in the greenhouse and transferred outside in late spring once ~2 months old.
155 Medium aged leaves were picked from non-flowering plants when plants were
156 approximately 8 months old.

157

158 *Experimental Set-Up and Measurements of Life-History Data*

159 The leaves of *B. rapa* offered to F2 females were checked daily for hatching larvae.
160 Hatched larvae were individually placed into Petri dishes (5.5 cm diameter) with
161 moistened filter paper and *B. rapa* leaf discs cut from 7-10 week old plants. Leaf
162 discs were provided *ad libitum* (except during starvation periods) and replaced daily.
163 Each dish was checked daily for the presence of exuviae to track the larval instar.
164 Larvae from each box (parental pair) in both the F3 and F4 generation were split
165 equally between one of two treatments, no starvation (N) or starvation (S). Starvation
166 individuals were twice starved for 24 h, first the day after moulting into 2nd instar (L2)
167 and second on the day of moulting into 4th instar (L4) to minimize early mortality
168 whilst mimicking the food deprivation larvae may experience when occurring in high
169 densities (Riggert 1939).

170 Eonymphs were placed in individual cups with soil for pupation. On the day of
171 emergence, adults were weighed (Sartorius AZ64, M-POWER Series Analytical
172 Balance, Göttingen, Germany), kept individually in Petri dishes and provided with

honey:water mixture. For the F3 adults, pairs of non-sib females and males reared under the same larval starvation treatments (N or S) were assigned to one of two reproductive environment treatments (C- or C+). C+ individuals were exposed to a leaf section (1 cm^2) of *A. reptans* for 48 h prior to mating, giving individuals time to take up clerodanoids (preprint Paul et al. 2021). Mated F3 females (2-9 days old) from each of these four treatments (NC-, NC+, SC-, SC+) and C- virgin females (NC-, SC-) were then placed in individual breeding boxes, as described above for the F2. The experimental generations (F3-F4) were reared to test the effects of parental (F3) and offspring (F4) larval starvation, and their interaction with the adult reproductive environment on the offspring phenotype (F4). In both generations, larval, pupal and total development time (from larva to adult) as well as the adult body mass at the day of emergence were taken for each individual. In total 358 F3 larvae (177 females, 181 males) and 486 F4 larvae (282 females, 202 males) reached adulthood, out of the 607 and 688 larvae, respectively, that were reared in each generation. Sample sizes for the F3 and F4 generation are given in Fig. 1. Experimental rearing and consumption assays were all carried out in a climate chamber (20 °C:16 °C, 16 h: 8 h light:dark, 70% r.h.).

Consumption Assays

Consumption assays were performed with F4 larvae at the start of L3 to measure the relative growth rate (RGR), relative consumption rate (RCR), and efficiency of conversion of ingested food (ECI). Each L3 larva was weighed (= initial body mass) (ME36S, accuracy 0.001 mg; Sartorius, Göttingen, Germany) and provided with four fresh leaf discs (surface area of 230.87 mm^2 per disc). After 24 hours, larvae were weighed again (= final body mass) and the leaf disc remains were scanned (Samsung SAMS M3375FD, resolution 640 x 480). The total area of leaf consumed

199 (mm^2) was then calculated as the difference between the average leaf area and the
200 remaining leaf area.
201
202 *Statistical Analyses*
203 All data were analyzed using R 4.0.2 (2020-06-22) -- "Taking Off Again". We set α =
204 0.05 for all tests and model residuals were checked for normality and variance
205 homogeneity. Stepwise backwards deletion using Chi-squared ratio tests
206 (package:MASS) was employed to reach the minimum adequate model (Crawley
207 2012). Posthoc analyses were carried out using the package 'multcomp' (Hothorn et
208 al. 2008). Post data entry, raw data were visually inspected thrice, all variables
209 plotted and outliers and possible anomalies in the data (e.g. strings of similar values)
210 interrogated (package:pointblank). Intragenerational effects of starvation on life-
211 history traits of F3 individuals were tested as described in S1.
212 Due to observations made during the experiment (no *a priori* hypothesis), we tested
213 in the F4 generation whether the likelihood of an additional larval instar (7 for females
214 and 6 for males) differed based on offspring larval starvation treatment (OS: N or S)
215 using a binomial generalized linear mixed model (glmm), where the predictor was OS
216 and parental pair was included as a random effect. The effects of the parental larval
217 starvation treatment (PS: N or S), parental clerodanoid exposure (PC: C- or C+), OS
218 and their interaction on larval, pupal and total developmental time as well as adult
219 mass of F4 individuals were assessed in separate linear mixed models (lmm), with
220 parental pair included as a random effect (controlling for non-independence of sibling
221 larvae). Female and male data were analyzed separately to enable model
222 convergence. Male adult mass was log-transformed to improve model fit.
223 RGR, RCR, and ECI (Castagneyrol et al. 2018) of F4 larvae were analyzed using
224 glmm. We excluded PC as a predictor variable from the consumption assay analysis
225 due to the low number of individuals in certain treatments (Fig. 1) and analyzed male

226 and female data separately as above. To assess variation in RGR, the change in
227 larval body mass [final mass — initial mass] was used as the response variable and
228 initial larval body mass, PS, OS, and both their three-way and two-way interactions
229 as the predictors. To assess variation in RCR, we used the total area of consumed
230 leaf material as the response variable and initial larval body mass, PS, OS, and both
231 their three-way and two-way interactions as the predictors. To assess variation in
232 ECI, we used the change in larval body mass as the response variable and the total
233 area of consumed leaf material, PS, OS, and both their three-way and two-way
234 interactions as the predictors. Parental pair was included in all three models as a
235 random effect.
236
237 *Sample Collection, Library Preparation, Sequencing and Alignment for RNAseq*
238 A total of 24 male larvae (L4, 9 d old), comprising six biological replicates per
239 treatment level (F3/F4: N/N, N/S, S/N, S/S, all C-, Fig. 2), were collected to
240 investigate the effects of parental and offspring starvation treatment on gene
241 expression in larvae of the offspring generation (F4). Individuals were chosen in a
242 way that maximized the equal spread of siblings across treatments and frozen at -80
243 °C prior to extraction. RNA was extracted with an Invitrogen PureLink™ RNA Mini Kit
244 (ThermoFisher Scientific, Germany), including a DNase step (innuPREP DNase I Kit,
245 analyticJena, Jena, Germany). RNA quality was assessed on a bioanalyzer 2100
246 (Agilent, CA, United States) and Xpose (PLT SCIENTIFIC SDN. BHD, Malaysia).
247 Library preparation (Ribo-Zero for rRNA removal) and sequencing (NovaSeq6000
248 and S4 Flowcells, Illumina, CA, United States) were provided by Novogene
249 (Cambridge, UK). Sequence quality before and after trimming was assessed using
250 FastQC (v. 0.11.9; Andrews 2010). After short (< 75 bp), low quality (Q < 4, 25 bp
251 sliding window) and adapter sequences were removed using Trimmomatic (Bolger et
252 al. 2014), more than 98% of reads remained. Reads were mapped to the annotated

253 genome of *A. rosae*, version AROS v.2.0 (GCA_000344095.2) with RSEM v1.3.1 (Li
254 and Dewey 2011), which implemented mapping with STAR v2.7.1a (Dobin et al.
255 2013).

256 Differential expression analysis was conducted with DESeq2 (version 1.28.1; Love
257 et al. 2014). RSEM results were passed to DESeq2 for gene-level analyses using
258 Tximport (version 1.16.1; Soneson et al. 2015). Prior to analysis, genes with zero
259 counts in all samples and those with low counts (< 10) in less than a quarter of
260 samples (6) were excluded. Model fitting was assessed by plotting dispersion
261 estimates and outliers were inspected using principle component analysis of all
262 expressed genes and pairwise-distance matrices between samples. DESeq2 allows
263 for both the normalization of read counts (accounting for differences in library
264 depth/sequencing depth, gene length, and RNA composition) and the shrinkage of
265 gene-wise dispersion (accounting for differences in dispersion estimates between
266 genes) whilst modelling a negative binomial glm of predictors for each gene to correct
267 for overdispersion (Love et al. 2014). Expression was modelled based on the entire
268 dataset, with the four levels representing the combination of parental and offspring
269 starvation treatments (F3/F4): N/N, N/S, S/N, and S/S. Differential expression was
270 based on four pairwise comparisons between the treatments: 1) N/N vs N/S and 2)
271 S/N vs S/S were used to assess the effects of offspring starvation treatment for
272 individuals whose parents experienced the same starvation treatment, whereas 3)
273 N/N vs S/N and 4) N/S vs S/S were used to assess the effects of differing parental
274 starvation treatment on individuals that experienced the same offspring starvation
275 treatment. Significance was based on a Wald Test and shrunken LFC values with
276 apeglm (version 1.10.1; Zhu et al. 2019). P-values were adjusted for multiple testing
277 using Benjamini-Hochberg (Benjamini and Hochberg 1995) procedure with a false
278 discovery rate (FDR) of 0.05. Afterwards, significantly differentially expressed genes
279 (relatively up- or downregulated) were extracted and filtered with a p-adjust of < 0.05.

280 Among these significantly differentially expressed genes we searched for the terms
281 linked to genes with known roles in stress and/or starvation response, using the
282 keywords: “heat shock protein” (or “hsp”), “cytochrome P450”, “octopamine” and
283 “tyramine” in the putative gene names. Venn diagrams (Venn.Diagram, version
284 1.6.20) were used to depict the relationship between the differentially expressed
285 genes for each pairwise comparison. The expression of the significantly expressed
286 genes of each comparison was visualized in a heatmap using normalized counts
287 scaled per gene (scale=“row”) (pheatmap, version 1.0.12).
288 Unmapped reads were extracted to check for the differential expression of genes
289 not present in the reference genome from the RSEM-produced BAM file using
290 samtools view (Li et al. 2009) and converted to fastq (using bamtools bamtofastq;
291 Barnett et al. 2011). The unmapped reads were mapped back to this reference and
292 expected read counts extracted using eXpress (Roberts et al. 2011). Transcripts with
293 >10,000 mapped reads were identified using BLASTN (default settings, limited to one
294 match per gene and $1e^{-1}$) against the NCBI nucleotide database. This process was
295 done jointly with reads from additional samples (preprint Paul et al. 2021) to optimize
296 coverage for the assembly. Differential expression of unmapped reads in the larval
297 samples was carried out as for mapped reads (see above).
298 For characterization of differential expression at the pathways level, KEGG (Kyoto
299 Encyclopedia of Genes and Genomes) terms were assigned to the predicted genes
300 in the *A. rosae* genome using the KEGG Automatic Annotation Server (KAAS; Moriya
301 et al. 2007) (S2). Of the annotated genes for which we had read counts, 65% were
302 assigned to at least one KEGG pathway. A gene set enrichment analysis was then
303 performed on the normalized counts using GAGE (Luo et al. 2009) and pathview
304 (Luo and Brouwer 2013) in R, applying an FDR-adjusted *P*-value cut-off of < 0.05 to
305 identify differentially expressed pathways. We derived the non-redundant significant

306 gene set lists, meaning those that did not overlap heavily in their core genes, using
307 esset.grp and a *P*-value cut-off for the overlap between gene sets of $10e^{-10}$.
308

309 **Results**

310 *Life-History Traits*

311 In the parental generation (F3), larval starvation had trait- and sex-specific effects on
312 life-history, leading to a prolonged total development time for both sexes and a lower
313 body mass in females (for details see S1). In the offspring generation (F4), both
314 females (X^2_1 = 11.06, P < 0.001) and males (X^2_1 = 29.25, P < 0.001) were
315 significantly more likely to have an additional larval instar prior to the eonymph stage
316 if they were starved than if they were not starved (Fig. 2A,B). The exposure of
317 parents to clerodanoids (PC) had no influence on any of the life-history parameters
318 measured in the offspring, whereas the effect of parental (PS) and offspring larval
319 starvation treatment (OS) were trait- and sex-specific. There was an interactive effect
320 of PS and OS on both female (PS * OS: X^2_1 = 10.37, P = 0.001) and male (PS * OS:
321 X^2_1 = 4.89, P = 0.027) total development time, such that intra-generational starvation
322 led to a longer development time for both sexes (Fig. 2C,D; S3A, S3B). Larval
323 development of F4 females was only affected by OS (OS: X^2_1 = 319.40, P < 0.001;
324 S4A), while there was an interactive effect of PS and OS on male larval development
325 time (PS * OS: X^2_1 = 10.37, P = 0.001; S3B, S4B). The reverse occurred for pupal
326 developmental time with an interactive effect of PS and OS on females (PS * OS: X^2_1
327 = 24.57, P < 0.001, S3A, S4C), but only a significant effect of OS on males (OS: X^2_1
328 = 17.23, P < 0.001; S4D). Both PS (X^2_1 = 4.02, P = 0.045) and OS (X^2_1 = 12.95, P <
329 0.001) independently influenced female adult mass (PS * OS: n.s.), with F4 females
330 having a lower mass if they had experienced starvation during development, but
331 those females whose parents were starved had a higher overall mass than when
332 parents had not starved (Fig. 2E). In contrast, F4 male adult mass was significantly

333 affected by the interaction between PS and OS (PS *OS: X^2_1 = 4.58, P = 0.030, Fig.
334 2F). Male offspring of starved parents that themselves were not starved during
335 development (S/N) had a higher mass than offspring that were starved (S/S)
336 (pairwise comparison: z = -3.47, P = 0.003, Table S3B).
337
338 *Consumption*
339 OS had a significant effect on the relationship between change in mass and initial
340 body mass (i.e. RGR) in offspring larvae in both females (initial body mass * OS:
341 $F_{1,60.75}$ = 5.96, P = 0.017) and males (initial body mass * OS: $F_{1,39.54}$ = 7.32, P =
342 0.009). The change in body mass of starved larvae increased more steeply with an
343 increase in initial larval body mass compared to non-starved larvae for both sexes
344 (Fig. 3A,B), indicating a higher growth rate (RGR) in starved larvae. There was no
345 effect of PS on RGR either independently or interactively for both sexes. In contrast
346 to RGR, OS did not have a significant effect on RCR either independently or
347 interactively for both sexes. For females, there was a significant interaction effect of
348 initial larval body mass and PS on the area of leaf consumed (initial body mass * PS:
349 $F_{1,62}$ = 6.15, P = 0.015), such that the area increased with initial body mass for
350 individuals from non-starved parents but did not change for individuals from starved
351 parents (Fig. 3C); i.e. larger larvae consumed more than smaller larvae when their
352 parents were not starved but not when their parents were starved. For males, initial
353 body mass had a significant positive effect on the area of leaf consumed (initial body
354 mass: $F_{1,41.45}$ = 21.97, P < 0.001) across all treatments (Fig. 3D); i.e. there was no
355 effect of either OS or PS on RCR in males. For ECI (change in mass per unit leaf
356 area consumed), leaf area consumed and OS independently influenced change in
357 body mass for both females (leaf area consumed * $F_{1,54.12}$ = 7.17, P = 0.009; OS:
358 $F_{1,54.77}$ = 11.36, P = 0.001) and males (leaf area consumed * $F_{1,44}$ = 31.55, P < 0.001;
359 OS: $F_{1,44}$ = 6.92, P = 0.011). The ECI was higher when larvae were not starved than

360 when they were starved, but under both conditions consuming more led to a higher
361 body mass increase for larvae (Fig. 3E, F).
362
363 *Gene Expression*
364 Sequencing of the 24 larvae resulted in a total of 1.4 billion reads with an average of
365 58 million reads per sample (SE: ±2 million reads) and an average GC content of
366 43%. Prior to normalization, the average number of mapped reads per sample was
367 33,675,600, equating to an average of 91% reads per sample aligned to the
368 reference genome (range 85-93%).
369 Offspring starvation had the strongest effect on gene expression (Figs 4, 5, S5).
370 The two comparisons between starved and non-starved larvae revealed 4727 (N/S vs
371 N/N) and 5089 (S/N vs S/S) significantly differentially expressed genes (LFC > 0,
372 adjusted $p < 0.05$) and a large number of these overlapped between the two
373 comparisons (4002). In contrast, there were far fewer significantly differentially
374 expressed genes in the two comparisons in which the larvae had the same starvation
375 treatment, namely 155 (S/N vs N/N) and 75 (N/S vs S/S). In terms of genes known to
376 be associated with stress response, in comparison to non-starved larvae when larvae
377 from non-starved parents were starved, there was significant downregulation of
378 putative heatshock proteins (8 downregulated and 1 upregulated) and upregulation of
379 putative cytochrome P450 genes (21 genes upregulated, 6 downregulated) as well
380 as upregulation of 1 octopamine and 1 tyramine gene. When parental starvation
381 conditions but not offspring starvation treatments differed, there was far less
382 differential expression of such genes with only one or two cytochrome P450 genes
383 being differentially expressed (S6).
384 While the majority of reads mapped to the reference genome, 247,997,896 reads
385 did not map; we assembled these into 334,717 transcripts using Trinity (default
386 settings) (Grabherr et al. 2011). Of those genes that we assembled *de novo*, 803

387 were putatively annotated against the NCBI nt database and of these a large portion
388 were mitochondrial (Table 1). DE analysis of unmapped reads showed that a lower
389 number of genes were differential expressed between the treatment pairs [1523 (N/S
390 vs N/N), 1860 (S/N vs S/S), 149 (S/N vs N/N) and 34 (N/S vs S/S)]. Many of these
391 were *A. rosae* genes linked to the mitochondria, particularly in the comparisons
392 between starved and non-starved offspring individuals.
393 The KEGG pathway level analysis showed that across all four comparisons the
394 ribosomal pathway was the most consistently differentially regulated, being
395 upregulated when offspring were starved and downregulated when they were not.
396 Interestingly, ribosomal regulation was affected not only by differences in offspring
397 starvation treatment but also by differences in parental starvation treatment. The
398 pathway was upregulated in starved larvae when compared to larvae that were not
399 starved (offspring treatment differed) and to larvae that were but whose parents were
400 also starved (parental treatment differed), with the reverse trend for non-starved
401 larvae (Table 1). The other significantly differentially expressed KEGG pathways only
402 occurred between individuals that differed in offspring but not parental larval
403 starvation treatments, mirroring the gene expression results. These pathways belong
404 to the four main categories: metabolic breakdown and protein processing (pancreatic
405 secretion, proteasome and protein processing in the endoplasmic reticulum, ER),
406 immune response (phagosome and antigen processing), energy production and
407 central metabolic processes (TCA/citrate cycle, glycolysis, thyroid hormone signalling
408 pathway), and ECM-receptor interaction. There was generally a downregulation of
409 these processes in larvae that were starved compared to non-starved individuals.
410

411 **Discussion**

412 We studied the influence of intergenerational effects on offspring phenotype in the
413 holometabolous insect *A. rosae*, which realizes distinct niches during its life-cycle.

414 Our results revealed that offspring starvation interacted with parental starvation in a
415 trait-and sex-specific manner for life-history traits. There was an interactive effect of
416 intergenerational (parental) and intragenerational (offspring) starvation treatment on
417 total development, male larval development, and female pupal development time.
418 The trend for faster development times in offspring that experienced a similar
419 environment as their parents was at least significant for female pupal development
420 time and may be suggestive of positive effects of matching conditions, i.e. a match-
421 mismatch scenario (Monaghan 2008). A matching of offspring phenotypes to
422 environments experienced by their parents is possible due to developmental plasticity
423 and potentially based on epigenetic effects (Le Roy et al. 2017). Such parental
424 effects have been found in a number of species in response to food availability
425 (Hibshman et al. 2016; Raveh et al. 2016) and can be sex-specific (Le Roy et al.
426 2017). However, other life history data of *A. rosae* did not reveal this match-mismatch
427 pattern, indicating a complex interplay between different intergenerational effects.
428 Adult mass showed a different pattern than development time, with female
429 offspring of *A. rosae* having a higher adult mass when their parents were starved as
430 larvae irrespective of their own starvation conditions, indicative of enhanced parental
431 provisioning to offspring of starved parents, i.e. parental buffering. Similar effects
432 have also been observed in the vinegar fly, *Drosophila melanogaster*, where females
433 reared on poor food had larger offspring than females reared on standard food
434 (Valtonen et al. 2012). This finding is consistent with life-history theory prediction that
435 under hostile conditions there is a shift towards fewer but better provisioned offspring
436 (Smith and Fretwell 1974; Roff 1992), although we did not examine the number of
437 offspring produced in our study. As neither larval developmental time for female *A.*
438 *rosae* nor their consumption (RGR and ECI) were affected by the parental starvation
439 treatment, other physiological effects (discussed below for the gene expression) may
440 mediate this kind of buffering, visible in the higher adult body mass of individuals

441 whose parents were starved. In contrast, for male *A. rosae*, only offspring of starved
442 parents differed significantly in adult mass, with lower mass in starved sons. Thus,
443 investment in body mass may be particularly low in males under repeatedly (across
444 generations) experienced poor environmental conditions, indicating "paternal
445 suffering" rather than buffering. Such trait-specific and sex-specific effects of
446 intergenerational treatment and interaction with intragenerational treatment have
447 been seen in previous studies (Zizzari et al. 2016; Le Roy et al. 2017; Wilson et al.
448 2019; Yin et al. 2019), suggesting that context-dependent effects of intergenerational
449 starvation may be common. We can particularly expect sex-specific differences as a
450 result of sex-specific directional selection on life-history traits (Tarka et al. 2018).
451 Overall, there was no clear indication of silver spoon effects for any of the life-history
452 traits, but such effects cannot be entirely ruled out (Engqvist and Reinhold 2016).
453 Consumption assays revealed that starved larvae had a marginally higher growth
454 rate (RGR) but a lower consumption efficiency (ECI) than non-starved larvae,
455 irrespective of their parental starvation treatment. Since we conducted the
456 consumption assays for all larvae at the same developmental stage, we were able to
457 disentangle the effect of starvation from effects due to differences in ontogeny
458 (Nicieza and Álvarez 2009), in contrast to earlier studies on this sawfly (Paul et al.
459 2019). Offspring that experienced starvation exhibited a steeper RGR than non-
460 starved larvae, suggesting compensatory growth (Hector and Nakagawa 2012).
461 Combining this compensatory growth with a longer development period potentially
462 allows starved individuals to become larger and to overcome size-based selection,
463 which can be an important determinant of individual fitness for many insect species
464 (Beukeboom 2018). While such compensatory growth has been seen in many
465 species, it can also pose consequent costs to individuals (Arendt et al. 2001;
466 Dmitriew and Rowe 2007; Auer et al. 2010). We found that individuals that were
467 starved had a lower ECI than non-starved individuals, which may suggest a

468 physiological cost of compensatory growth. For the RCR we found differences in
469 responses between sexes, with female offspring consuming different amounts of leaf
470 material depending on their parental starvation treatment, while male RCR was solely
471 influenced by body mass. Considering the larger body mass of female offspring from
472 starved parents, we would have expected such individuals to consume more leaf
473 material, although the opposite pattern was observed. Adjustments in consumption
474 patterns to different starvation regimes have been found to be expressed in the next
475 generation (McCue et al. 2017). However, patterns may differ depending on the
476 developmental stage in which an individual is facing starvation, which needs to be
477 further explored in *A. rosae*.

478 Larval starvation led to an additional larval instar in *A. rosae* of both sexes. For this
479 sawfly species, usually 6 instars for female and 5 instars for males are found (Sawa
480 et al. 1989). Increasing the number of larval instars is potentially an adaptive strategy
481 that allows individuals to recover from starvation via the prolongation of
482 developmental time, e.g. by catch-up growth (Hector and Nakagawa 2012). Such
483 intraspecific variation in the number of larval instars is common in insect species and
484 can occur in response to various biotic and abiotic factors such as injuries, food
485 availability and quality, temperature, and humidity (Esperk et al. 2007). Intraspecific
486 variability in instar numbers in response to food availability and quality can be in the
487 range of one to three instars, including tenthredinid species (Esperk et al. 2007), but
488 has not been described, to our knowledge, in the tenthredinid species *A. rosae*.

489 In contrast to our expectation, clerodanoid uptake, which is known to enhance
490 mate attractiveness in *A. rosae* (Amano et al. 1999; preprint Paul and Müller 2021),
491 had no effect on the measured traits in offspring of both sexes. This is surprising,
492 because partner attractiveness has been shown to affect investment into offspring
493 or/and offspring traits across a wide variety of species (Robart and Sinervo 2019),
494 including also multigenerational consequences (Gilbert et al. 2012; Wilson et al.

495 2019). In other species, mating with more attractive partners has mostly direct effects
496 for the partner, but not necessarily for the offspring. For example, in the field cricket,
497 *Gryllus firmus*, mating with more attractive males led to a higher number of eggs laid
498 by females but offspring did not show any fitness benefits (Kelly and Adam-Granger
499 2020). Instead other partner traits, such as body size, may influence offspring
500 (Booksmythe et al. 2017; Young and Long 2020), which needs further testing in *A.*
501 *rosae*. Alternatively, parental clerodanoid exposure may affect other traits, such as
502 immunity (Bozov et al. 2015), lifespan (Zanchi et al. 2021), or traits exhibited in
503 adulthood, e.g. mating success (Amano et al. 1999; preprint Paul and Müller 2021).
504 Parental effects that occur via differential allocation may also be context-dependent.
505 Transcriptome analysis of male larvae of the F4 generation also revealed stronger
506 intra- than intergenerational effects of larval starvation on gene expression, in line
507 with our hypothesis. If F4 larvae were starved, about half of the genes were
508 differentially expressed compared to non-starved larvae. As typical stress response
509 indicators, genes potentially encoding heat shock proteins and cytochrome P450s
510 were differentially expressed, being partly up- and partly downregulated in *A. rosae* in
511 response to starvation. Regulation of heat shock proteins in response to starvation
512 has been reported elsewhere, including larvae of Lepidoptera and Hymenoptera
513 (Farahani et al. 2020; Wang et al. 2012). These proteins act to stabilize and protect
514 other proteins in the face of both abiotic and biotic stresses (Sørensen et al. 2003;
515 Farahani et al. 2020). Cytochrome P450s are best known for their role in metabolism
516 of xenobiotics by insects (Feyereisen 2012). However, they were also found to be
517 more highly expressed in *D. melanogaster* flies selected for starvation resistance
518 compared to controls (Doroszuk et al. 2012). In addition, genes putatively encoding
519 the monoamines octopamine and tyramine were upregulated in starved larvae of *A.*
520 *rosae*, both of which play a key role in modulating behavioral and physiological

521 processes in invertebrates and can be involved in starvation resistance (Li et al.
522 2016).
523 Furthermore, starvation of the offspring led to differential gene expression of
524 pathways involved in metabolism, including pancreatic secretion, which is also found
525 in starved larvae of the cotton bollworm, *Helicoverpa armigera* (Lepidoptera:
526 Noctuidae) (Jiang et al. 2019). In starved larvae of *A. rosae*, differential regulation of
527 metabolic pathways may also explain why these larvae showed an overall lower ECI
528 compared to non-starved larvae. Expression of metabolic genes likely also changes
529 with the duration of starvation. For example, in *D. melanogaster* a 16 h starvation
530 period led to a downregulation particularly of the endopeptidase gene family
531 (Moskalev et al. 2015), but extended periods of starvation may also induce an
532 activation of proteolysis genes, as found in different microorgansims (Nakashima et
533 al. 2006; Michalik et al. 2009). However, little is known about long-term effects,
534 including cross-generational, on gene expression patterns in response to starvation.
535 Moreover, many of the genes differentially expressed in starved vs non-starved
536 individuals of *A. rosae* linked to the mitochondria, reflecting wholescale disruption of
537 homeostasis caused by starvation (Gilbert 2012).
538 We found that parental starvation also caused changes in differential gene
539 expression of about 1 % of the genes of their offspring, indicating that subtle
540 intergenerational imprints on gene expression can occur. Ribosomal pathway genes
541 were enriched among downregulated genes when parents were starved vs non-
542 starved but offspring was not starved and upregulated when offspring were starved.
543 These results indicate that physiological buffering differs in dependence of the
544 parental as well as offspring starvation treatment. Larvae that were starved but
545 whose parents were not starved may have suffered more than starved larvae from
546 starved parents, as indicated by the upregulation of putative ribosomal pathway
547 genes. Differential expression of genes of the ribosomal pathway has been observed

548 in a wide variety of taxa in response to thermal stress (Paraskevopoulou et al. 2020;
549 Schwanz et al. 2020; Srikanth et al. 2020), demonstrating the broad role of this
550 pathway in stress response. Differential expression of genes involved in stress and
551 other physiological and metabolic responses may have energetic costs, which may
552 lead to a prolonged development time of starved larvae in *A. rosae*.

553 Alteration in ribosomal expression may also be an important mechanism mediating
554 intergenerational effects of starvation (Aldrich and Maggert 2015; Bughio and
555 Maggert 2019). In *Caenorhabditis elegans* starvation led to the generation of small
556 RNAs, and small RNA-induced gene silencing persisted over up to three generations
557 (Rechavi et al. 2014). Such mechanisms may also explain the intergenerational
558 changes in gene expression observed in *A. rosae* in the present study; future work
559 comparing small RNA expression in relation to starvation could address this. Further
560 work could also utilize tissue-specific gene expression to determine, where gene
561 expression changes are most prominent. For example, larvae of the soldier fly,
562 *Inopus falvus* (Diptera: Stratiomyidae), differentially express numerous genes in the
563 salivary glands in response to starvation stress (Etebari et al. 2020). Other tissues of
564 interest may be different sections of the gut.

565 In summary, our findings highlight that intragenerational starvation effects were
566 somewhat stronger than the intergenerational effects of starvation on various traits
567 investigated here, including life-history, consumption, and gene expression patterns.
568 Shaping of offspring occurred via parental effects including match-mismatch and
569 parental buffering, while there was no clear evidence for silver spoon effects. The
570 parental reproductive environment left no signature on the measured offspring traits.
571 Overall, due to different trajectories and environments experienced during different
572 stages, distinct niches are realized, which are expressed in a diversity of phenotypes.
573 In terms of wider consequences these results suggest that periods of starvation may

make individuals even more robust when facing another food shortage in the next generation and may thus lead to enhanced damage of crops.

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Data availability

All data in this manuscript will be deposited online on Zenodo.

Author contributions

Conceptualization and funding acquisition: CM; methods development/experimental design: SCP, CM; data collection: SCP, data validation and analysis: life history data: SCP, consumption assay data: SCP, PS, gene expression data: SCP, ABD; data visualization: SCP, PS; writing original draft: SCP, CM, PS; reviewing and editing: SCP, PS, ABD, CM.

Supplemental Material

S1. Effects of Starvation on Life-History Traits in F3 Individuals

S2. KEGG Term Analysis

S3. Results of Posthoc Analyses for F4 Generation

S4: Influence of Parental and Offspring Larval Starvation Treatments on Developmental Times of *Athalia rosae* in F4 Generation

601 S5. Percentage of Differentially Expressed Genes.

602 S6. Numbers of Differentially Expressed Genes.

603

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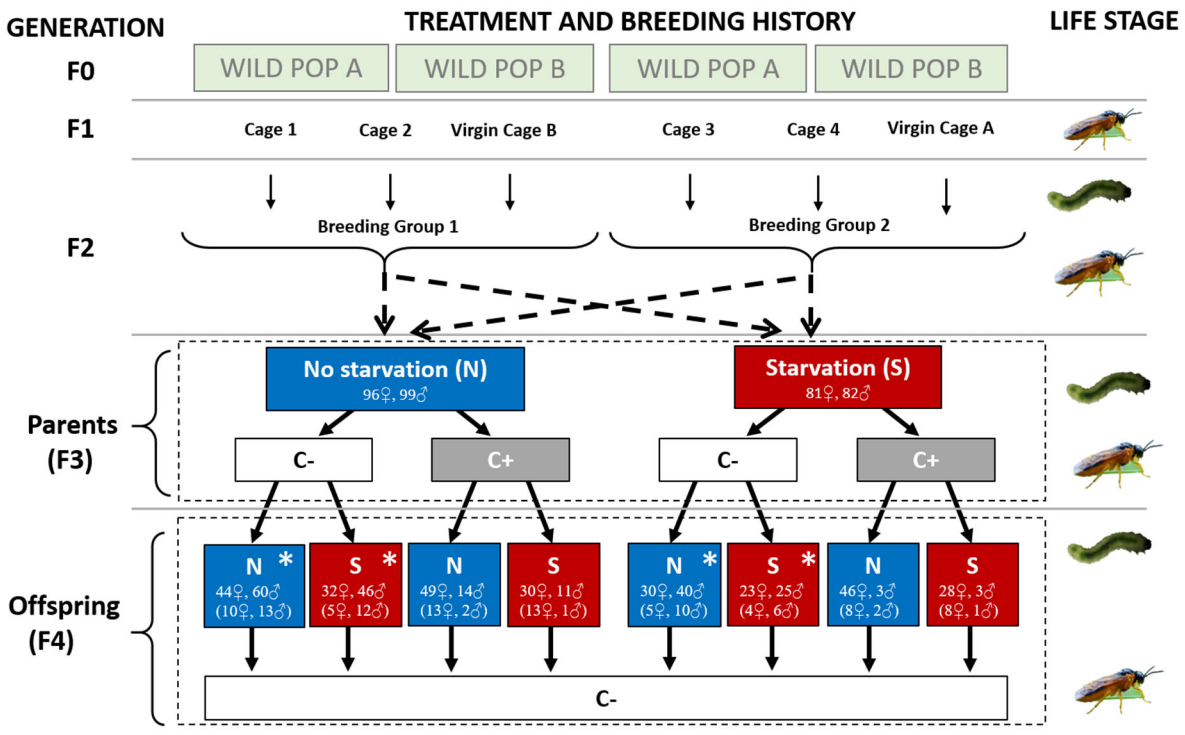
843

844 **Figures**

845 **Figure 1:**



846

847 **Figure 1:** Full experimental design showing outline of breeding structure and

848 experimental treatments from F0 to F4 generation in *Athalia rosae*; * indicates

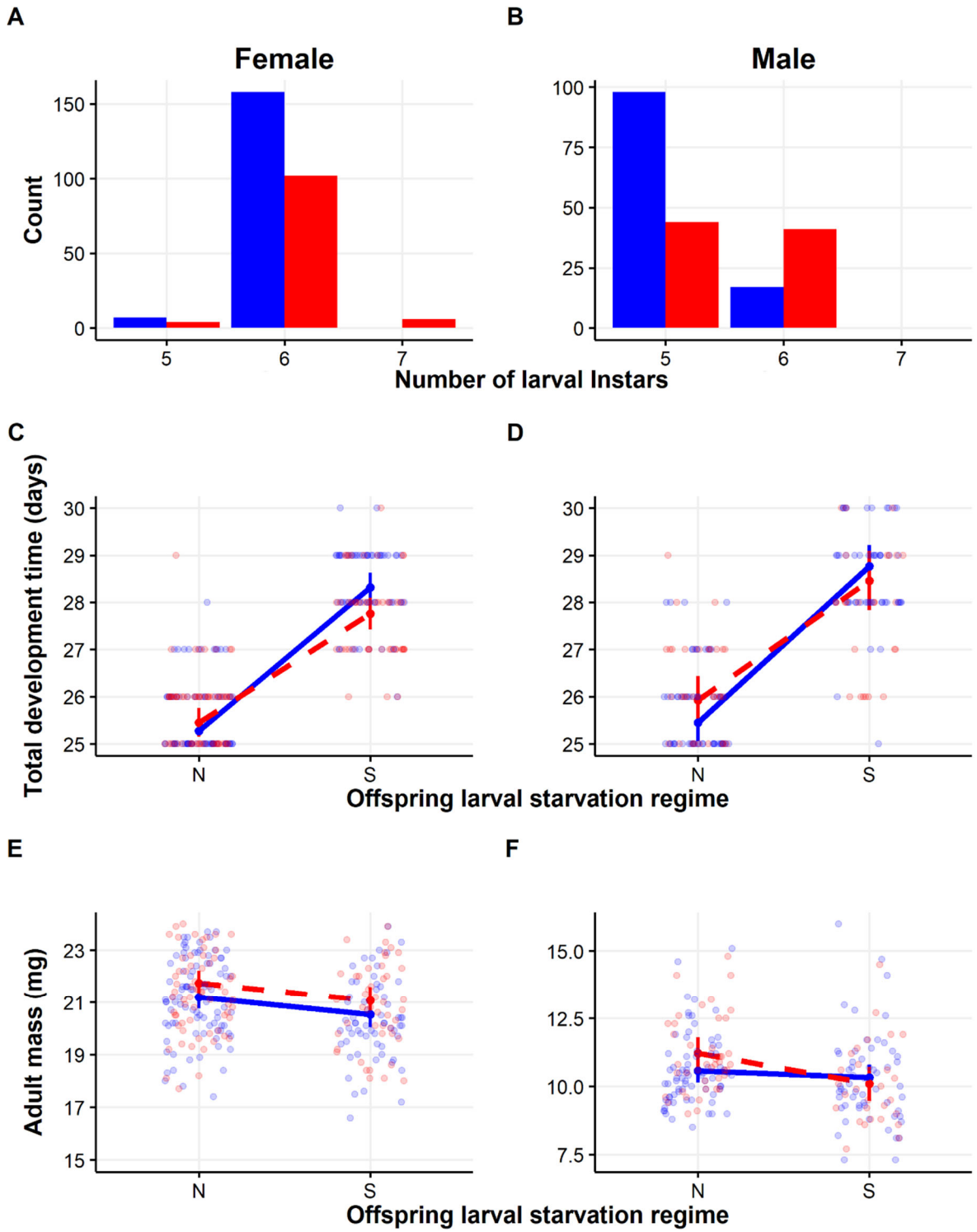
849 individuals taken for RNASeq analysis. Sample sizes split by sex are given in boxes

850 for F3 and F4 generation (numbers in brackets refer to sample sizes for consumption

851 assay).

852

853 **Figure 2**



854

855 **Figure 2:** Number of larval instars in F4 in dependence of offspring starvation

856 treatment (blue: non starved, red: starved) and influence of offspring larval starvation

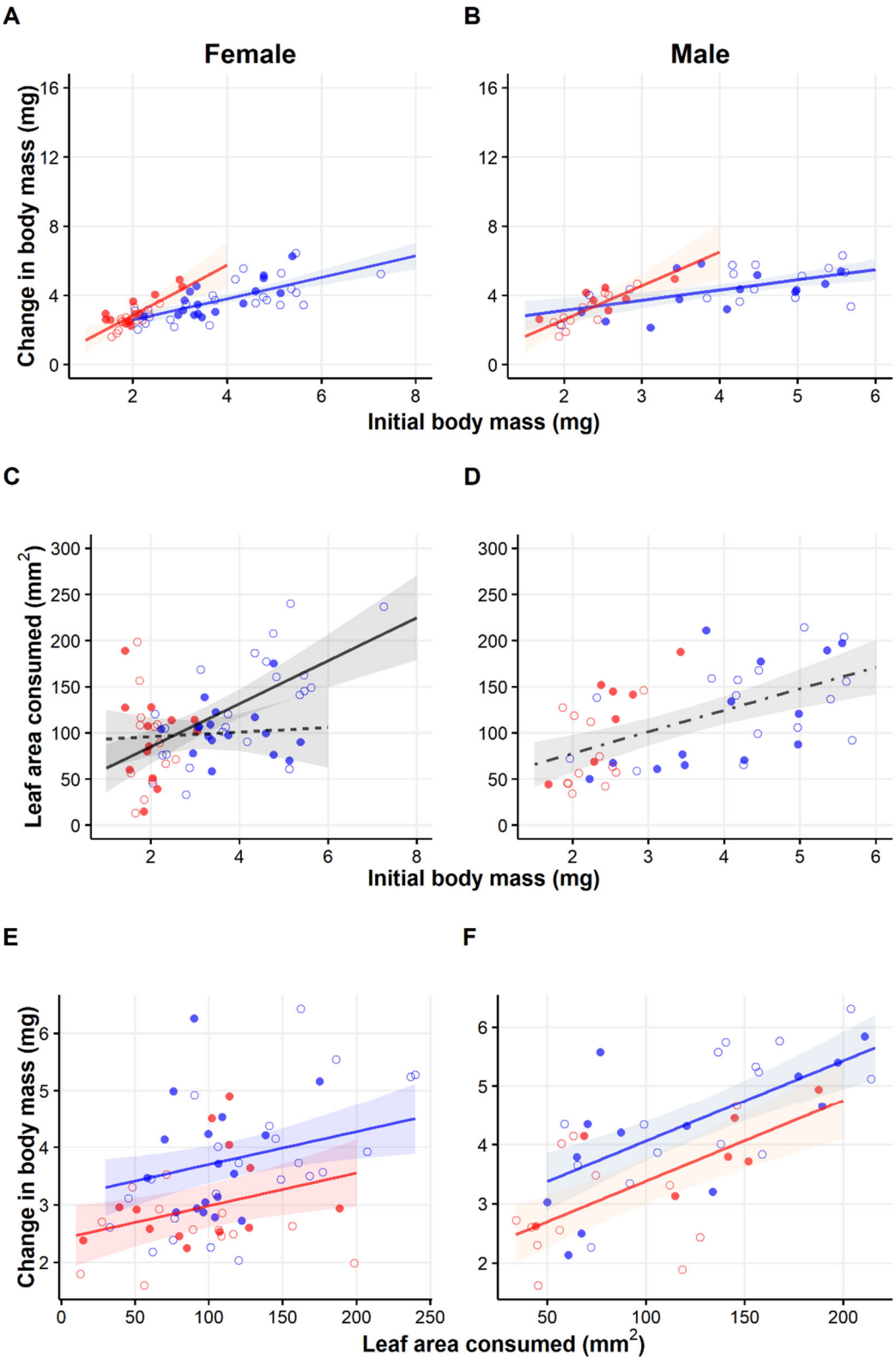
857 treatments (N = no starvation, S = starvation) and parental larval starvation

858 treatments (blue solid line = no starvation, red dashed line = starvation) on offspring

859 total development time (C, D) and adult body mass (E, F) of *Athalia rosae*. Data are

plotted separately for females (A, C, E) and males (B, D, F); individuals were sexed on emergence. Points are model predictions with associated confidence intervals and colors of points and lines correspond to parental starvation treatment. Raw data are plotted in transparent colors in the background.

865 **Figure 3**



866

867 **Figure 3:** Influence of offspring (blue line = no starvation, red line = starvation) and

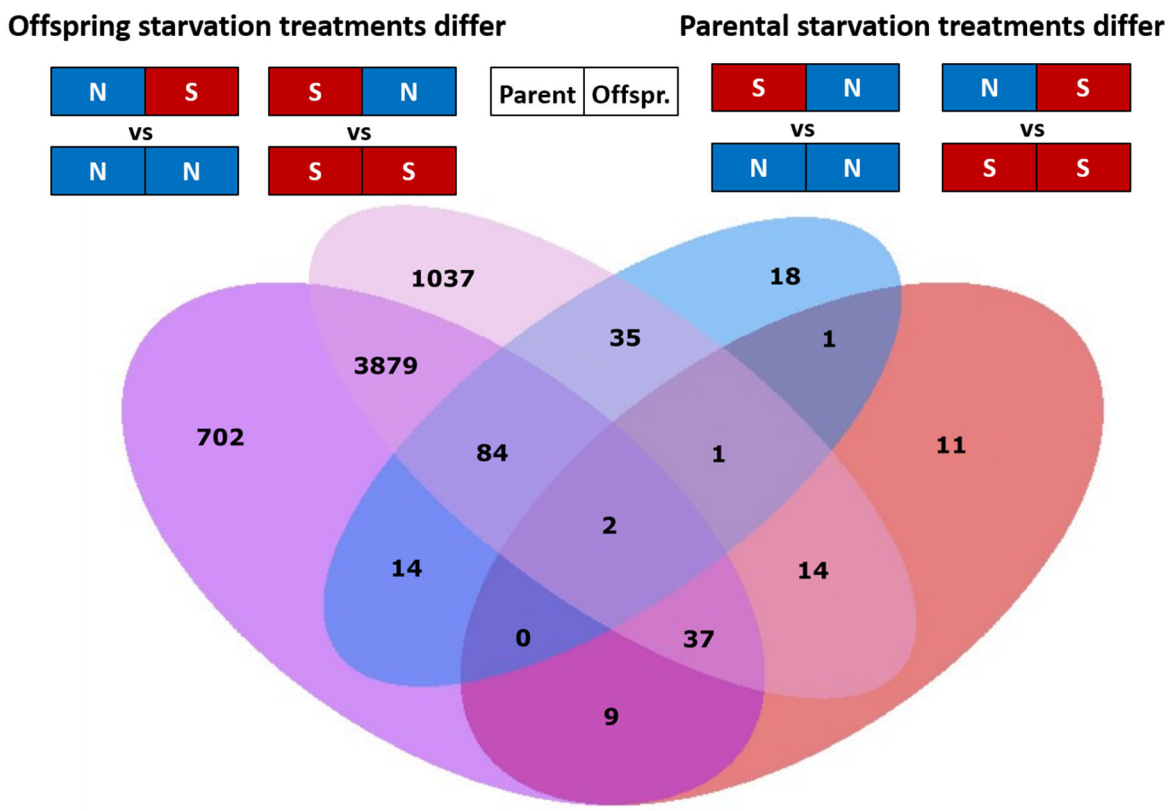
868 parental larval starvation treatments (solid line = no starvation, dashed line =

869 starvation) in F4 on relative growth rate, RGR (A, B); relative consumption rate, RCR

870 (C, D); and efficiency of conversion of ingested food, ECI (E, F) of *Athalia rosae*.

Note that neither offspring starvation treatment nor parental starvation treatment had an effect on RCR in males. Data are plotted separately for females (A, C, E) and males (B, D, F); individuals were sexed on emergence. Lines depict model predictions with shaded regions showing the 95% confidence interval range. Raw data representing offspring larval starvation treatment (blue = no starvation, red = starvation) and parental larval starvation treatment (open circles = no starvation, filled circles = starvation) are plotted in the background.

879 **Figure 4**



880

881

882 **Figure 4:** Venn diagram illustrating the number of unique and shared differentially

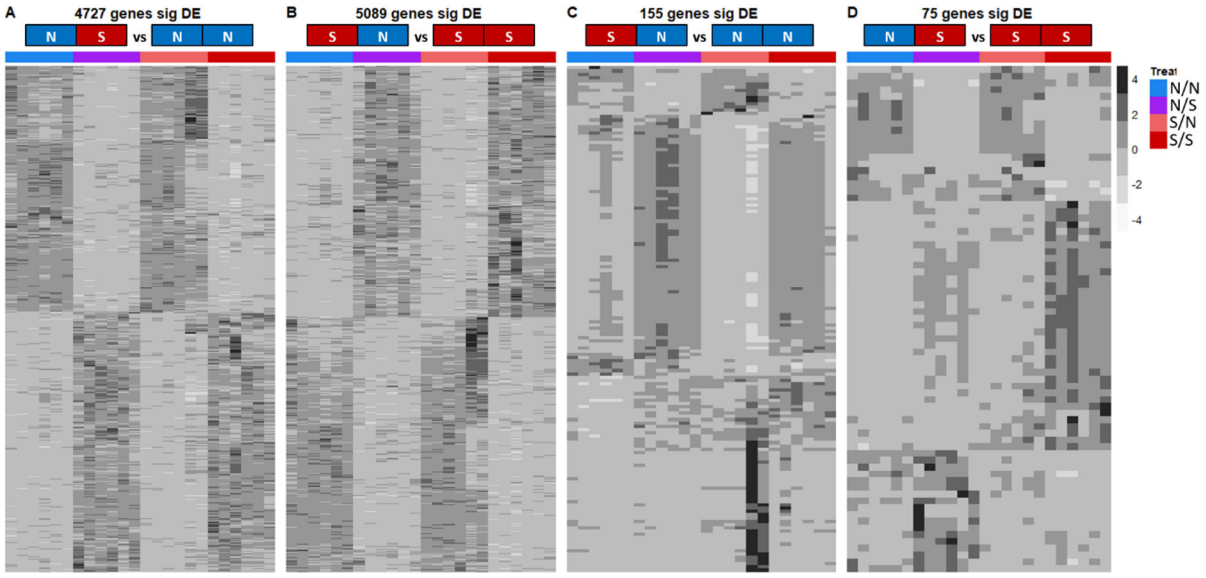
883 expressed genes in male offspring larvae (F4) of *Athalia rosae* resulting from each of

884 the four pairwise comparisons, (N = no starvation and S = starvation; left box F3,

885 right box F4).

886

887 **Figure 5**



888

889 **Figure 5:** Heatmaps showing the expression (normalized counts) of genes in male

890 offspring larvae (F4) of *Athalia rosae* across all samples for those genes that were

891 differentially expressed when offspring starvation treatment differed (A, B) or parental

892 starvation treatment differed (C, D), (N = no starvation and S = starvation; left box F3,

893 right box F4). Plotted values are *z*-scores computed from normalized counts post

894 clustering.

895

896 **Table 1.** Significantly differentially expressed KEGG gene sets/pathways male
897 offspring larvae (F4) of *Athalia rosae* that differed in either their own or their parent's
898 (F3) larval starvation regime (N = no starvation and S = two periods of starvation for
899 24 hours during larval development; left box F3, right box F4). The combination listed
900 first (on top) is the 'treatment' the one listed second (underneath) is the 'control' in
901 each comparison, meaning that pathways are up- or downregulated in 'treatment'
902 relative to 'control'. Larvae undergoing starvation (S) were subjected to two periods of
903 starvation for 24 hours during larval development, one in L2 and one in L4.

KEGG pathway	Offspring starvation treatment differs					Parental starvation treatment differs			
	N	S	S	N		S	N	N	S
	vs		vs			vs		vs	
	N	N	S	S		N	N	S	S
Ribosome	↑ up		↓ down			↓ down		↑ up	
Pancreatic secretion	-		↓ down			-		-	
Protein processing in endoplasmic reticulum	↓ down		↑ up			-		-	
Proteasome	↓ down		↑ up			-		-	
Phagosome	-		↑ up			-		-	
Citrate (TCA) cycle	↓ down		-			-		-	
Glycolysis / Gluconeogenesis	↓ down		-			-		-	
Antigen processing and presentation	↓ down		↑ up			-		-	
Protein export	↓ down		-			-		-	
ECM-receptor interaction	-		↑ up			-		-	
Thyroid hormone synthesis	-		↑ up			-		-	

904