Sociogenomics of maternal care and parent-offspring coadaptation in the European earwigs *(Forficula auricularia)*

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SUMMARY

Conflict and cooperation are ubiquitous in nature and in animal families where parents and offspring reciprocally influence each other's behavior and fitness. Evolutionary models predict selection for parent-offspring coadaptation that strike balance between parents pursuing self-fitness versus offspring demanding parental investment. Ultimately, it facilitates well-coordinated parenting and optimized cooperation with their offspring in the face of sexual reproduction and genetic recombination which cause genetic conflict. However, the genomic basis of parent-offspring coadaptation is poorly understood. My dissertation focused on the sociogenomics of materanl care and parent-offspring coadaptation in the European earwig (*Forficula auricularia),* a facultative uni-parental female care insect.

In the first chapter, we sequenced the transcriptome of the European earwig from various tissues and developmental stages of female and male applying Roche 454 pyrosequencing and Illumina HiSeq. The reads were *de novo* assembled independently and screened for possible microbial contamination and repeated elements. Hybrid assembly of these data yield comprehensive transcriptome with a low level of fragmentation comparing to the eukaryotic core gene dataset. More than 8,800 contigs of the hybrid assembly show significant similarity to insect-specific proteins and those were assigned for Gene Ontology terms. Finally, I validated the transcriptome and established a quantitative PCR method and applied it to homologs of five known sex-biased genes of the honeybee. The qPCR pilot study confirmed sex specific expression and also revealed significant expression differences between the brain and antenna tissue samples. The transcriptome presented here offers new opportunities to study the molecular bases and evolution of parental care and sociality in arthropods.

In the second chapter, I identified two parent-offspring coadapted genes, *PebIII* and *Th,* in the European earwig, based on comparative transcriptomics from experimentally manipulated mother-offspring interactions. Functional study applying RNAi revealed that *PebIII* in offspring enhances survival, in mothers enhances their relative investment in future reproduction and indirectly delayed offspring development; *Th* in mothers enhanced food provisioning, in offspring indirectly enhanced the likelihood of maternal future reproduction. These results suggested *PebIII* being reciprocally selfish while *Th* being reciprocally altruistic in both mothers and offspring. Metabolic pathway analyses

further indicated the role of *Th*-restricted dopaminergic reward, *PebIII* mediated chemical perception and regulations between insulin signaling, juvenile hormone and vitellogenin in parent-offspring coadaptation and social evolution.

In the third chapter, I manipulated the interaction between earwig mothers and offspring over two generation and investigated transgenerational effects of maternal care on the expression of the two parent-offspring coadapted genes found in chapter2 and the fitness consequences in mothers and offspring. Significant transgenerational effects were found for the expression of *PebIII* and *Th* in the head of mothers. The expression of *PebIII* in the whole body of offspring showed significant effects of transgeneration treatment, current generation treatment and current generation by transgeneration treatments interaction. Significant transgenerational effect was found for relative maternal investment in future reproduction and offspring growth rate. Maternal future reproduction and latency for maternal future reproduction showed significant effects of current generation parental care treatment. Our results indicates an epigenetic regulation of gene expressions underlying parent-offspring coadaptation.

In the last chapter, the expressions of parent-offspring coadapted genes were validated using Fluidigm gene expression dynamic array. An additional treatment was included to control for time effect. We found the regulation of *Th* and *PebIII* were not influenced by the interaction between parent and offpsirng *per se*, but rather controlled by the reproductive stage of mothers suggesting preprogrammed expression in earwig. Such regulation of parenting genes in the sub-social species might be ancestral to the age-dependent division of labor in eusocial system.

These four chapters of my thesis were a series of continuous work and provided significant insights into the genomic basis of parent-offspring coadaptation. I established qPCR method to validate the *de novo* hybrid assembled transcriptome of the European earwig. I identified candidate parent-offspring coadapted genes using comparative trascriptomics. I established the method of Fluidigm gene expression dynamic array for earwigs to validate the RNA-Seq results. I established the RNAi techonology for earwigs to manipulate gene expressions and to study the social function of candidate genes. I demonstrated that *PebIII* and *Th* are two parent-offspring coadapted genes, which are co-regulated in mothers and offspring during active post-hatching parental care. Their expression were preprogrammed in mothers, reflecting the reproductive stage of females. Both genes showed causal

effects on the behavior and fitness of earwig mothers and nymphs, coordinating the selfishness and altruism in family life. I showed transgenerational effects of maternal care on the expression of *PebIII* and *Th*, and opened the door for future studies of the epigenetic mechanisms regulating gene expression over generations and maintaining parent-offspring coadaptation in earwigs.

GENERAL INTRODUCTION

Life on earth has evolved from cells to multicellular organisms and then organized social systems [1]. Altruism or cooperation as well as conflict are ubiquitous features in social interactions, where an individual benefit others at the cost of its own. Sociogenomics is the study of social life in molecular terms from a genomic perspective [2]. Many genes have been identified related to animal social behavior and social evolution, either through candidate gene studies based on current knowledge of well studied model organisms [3], or through genomic approaches for none-model organism [4]. The taxa range from bacterium *Myxoccocus xanthus* [5], honeybee [3], crayfish [6], song bird [7], [8], to rats and human [9], [10]. The functions of genes vary from chemical signal, brain development and function, immunity, reproduction, metabolism and nutrition.

Animal sociality is characterized by a continuum of social complexity ranging from eusociality to simpler forms of family living namely parental care [11]. Current evidence is consistent with the hypothesis that eusociality originally evolved from such simpler family living [12], [13]. This hypothesis posits that genes involved in the regulation of parental care were evolutionarily coopted, and ultimately form the genomic building blocks of complex animal sociality [14]. If true, genes underlying caste differentiation in eusocial systems should be conserved and have their original function in the regulation of parental care; genes mediating the social interactions between parents and their offspring would be the core genes of social evolution.

Parent-offspring coadaptation

Parents from a broad range of taxa provide parental care including food provisioning and antipredator defence to the offspring, at the cost of their own fitness such as future reproductive success and survival [15], [16]. Offspring who tend to aggregate with their parents are more likely to benefit from parental care and convert it into their own fitness, for instance, higher survival and growth rate [17], [18]. However, offspring could also affect parental care through behavioral demanding for resource as well as chemical signals [19]. Therefore, the evolution of traits for parent-offspring communication and regulations of parental care are believed to be under positive selection [20]–[23].

Many correlated traits have been reported with positive covariance from various species: the offspring

growth and parental effects on offspring growth in mice *(Mus musculus)* [24] and pigeons *(Columba livia)* [25], the maternal sensitivity to begging calls and the intensity of offspring begging calls in great tits (*Parus major*) [26], the food provisioning and the begging rate of burying beetle *(Nicrophorus vespilloides)* [27], the maternal milk letdown and the sucking efficiency of mice [28]. What is not clear yet is the genetic basis of parent-offspring coadaptation, whether it is due to pleiotropy, physical gene linkage disequilibrium or transgenerational phenotypic plastisity [16].

The quantitative genetic model of parent-offspring co-adaptive evolution was originally proposed by Feldman and Eshel in 1982 [29] , and it was developed by Wolf and Brodie in 1998 [30]. The limitation of Wolf and Brodie's model is the assumption that only offspring trait is under selection and the offspring are passively affected by the parents. The model was extended by Kölliker [23], [31], taking into account the reciprocal interactions between parent and offspring . This model predicted that, selection favors parent-offspring coadaptation which is a combined optimization of the correlated traits from the both sides. Such coadaptation is reflected by their co-regulation in parents and offspring either through physical linkage in the genome or coopted regulatory network [32], [33]. coadaptation must strike balance between parents pursuing self-fitness versus offspring demanding parental investment. Ultimately, it facilitates well-coordinated parenting and optimized cooperation with their offspring in the face of sexual reproduction and genetic recombination [32], [33] which cause genetic conflict [21], [34].

A key mechanism maintaining parental care and driving parent-offspring coadaptation is the co-regulation of genes expressed in mothers and in offspring over generations, where the care and the effects of care are genetically correlated [35]. From an epigenetic perspective, coadapted traits are expected to evolve with similar genomic imprinting patterns inherited from the caring parent [36], as the nymphs who received maternal care when they were young would provide similar care to their own offspring when they grew up. Contrary to this prediction, the kinship theory predicted the inheritance of genomic imprinting patterns from the non-caring parent (usually the male, if there is multiple mating) [37]. In mammalian placenta, the high frequency of imprinted genes of both maternal origin and paternal origin was speculated as the selection for genetic conflict on some loci and for coadaptation on other loci [36]. In rodents, post-natal maternal care influence the expression of estrogen receptor-α gene, DNA methylation in the promoter of this gene and maternal behavior of

female offspring [38], [39]. Such maternal effect on DNA methylation and maternal behavior could be transmitted over two generations [40].

European earwig as a model system

The European earwig (*Forficula auricularia*) is a sub-social insect species, which provide uniparental female care to the offspring in terms of food provisioning and protection against natural enermies, but the nymphs could also survive independent of their mother after hatching [41], [42]. The interactions between the mother and nymphs are reported through chemical signals such as cuticular hydrocarbon componds [19], [43].

The facultative nature of maternal care makes the earwig an ideal model to test for the sociogenomic bases of parenting and family living. First, the presence of the mother can be experimentally manipulated without causing unnatural and detrimental effects on offspring. And second, facultative forms of family living in earwigs are considered to be close to an ancestral form of family living. Thus, the co-regulated genes we found may more likely represent original genes that evolved for the formation of maintained mother-offspring associations than in systems with fully dependent offspring and highly derived forms of maternal care like in mammals or birds. If the identified genes turn out to be the same as those found in eusocial systems, this would provide more compelling evidence for co-option of the original mother-offsrping interaction genes and their evolutionarily conserved function.

As a non-model organism, the genome of *F. auricularia* is not yet available. No microarray has been developed for gene expression studies in such species either. One transcriptomes was published for the purpose of insect phylogenomic reconstruction [44]. However, the completeness of that transcriptome is rather poor with less than 30% completeness according to The Core Eukaryotic Genes Mapping Approach [45]. Therefore, in order to get an overview of expressed genes in the European earwig a comprehensive transcriptome of the earwig is essential for in-depth sociogenomic studies.

Thesis outline

Chapter 1

To obtain a comprehensive transcriptome, we sequenced mRNA from various tissues and developmental stages of female and male earwigs using Roche 454 pyrosequencing and Illumina

HiSeq. The reads were *de novo* assembled independently and screened for possible microbial contamination and repeated elements. The remaining contigs were combined into a hybrid assembly and clustered to reduce redundancy. A comparative analysis revealed that more than 8,800 contigs of the hybrid assembly show significant similarity to insect-specific proteins and those were assigned for Gene Ontology terms. Finally, we established a quantitative PCR method and tested the expression of homologs of five known sex-biased genes of the honeybee. The qPCR pilot study confirmed sex specific expression and also revealed significant expression differences between the brain and antenna tissue samples.

Chapter 2

In this chapter, we focused on sociogenomics of maternal care and parent-offspring coadaptation. Based on the comparison of RNA-seq data from different mother-offspring interactions, we identified two possible parent-offspring coadapted genes (*PebIII* and *Th*) in the European earwig *and* confirmed their expression in an independent experiment. Functional study of these genes via RNAi revealed causal effects of *PebIII* on offspring development, survival and relative maternal investment in future reproduction; *Th* influence maternal food provisioning and likelihood of maternal future reproduction. Our results suggested *PebIII* being a “selfish” gene while *Th* being an “altruistic” gene in both mothers and offspring. Metabolic pathway analysis suggested the role of *Th*-restricted dopamine reward, *PebIII* mediated chemical perception, regulation between insulin signaling, juvenile hormone and vitellogenin in parent-offspring coadaptation.

Chapter 3

In this study, we manipulated the interaction between earwig mothers and offspring over two generations and found transgenerational effects on the expression of these two coadapted genes and on the the fitness in mothers and offspring. Our results indicate an epigenetic regulation of genes underlying parent-offspring coadaptation.

Chapter 4

In the last chapter, we validated the expression pattern of *Th* and *PebIII* found in chapter 2 with a replicate experiment and Fluidigm gene expression dynamic array. An additional treatment controlling for time effect of females revealed the preprogrammed expression of both genes in earwig mothers

when they were caring for their broods. This result suggested that the regulation of parent-offspring coadapted genes according to females' reproductive stage instead of the interaction between parent and offspring *per se* in the sub-social earwigs is the first step of the evolution from solitary life form to division of labor in eusocial species.

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CHAPTER 1

De Novo Transcriptome Hybrid Assembly and Validation in the European Earwig (*Dermaptera, Forficula auricularia*)

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Abstract

Background: The European earwig (*Forficula auricularia*) is an established system for studies of sexual selection, social interactions and the evolution of parental care. Despite its scientific interest, little knowledge exists about the species at the genomic level, limiting the scope of molecular studies and expression analyses of genes of interest. To overcome these limitations, we sequenced and validated the transcriptome of the European earwig.

Methodology and Principal Findings: To obtain a comprehensive transcriptome, we sequenced mRNA from various tissues and developmental stages of female and male earwigs using Roche 454 pyrosequencing and Illumina HiSeq. The reads were de novo assembled independently and screened for possible microbial contamination and repeated elements. The remaining contigs were combined into a hybrid assembly and clustered to reduce redundancy. A comparison with the eukaryotic core gene

dataset indicates that we sequenced a substantial part of the earwig transcriptome with a low level of fragmentation. In addition, a comparative analysis revealed that more than 8,800 contigs of the hybrid assembly show significant similarity to insect-specific proteins and those were assigned for Gene Ontology terms. Finally, we established a quantitative PCR test for expression stability using commonly used housekeeping genes and applied the method to five homologs of known sex-biased genes of the honeybee. The qPCR pilot study confirmed sex specific expression and also revealed significant expression differences between the brain and antenna tissue samples.

Conclusions: By employing two different sequencing approaches and including samples obtained from different tissues, developmental stages, and sexes, we were able to assemble a comprehensive transcriptome of *F. auricularia*. The transcriptome presented here offers new opportunities to study the molecular bases and evolution of parental care and sociality in arthropods.

Introduction

Earwigs are widely distributed geographically and are important in ecology and agriculture as predatory and detritivorous insects. Some species are invasive and have successfully colonized non-native grounds after anthropogenic dispersal and have become pests (reviewed in [1]). Most earwigs are cosmopolitan foragers feeding on plant material including pollen, fruits, and detritus, but they also represent important predators of other invertebrates and their eggs. As a consequence, numerous earwig species are studied for their role in agricultural food webs to improve their efficacy as a biocontrol for pests such as aphids and the fall armyworm, Spodoptera frugiperda [2,3]. Earwigs form part of the Polyneoptera, an insect lineage still rather poorly resolved phylogenetically [4], and are a phylogenetically ancient insect order (the Dermaptera). The earliest earwig fossils date back to the Jurassic and lowermost Cretaceous (i.e. more than 200 Mya, [5]). The order is characterized by the conspicuous sexually dimorphic un-segmented cerci (‘‘forceps’’, [6]), a typically ground-living, often gregarious and nocturnal life-habit, and the ubiquitous occurrence of forms of maternal care [1]. The order comprises approximately 1,800 species that are consistently organized in 11 families [7]. While the major phylogenetic position and structure of the order are now roughly established [7,8], the details of the phylogenetic relation- ships among earwig species have not been fully resolved, partly due to lack of genomic data.

The European earwig (*Forficula auricularia*) is probably the most common and widely distributed

earwig species in Europe. Native to the western Euroasian region, it was introduced by human activity in Northern America, Australia and New Zealand where it quickly established and is sometimes regarded as an invasive species and a pest in gardens and agricultural settings [1]. The European earwig is also the scientifically best-studied earwig species and has been used as experimental system in various evolutionary contexts, including sexual selection and the evolution of reproductive tactics, maternal care and family interactions [9– 11]. Females show pronounced maternal care; they protect and clean the eggs, and they provide food and protection to hatched nymphs. While maternal care for the eggs is mandatory, it is facultative for later life stages since the nymphs are mobile and can survive without maternal care by self-foraging (reviewed in [1]). These conditions are thought to approximate ancestral conditions under which parental care originally evolved. Therefore, the European earwig (and other earwig species like *Anisolabis maritima* and *Euborellia annulipes*) is increasingly used as an experimental system to study the evolutionary origin and genetics of parental care and social behavior.

Yet, despite the scientific interest in earwigs, only little knowledge and data are available at the genomic or proteomic level. The first transcriptomic data of the European earwig was recently published in an attempt to improve the polyneopteran phylogeny [8]. Even though this transcriptome is a first step in the establishment of genomic/transcriptomic resources to study earwig biology in molecular terms, it was based on RNA extracted from only adult stage and yielded fragmented and incomplete sequence data. Thus, towards the improvement of the genomic resources needed to study for example gene or genome evolution, gene expression, or insect systematics, we aimed to establish a more comprehensive transcriptome of the European earwig. Here, we present and validate the draft transcriptome based on a hybrid assembly of Roche 454 and Illumina HiSeq data. In order to obtain a more exhaustive representation of transcripts, we combined different tissues (heads, thoraxes, abdomens, brain, and antenna) and developmental stages (eggs, nymphs and adults) from both males and females. As our analysis showed that the published transcriptome is fragmented, incomplete and lacking quality information, we deliberately did not use these published data for our hybrid assembly. After the assembly, we screened our transcriptome for putative microbial contamination. We also annotated transposable elements and removed redundancy, keeping alternative-splice variants. We then estimated the completeness and the fragmentation of our dataset by applying the core Eukaryotic gene mapping approach (CEGMA, [12]). Our transcriptome was also compared against other insect protein databases to determined protein-coding genes shared with eu- social and non-social insects. This sub-

sample was annotated using Gene-Ontology (GO). We eventually established and validated qPCR by studying expression differences in males and females for 5 genes reported as being sex-biased in the honey bee [13]. We could confirm that some of these genes show expression differences between males and females but also between brain and antenna tissue in earwig. This method will allow us to study the expression of candidate genes putatively involved in maternal care and social behavior in the future. Further information on the assembly and links can be found at http://evolution.unibas.ch/walser/dermaptera.htm.

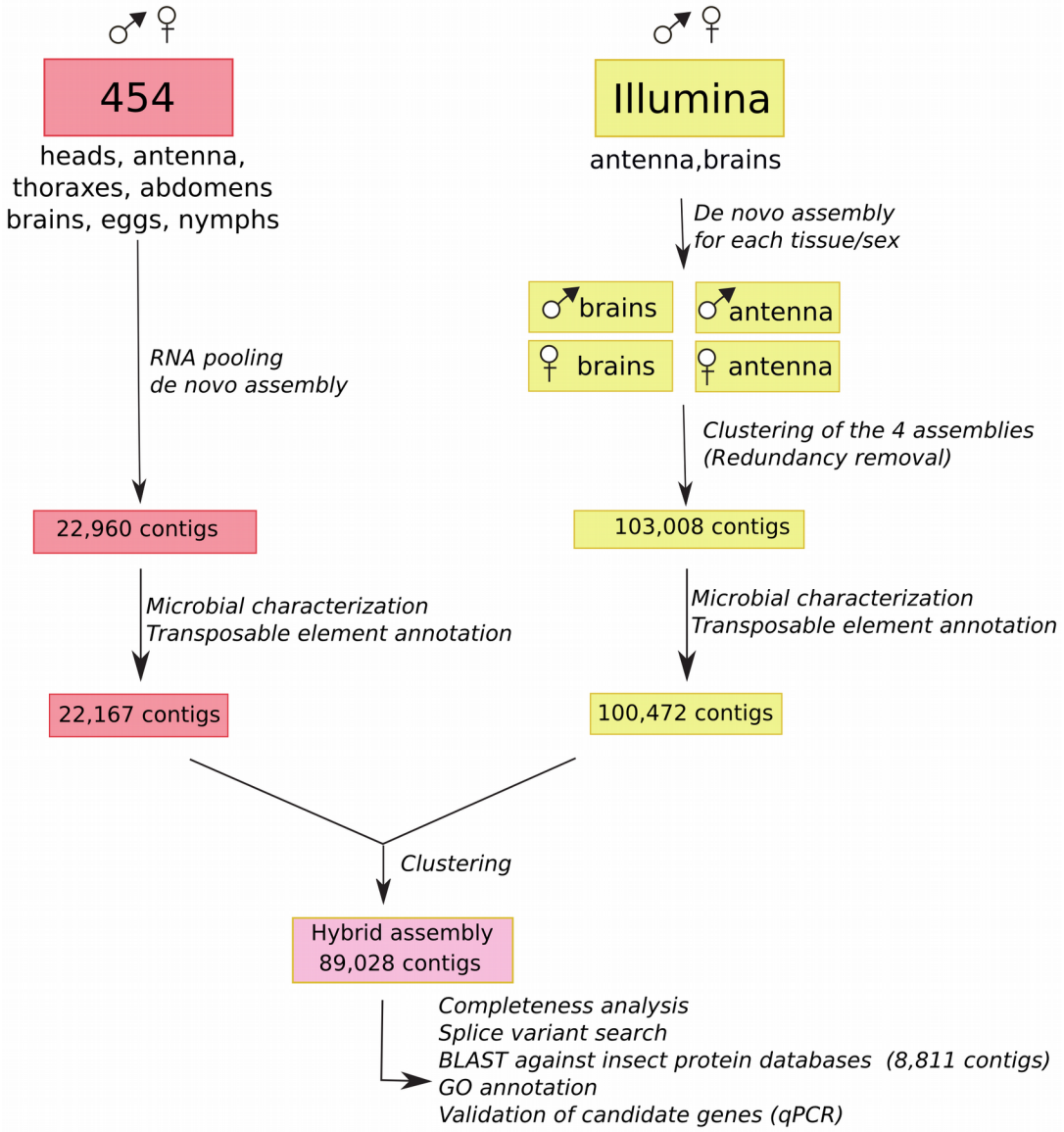

Figure 1. Flow chart of the hybrid assembly process.

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Results and Discussion

A recent study showed that higher quality assemblies could be obtained when 454 and Illumina contigs are combined [14]. Following these guidelines, the Illumina and 454 reads were independently pre-assembled make use of an optimized de novo assembler platform. The initial Illumina and Roche 454 pre- assemblies (Fig. 1) resulted in 103,008 and 22,960 high quality contigs, respectively. The not assembled reads from the Roche 454 run, called singletons, were adapter trimmed, quality, and size selected but not included for further analysis. In a first step, the contigs were screened for possible contaminants and transposable elements. The remaining contigs were combined in a hybrid assembly resulting in 89,028 unique contigs.

Characterization of non-earwig and transposable element sequences in the pre-assemblies

Microbiota screening. Earwigs, as many other organisms, live in close contact to microbial communities. Thus, we carefully prepared the samples in order to reduce level of possible contaminants (see Materials and Methods). In addition, the library preparation discriminated against non-polyadenylated molecules (poly-A enrichment, see Materials and Methods) and further reduced potential bacterial contaminants. Both steps reduced but did not entirely remove microbial contamination. To assess the level of potential remaining contaminants, we applied Pauda [15] to align the two pre-assemblies against a database of 56 million known proteins from Alveolata, Amoebozoa, Archaea, Bacteria, Fungi, Nematoda, Platyhelminthes and Viruses (Table S1). In total, 468 sequences (i.e. about 0.5% of all contigs) were putative homologs of microbial proteins. In addition, we identified 152 contigs corresponding to the small (SSU: 16S or 18S rRNA) or large ribosomal subunit (LSU: 23S or 28S rRNA), including 21 contigs specific to arthropods and therefore putatively of earwig origin (Table S1). Overall, we could assign about 23% of those contigs to a bacterial origin and 60% to a fungal origin (Fig. 2, Fig. S1 and Table S1). Out of the 50 top genera identified, 39 corresponded to fungi, 4 to bacteria and 1 amoeba all commonly found in soil samples. Interestingly, one of the identified fungi species is an already known parasite isolated from the habitat of the European earwig [16]. With this screening, it is likely that we identified part of the native microbiota of the earwig. Those sequences were removed from the pre-assemblies.

Transposable element screening. Numerous studies documented that transposable elements (TEs) are pervasive and often constitute a substantial component of the size of a genome [17]. An unknown

proportion of full-length TEs are transcriptionally active (i.e. transcribed) in a given genome at a given time [18]. Our approach does not discriminate against all TEs especially the retrotransposons which are polyadenylated [19]. Therefore, active TEs could inflate the number of contigs found in our assemblies and need to be identified and excluded from the final transcriptome. Therefore, we screened our preliminary assemblies for TE specific proteins using RepeatMasker [20]. We identified 2,076 and 694 contigs with significant similarity to known TE protein (Fig. 3 and Table S2). The fraction of retrotransposons (class I) and DNA transposons (class II) identified is similar to other transcripome studies in insects (e.g. [21]). In particular, Mariner and Gypsy elements seem to be common in the earwig transcripome. This finding is in agreement with previous work, which described the ubiquitous presence of these elements in insects [22–25] including earwigs [26].

Completeness of the hybrid assembly

The 454 and Illumina pre-assemblies cleaned of microbial and transposable element sequences were combined and clustered to result in a hybrid assembly comprising 89,028 contigs (Fig. 1). To estimate the completeness of the hybrid assembly (hereafter designated as the earwig transcriptome), we compared the 89,028 contigs to a set of highly conserved and reliable annotated core proteins (n=458) of Drosophila melanogaster and Aedes aegypti [12]. The Core Eukaryotic Genes Mapping Approach (CEGMA) showed that the 458 proteins of the core dataset could be unambiguously identified in our transcriptome, with a median value of completeness of 97%. Among those, 252 proteins were fully present (completeness 95%, Table S3). In order to put this into prospective, the previously published earwig transcriptome used for phylogenetic analysis (Simon et al. 2012) harbors a median value of completeness of 30%, with 20 full proteins only (Table S3). This comparison shows that our dataset contains a larger and/or less fragmented fraction of the earwig transcrip- tome. For this reason, the published transcriptome was not included in our hybrid assembly. This interpretation is also supported when comparing the CEGMA analysis of our transcriptome with the one from other published de novo transcriptome assemblies [27,28].

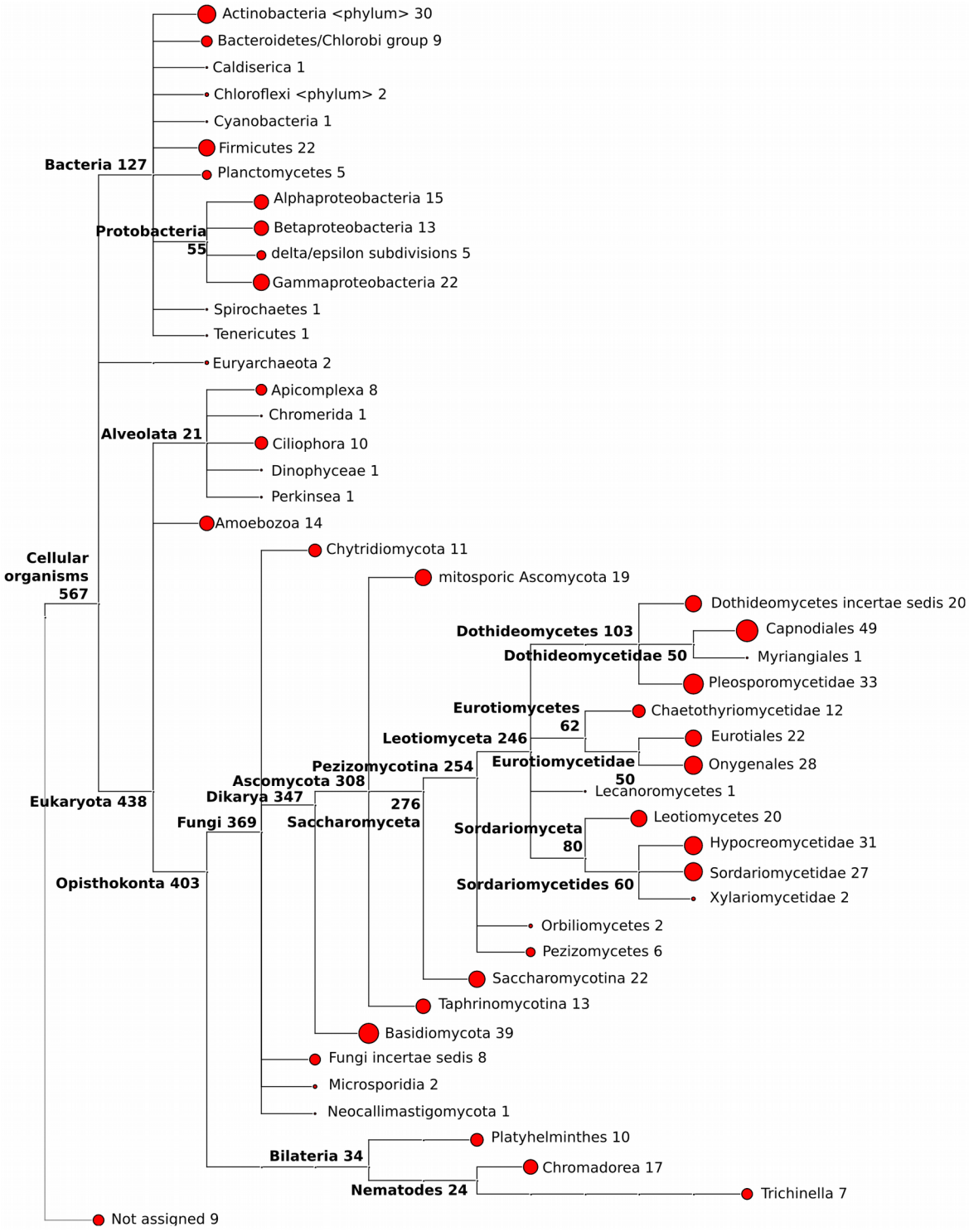

Figure 2. Taxonomic assignments of microbial contaminants using MEGAN. Assignment of all microbial SSU-LSU rRNA and mRNA sequences to the least common ancestor of their blastn and blastx hits, respectively. The red circle size are proportional to the number of sequences assigned to that node (maximum 49 reads), whereas the numbers are the accumulative sum of sequences assigned within subclades.

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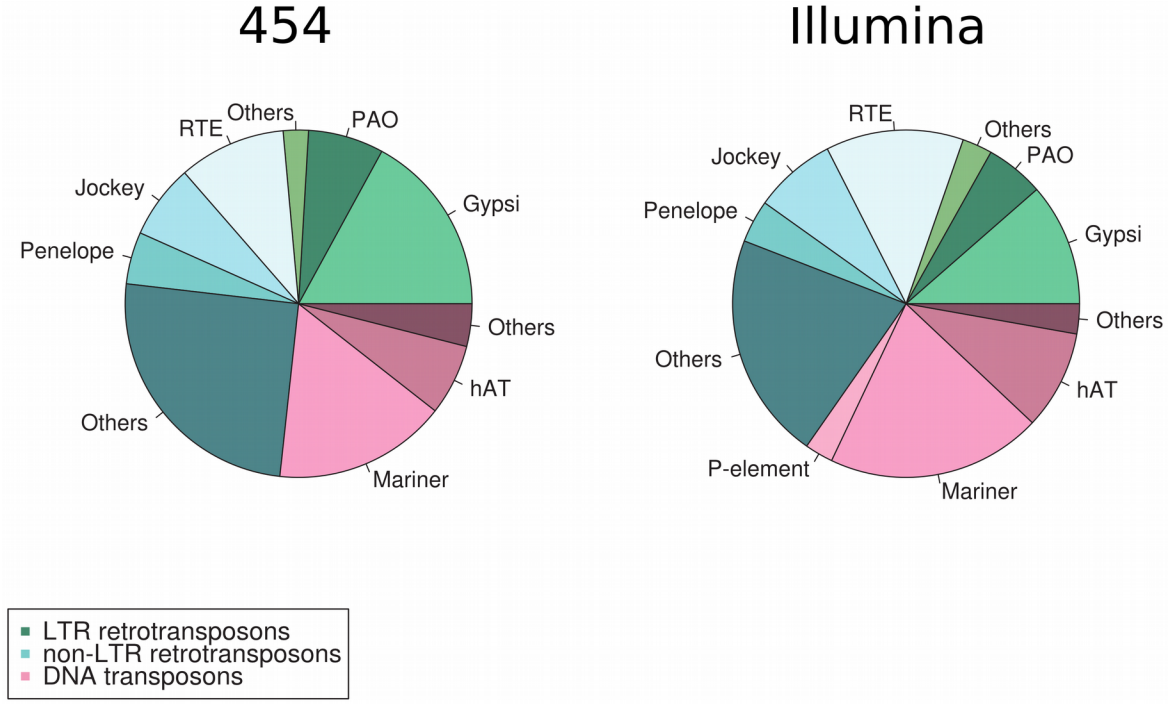

Figure 3. Most common transposable element distribution in the 454 and Illumina pre-assemblies.

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Identification and annotation of the earwig protein core set

Based on comparison with other insect species and the observation that gene number and average gene length are highly conserved among eukaryotes [27], we assume that approximately 200 Mb of the F. *auricularia* genome is organized in exons. Although we carefully removed potential microbial contamination, diminished TEs sequences, and even reduced redundant tran- scripts (see Materials and Methods), we believe that our dataset overestimates the number of protein coding genes, a common problem of RNAseq based transcriptome studies. The high number of contigs might also indicate the presence of non-coding transcripts (nc-RNA [29]), pseudogenes [30] or sequences errors (e.g. chimeras, [31]). It is also likely that a less stringent clustering could have reduced the number of contigs but also removed potential splice variants. In fact, we found evidence of putative variable transcripts. For example, we found two possible isoforms of the RhoGAP-like gene (TextS1). The mapping of the Illumina short reads using both isoforms as a reference supports this idea. Even though these preliminary results would need to be confirmed by qPCR, it indicates that one of the variants is more abundant than the other in the brain sample (data not shown).

A BLAST search using our contigs as query against 2 social and 3 non-social insect databases i.e *Apis mellifera* (honey bee), *Acromyrmex echinatior* (leaf-cutting ant), *Drosophila melanogaster* (fruit fly), *Tribolium castaneum* (red flour beetle) and *Nasonia vitripennis* (jewel wasp) revealed 8,811 contigs shared between our transcriptome and a least one of the five reference insect genomes (Fig. 4). Among those, only 2,400 could be found in the previous published transcriptome [8], which further confirms the completeness of our hybrid assembly. The completeness analysis was performed again using the 8,811 identified contigs. The same results as with the whole transcriptome were obtained (458 proteins identified, completeness of 97%), suggesting that these contigs, even though not representative of the whole transcriptome, constitute the earwig core protein dataset.

This subset of 8,811 contigs was then assigned for Gene Ontology terms (GO; [32]) using Blast2GO and based on blastx hits against the Swiss-Prot database. We were able to assign the contigs to the following categories (in terms of their numbers): molecular function: 1,046; cellular component: 2,021; biological process: 7,018 (Fig. S2). Altogether, the binding proteins and catalytic activity represent the vast majority of the molecular function category. Most of the contigs associated with the cellular component were assigned to the cell and the organelle part while those associated with a biological process were mainly involved in the cellular and metabolic process. Although GO term annotations are more relevant in the context of comparative analysis (between developmental stages for example), these results are congruent with findings in other insect transcriptome studies [33,34] and confirm that we obtained the sequences of genes involved in central pathways. This was further illustrated by the KEGG metabolic pathways analysis (see Table S4), which allowed us to identify pathways involved for example in the purine (189 genes), pyrimidine (76 genes), or inositol-phosphate (45 genes) metabolisms.

Our comparative analysis also indicates that 124 (1.4%) of the identified 8,811 contigs might be specific to social insects (e.g. *A. mellifera* and *A. echinatior, F. auricularia*) and absent from non-social insects (e.g. *D. melanogaster, T. castaneum* and *N. vitripennis*). 75 transcripts could be assigned to a molecular function, the most prevalent categories being protein-binding (52 transcripts) and proteins associated with a catalytic activity (23 transcripts, data not shown). These 124 contigs constitute possible candidates to further investigate the genetic bases of maternal care and extended social behavior (i.e. caste determination and task-specialization).

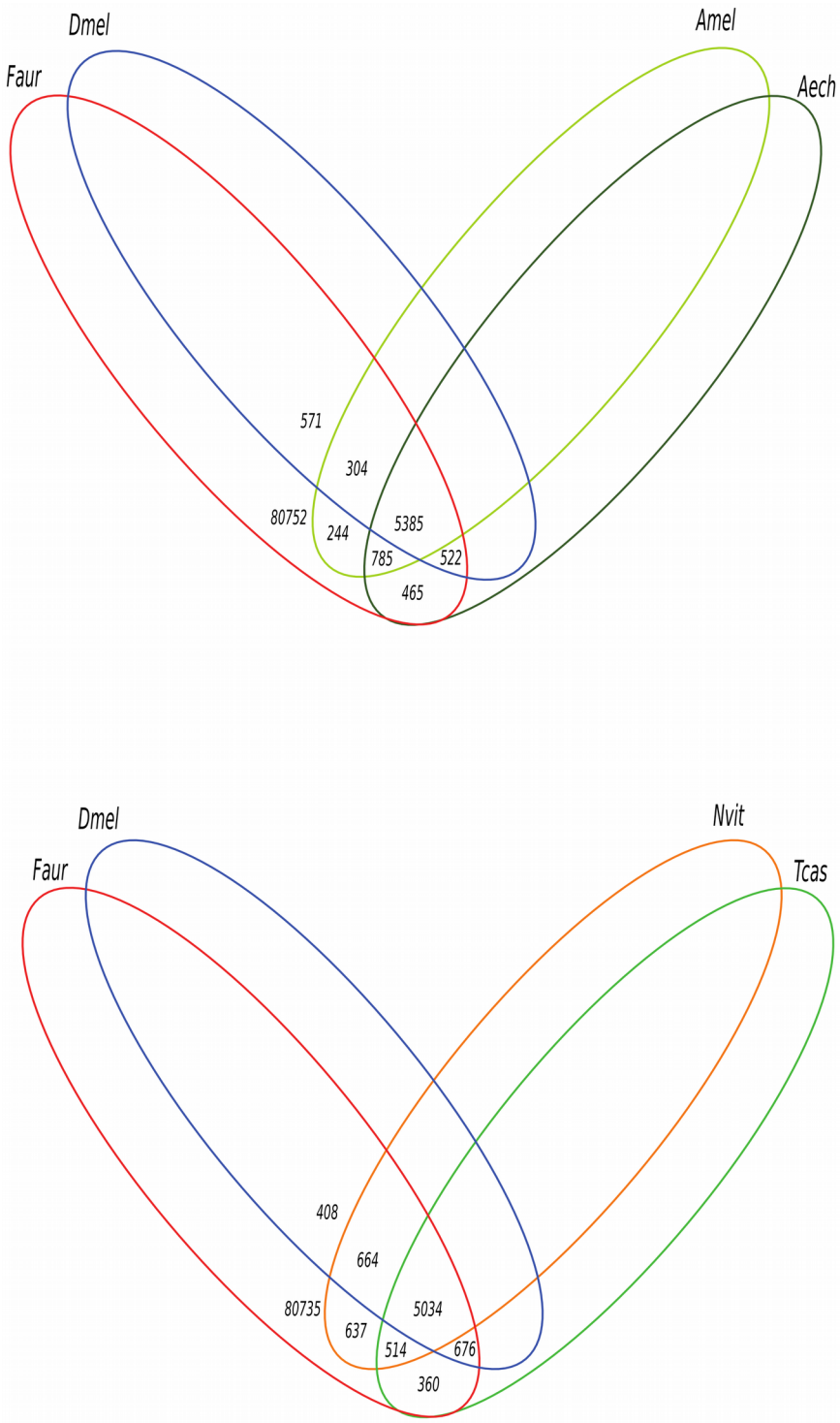

Figure 4. Venn-diagram of protein sequences shared by *F. auricularia* and 5 insect species. Numbers represent the number of proteins specifically shared by the particular combination of species. A) between *F. auricularia*, *D. melanogaster* and the social insects *A. mellifera* and *A. echinatior* B) between *F. auricularia*, *D. melanogaster* and the non-social insects *N. vitripennis* and *T. castaneum*.

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Validation of the transcriptome and candidate gene expression analysis

We selected 7 housekeeping genes (*actin, EF1, mnf, rpl32, rpl20, tubulin* and *18S*) used as qPCR internal standards in *Drospohila melanogaster* [35]. Five of the selected housekeeping genes (*actin, EF1, mnf, rpl32* and *tubulin*) showed homologous sequences in our transcriptome and four of them (*actin, EF1, mnf* and *rpl32*) could be successfully amplified with earwig specific primers (Table S5). Using primers specific for the *18S* from *D. melanogaster* [36], we also successfully amplified this gene in our earwig samples. Yet, the stability test (see Materials and Methods) indicated that the *EF1* and *18S* genes could not be used as potential standards. In addition, because *mnf* showed significant sex-biased expression in both brain and antenna (wilcoxon test p,0.5, Table S6), the *actin* and *rpl32* genes were the only standards kept for further analysis (Fig. 5).

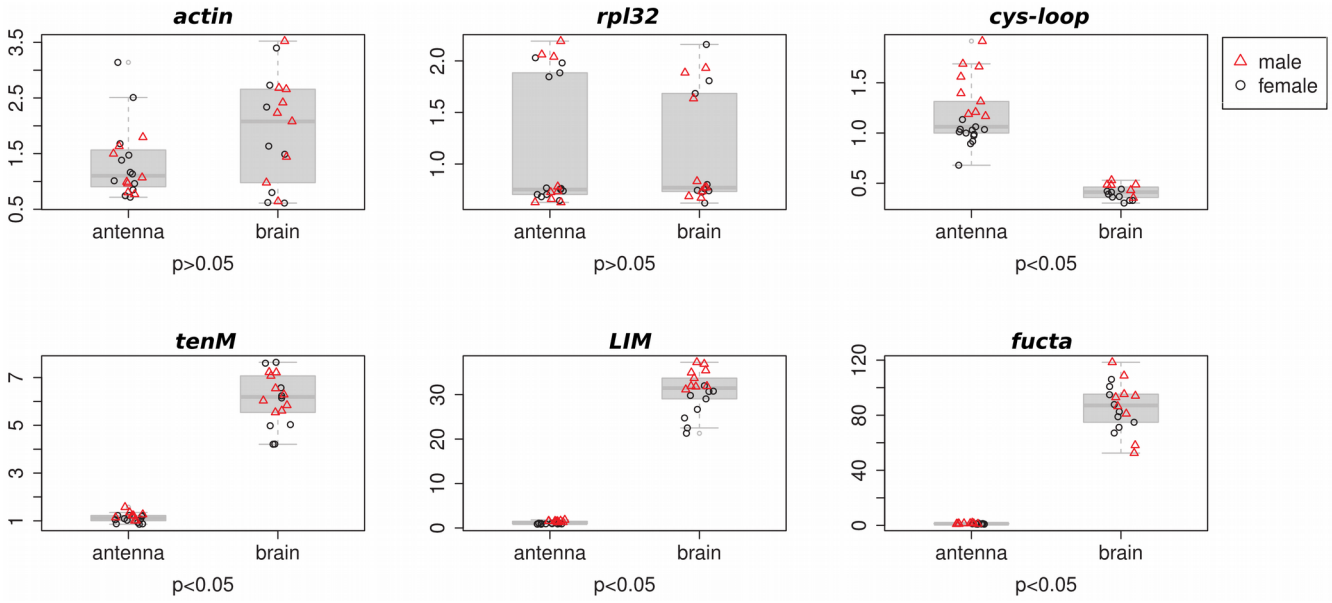

Figure 5. Gene expression for 2 housekeeping genes (actin and rpl32) and 4 candidate genes. Red triangles display male samples. Black circles display female samples. P-values indicate whether the gene is significantly differentially expressed between brain and antenna samples. * display genes which harbor a sex-biased expression.

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We also selected 5 candidate genes (*cys-loop, NAD-like, LIM, tenM* and *fucta*) for which sex biased expression has been reported in the honey bee, *A. mellifera* [13], and compared their expression level between sexes (adult males versus females) and tissues (antennae versus brain). *NAD-like* was excluded from further analysis because most of the *NAD-like* samples did not meet the Ct8 criteria (See Materials and Methods). However, we confirmed sex-biased expression for the *cys-loop* and *LIM* genes

in our system (Fig. 5, Table S6). In addition, significant expression differences between brain and antenna samples could be observed for the 4 selected genes (Fig. 5, Table S6). Interestingly, the *cys-loo*p gene showed higher expression in antenna than in brain. This gene has been described as a ligand-gated ion channel, i.e. a receptor that converts chemical signals to electrical signals. It is therefore not surprising to observe such an expression pattern between the olfactory tissue antenna [36] and the central system (brain). These results demonstrate that our transcriptome can further be used to develop gene primers and to study candidate gene expression. The established qPCR approach presented here will allow and thus enhance the study of the molecular evolution of social behavior in our system.

Database

In order to facilitate the search of sequences of interest, we provide a searchable database at http://evolution.unibas.ch/ walser/dermaptera.htm. This database allows to perform BLAST searches separately on the different data-sets described in the manuscript, i.e. the complete hybrid assembly (n=89,000 contigs), core earwig proteins (n=8,811 contigs), transposable elements (n=2,076), microbitoa (N=620), unassembled 454 reads single- tons (n=124,630).

Conclusion

The European earwig, *Forficula auricularia*, is an organism studied in evolutionary, ecological and agricultural research. It is an important and very interesting insect system for the study of the evolution of reproductive tactics [9], and the early evolution of parental care and family interactions [37]. Despite the broad interest in earwigs, only limited and incomplete data existed at the molecular level. In this study, we showed that our transcriptome provides a substantial portion of the genes present in the European earwig, which is an important first step to enhance our ability to investigate the genetics and genomics of this species as well as other Dermaptera and insects.

Materials and Methods

Ethics statement

No specific permits were required for the described experiments. The European earwig is not an endangered or protected species.

Earwig sample

The earwigs used for this study were part of a breeding line that originated from the progeny of three earwig females caught in Dolcedo (region Liguria), Italy in July 2008. These females were among a group of six females and six males caught on two adjacent olive trees. The females probably had already mated upon capture, but to ensure mating, the six females were set-up jointly with six males in the laboratory for continued mating until oviposition. The offspring of the selected females were used to establish a laboratory breeding line (line FaDo-08i). For mating, the offspring were set-up in containers of about 120 individuals each (approximately 60 males and 60 females). For each subsequent generation offspring of 5–10 females were chosen to continue the line. At the time when the individuals were sampled for the current study on May 5–6th, 2011, the line had been kept for four (adult tissues) and five (eggs/juveniles tissues) generations, respectively. For more details about rearing conditions, see [38].

RNA isolation and sequencing procedure

Male and female adult earwigs, eggs and whole nymphs from all five juvenile stages (eggs, juvenile instars L1–L4) were selected from the breeding line FaDo-08i for total RNA isolation. Prior to dissection, the animals were exposed to petroleum ether (Sigma- Aldrich #77379) vapor. The digestive tract was carefully removed from adult animals to minimize possible contamination from gut content and microbes. We collected whole heads, antenna, thoraxes, abdomens, and dissected brains of five adult females and five adult males. We further sampled about 15 oocytes from one female, collected 10 nymphs from the L1 and L2 develop- mental stages, and five nymphs from the L3 and L4 stages. All samples were stored in RNAlater (Qiagen), a RNA stabilizing reagent, after dissection. A TRIzol (Invitrogen) protocol was used to isolate total RNA. The Roche 454 run was split into two half plates and two libraries from pooled samples were prepared. Equal amounts of RNA from the whole heads and thoraxes of female and males were pooled for the first library. For the second library the abdomens of female and males, the oocytes, and the nymphs were combined in equivalent amounts. Approximately 2 mgof total RNA from the pooled samples was used for the cDNA library construction and subsequent sequencing. The library preparation and run was performed at the Functional Genomic Center in Zurich (For more details see Text S2). For the Illumina HiSeq run libraries for the brain and antenna tissues from females and males were prepared separately using Illumina TruSeq kit with index following the manufacture's protocol. The single read (SR) 100 nt and 150 nt multiplex HiSeq run was

performed at the Quanti- tative Genomics Facility (QGF) in Basel.

De novo pre-assemblies

The Roche 454 and the Illumina datasets were assembled separately. A detail schematic of the sample design and the different assembly steps are provided in Figure 1. For the 454 data the quality filtering, the read trimming, and the transcriptome assembly were generated using GS De Novo Assembler (version 2.7; Roche, Switzerland). Because the unas- sembled reads (i.e. singletons) still contain the adaptor sequences, the reads were trimmed and size selected using cutadapt [39] version 1.2. PRINSEQ lite [40] was used for quality assessment and filtering of the SR100 and SR150 Illumina reads prior to the de novo assembly performed with CLC Genomic Workbench (Version 6.0.1). The four individually assembled transcriptomes (e.g. female brain, male brain, female antenna, and male antenna) were concatenated and usearch (version 7.0, [41]) with a 95% identity clustering to reduce redundancy was applied.

Contamination analysis

Initial 454 and Illumina contigs were submitted to Bowtie2 v2.1.0 [42] and Pauda v1.0.1 [15], where they were mapped to reference proteomes. These latter were downloaded as of May 2013 from the NCBI website (http://www.ncbi.nlm.nih.gov/ Taxonomy/Browser/wwwtax.cgi, Accessed 2014 March 15) by independently selecting all proteins sequences from Amoebozoa (about 0.2 million of proteins), Alveolata (0.5 m), Archaea (1.7 m), Bacteria (46.5 m), Fungi (2.9 m), Platyhelminthes (0.1 m), Nematoda (0.3 m) and Viruses (2.2 m) (total of about 56.4 m). Briefly, individual contigs were translated using all six reading frames into proteins and fast aligned, using default parameters, to the above reference proteins. The blastx scores were parsed using local perl scripts and used to rank the microbiota. Only blastx results with an alignment length over 33 amino acids to the reference proteins, a similarity over 75% and e-value below 10210 were considered as positive hits. Results were visualized in MEGAN v4.0.1 [43]. While inspecting the data we ignored reads unassigned to taxa. Sequencing reads were also submitted to the r115 database of ARB-SILVA (release date: August 2013, https://www.arb-silva. de/no_cache/download/archive/release_115/Exports/) [44] to a local blastn search to identify small (SSU: 16S and 18S) and large (LSU: 23S and 28S) subunits of ribosomal RNAs of Bacteria, Archaea and eukaryotic organisms. Only blastn hits with an alignment length over 100 nt to reference rRNA sequences, an identity over 75% and e-value below 10215 were considered as positive

SSU and LSU.

Transposable element identification

Contigs from the 454 run and the combined Illumina data were screened for the presence of transposable elements using the protein based database search provided by RepeatMasker [20]. Contigs whose 90% of the total length showed homology with a TE protein were excluded from the hybrid assembly (see Fig. S3 for distribution). Singletons were deliberately not analyzed.

Clustering and hybrid assembly

Possible redundancy of the combined contamination-reduced 454 contigs and Illumina dataset as well as the singletons was reduced using usearch (version 7.0, [41]) and CAP3 [45]. The hybrid assembly of the combined 454 contigs and the Illumina contigs resulted in a total of 89,028 sequences. The hybrid assembly together with the clustered singletons (deliberately not included for further analysis) builds the transcriptome of the European earwig. A BLAST server will be made available upon acceptance of the manuscript for publication. The parameters for the clustering were carefully determined in order to reduce redundancy without removing possible alternative transcripts. In order to identify putative splice-variants, contigs of the hybrid assembly were BLAST searched against the *D.melanogaster* Exon Database (http://proline.bic.nus.edu.sg/dedb/, Accessed 2014 March 15). Contig pairs showing homologous relationship with the same gene of D. melanogaster but with different exons and showing 100% of sequence identity with each other for a 300 bp region were considered as potential gene isoform.

Completeness analysis

The completeness of the hybrid assembly and of the published transcriptome was determined by performing a tblastn search using our transcriptome contigs as query against the CEGMA core genes dataset of D. melanogaster and A. Aegypti (http://korflab. ucdavis.edu/datasets/cegma/, [12], Accessed 2014 March 15). Custom Perl scripts were used to assess the completeness of our transcritpome (% coverage between query and core protein alignments). Only local alignments with e-value,1026 were taken into account. Only the best BLAST hit results were kept (allowing only 1 contig per protein) so that the completeness analysis also reflects the transcritpome fragmentation.

Protein comparison with insect databases, GO term analysis

Contigs were used in a reciprocal best-hits BLAST approach [46] to find homologues with *Apis mellifera* (honeybee, [22], http:// hymenopteragenome.org, Accessed 2014 March 15), *Acromyrmex echinatior* (leaf-cutter ant, [47], http://www.antgenomes.org, Accessed 2014 March 15), and *Drosophila melanogaster* (fruit fly, [48], ftp://ftp.flybase.net, Accessed 2014 March 15), *Tribolium castaneum* (flour beetle, [49], http://beetlebase.org/, Accessed 2014 March 15) and *Nasonia vitripennis* (parasitic wasp, [50], http:// hymenopteragenome.org/nasonia/, Accessed 2014 March 15). BLAST hits with a score ,50 and e-values . than 1026 were not considered for further analysis.

Gene ontology (GO) annotation was performed using Blast2GO version 2.5.1 [32], using the NCBI Blast service and a cut-off value of 10e26 for the blastx search against the Swiss-Prot database. Categories represented by more than 15 sequences were taken into account. Blast2GO was also used to identify the metabolic pathways based on the Kyoto Encyclopedia of Genes and Genome (KEGG; [51]) and the Swiss-Prot database.

qPCR establishment and validation of candidate gene expression

Earwigs from the same breeding line as the ones used for Illumina sequencing (from the eighth generation since the line was established) were used to extract RNA from both male and female brains and antenna. The experiment consisted of 40 females and 40 males and the RNA was extracted from brains and antenna at the stage when females were guarding their clutch of eggs. As before, the insects were sacrificed before dissection by exposure to petroleum ether. The protocol of RNA extraction is the same as described above. In order to obtain sufficient amount of RNA for qPCR, the extracted RNA from 10 males or 10 females were pooled for each tissue resulting in 4 biological replicates per sex and tissue. The extracted RNA was treated with DNaseI (Fermentas) to remove genomic DNA, and quantified in Qubit 2.0 Flurometer with RNA BR (Broad-Range) Assay Kit (Invitrogen). The quality of the extracted RNA was then controlled with the 8-capillary NanoDrop 8000 (Thermo Scientific). The cDNA library was prepared using GoScript Reverse Transcription System (Promega). An intron control PCR was run to confirm that the RNA samples were free of genomic DNA. The 56HOT FIREPol EvaGreen qPCR Mix Plus (ROX) were used for runs on Applied Biosystems 7500 Fast Real-Time PCR System.

5 candidate genes (*cys-loop, NAD-like, LIM, tenM* and *fucta*), known to harbor sex-biased expression in honey bee (*A mellifera* [13], and showing homologous sequences in our transcriptome (Table S5) were chosen for the analysis. For internal control, we selected 7 commonly used housekeeping genes (*actin, EF1, mnf, rpl32, rpl20, tubulin* and *18S* [35]). Primers were designed to discriminate potential genomic DNA (Table S5). The amplification efficiency was calculated in LinRegPCR (11.4 [52]) and genes with an efficiency range between 1.8 to 2.0 were kept for further analyses. The expression stability of the housekeeping genes was tested in each RNA pool (brain and antenna in both male and female) using geNorm, which is implemented in qbasePLUS [53]. The expression of candidate genes was calculated using 2DDCt method [54]. For each of the 4 biological replicates, 3 technical replicates were used. Melting curves were used to control the quality of the PCR products. Samples that did not meet the Ct8 value criteria (e.g. difference between the no reverse transcriptase control and the tested sample values greater than 8) were excluded from further analysis. The significance of expression differences between male and female or brain and antenna samples were tested in R (v.2.14.1 [55]) with a Wilcoxon test.

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Data deposition

The Roche 454 and Illumina reads of *F. auricularia* have been deposited to the NCBI Sequence Read Archive (SRR1043671, SRR1048074, SRR1051467).

Author Contributions

Conceived and designed the experiments: JCW MK. Performed the experiments: JCW RA SKB MW. Analyzed the data: JCW ACRMWSP. Wrote the paper: ACR MW JCW MK.

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Figure S1. Pie-charts of microbial contaminant taxonomic assignments at the phylum, class and family level.

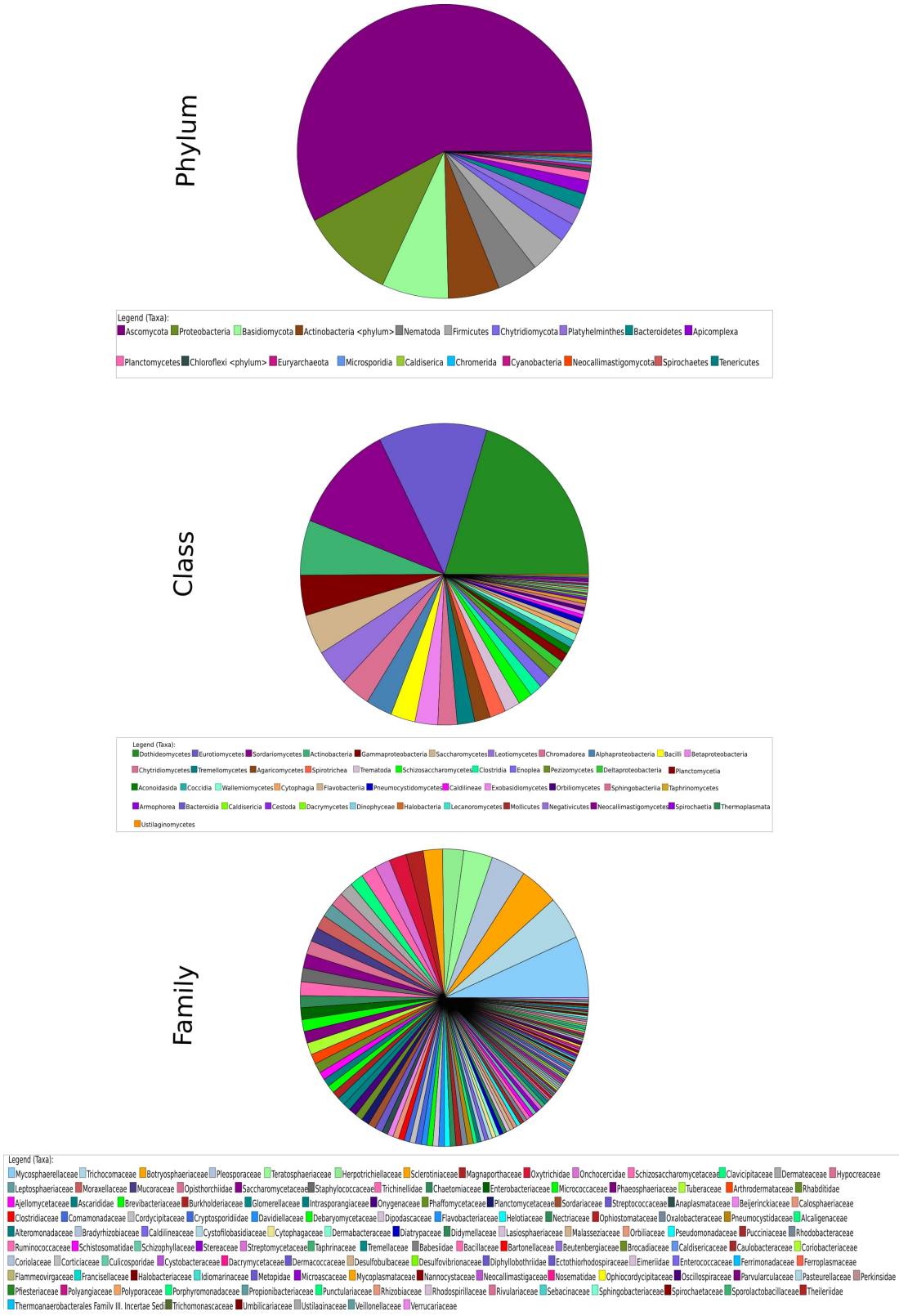

Figure S2. Gene ontology annotation (molecular function, cellular component and biological process) of the 8,811 contigs conserved among insects.

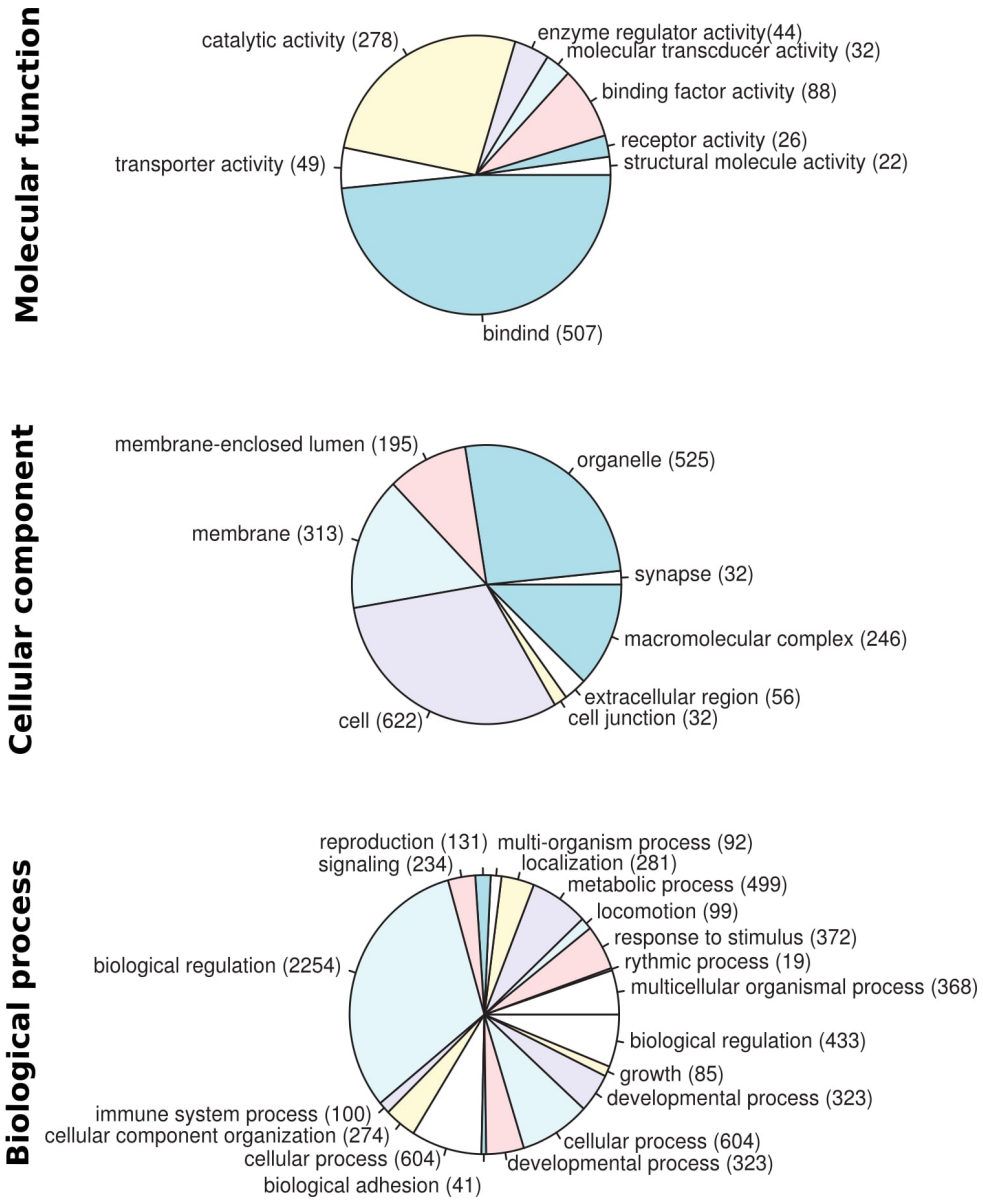

Figure S3. Distribution of the proportion of the protein masked by repeat masker. Red bars show contigs which have been removed from the assembly, *e.g.* sequences for which 90% of the length is masked (TE sequences).

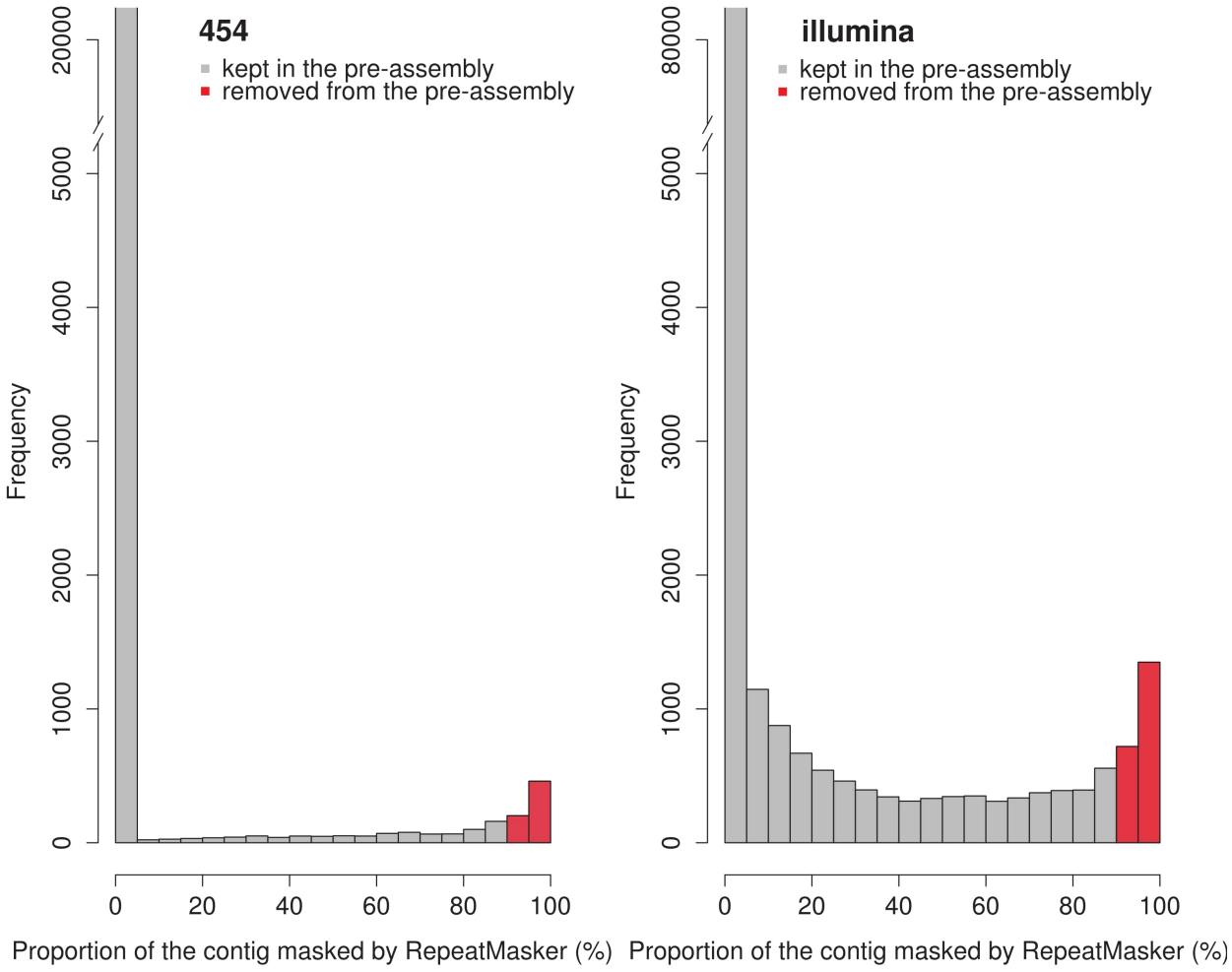

Table S1: Counts of mRNAs encoding microbial proteins and rRNAs (SSU/LSU, counts in brackets) and their taxonomy assignments.

Taxonomy assignments	454 contigs	Illumina	Total
Alveolata	9 (1)	11 (0)	20 (1)
Amoebozoa	8 (0)	6 (0)	14 (0)
Archaea	0 (0)	2 (0)	2 (0)
Arthropoda	nd (10)	nd (11)	nd (21)
Bacteria	31 (2)	38 (74)	69 (76)
Fungi	35 (2)	295 (39)	330 (41)
Nematoda	12 (0)	11 (1)	23 (1)
Platyhelminthes	4 (0)	6 (0)	10 (0)
Viridiplantae	nd (1)	nd (8)	nd (9)
Virus	0 (0)	0 (0)	0 (0)
Other eukaryotes	nd (1)	nd (2)	Nd (3)
Total	99 (17)	369 (135)	468 (152)

Table S2: Transposable element families identified in the 454 and Illumina assembly

454

Class	Order	Superfamily	number (contigs)	total
Class 1 (retrotransposons)	LTR	copia	13	180
		gypsi	116	
		ERV	2	
		PAO	48	
		Other	1	
	LINE	RTE	68	319
		Jockey	47	
		Penelope	33	
		LINE/I	28	
		R1	40	
		L2	30	
		R2	10	
		other	63	
Class 2 (DNA transposons)	TIR	Mariner	110	182
		hAT	45	
		P	13	
		MuDr	3	
		PiggyBac	5	
		other	6	
	Helitrons		13	

ILLUMINA

Class	Order	Superfamily	number	total
Class 1 (retrotransposons)	LTR	copia	51	391
		gypsi	223	
		PAO	111	
		DIRS	4	
		ERV	2	
	LINE	RTE	262	931
		Jockey	156	
		Penelope	81	
		LINE/I	86	
		R1	61	
		L2	72	
		R2	40	
		other	173	
Class 2 (DNA transposons)	TIR	Mariner	409	708
		hAT	188	
		P	54	
		PiggyBac	29	
		MuDr	5	
		other	23	
	Helitrons			46

Table S6: Wilcoxon test results for biased expression in sex and tissues

	tissue	**sex~brain**	**sex~antenna**
actin	0.1174	0.4807	0.7921
rpl32	0.6863	0.9314	0.9723
mnf	0.1257	4.11E-005	0.02271
cys_loop	4.70E-007	0.02144	6.80E-006
fucta	3.21E-011	0.6665	0.193
LIM	3.21E-011	4.94E-004	6.80E-006
tenM	5.96E-011	0.4894	0.0691

Table S3: Completeness analysis (Separate file)

Table S4: KEGG analysis results (Separate file)

Table S5: qPCR candidate and housekeeping gene sequences and primers (Separate file)

CHAPTER 2

The Genetic Mechanism of Selfishness and Altruism in Parent-Offspring Coadaptation

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Abstract

The social bond between parents and offspring is characterized by coadaption and balance between altruistic and selfish tendencies. Yet the underlying genetic mechanism remains unknown. Using transcriptomic screen in the sub-social European earwig, *Forficula auricularia,* we identified two genes, *Th* and *PebIII*, both synergistically up-regulated in mothers' heads and offspring during mother-offspring interaction. *In vivo* RNAi experiments confirmed the direct and indirect genetic effects of *Th* and *PebIII* on behavior and fitness, including maternal food provision and reproduction, offspring development and survival. The direction of the effects in mothers and offspring consistently indicated a reciprocally altruistic function for *Th* and selfish function for *PebIII*. Metabolic pathway analysis suggested roles for the *Th*-restricted endogenous dopaminergic reward, *PebIII*-mediated chemical perception and insulin signaling-juvenile hormone-vitellogenin regulations in parent-offspring coadaptation and social evolution.

Parents and offspring influence each other's behavior and evolutionary fitness through reciprocal interactions. As an altruistic trait parental care is beneficial to the survival and development of offspring but costly for the parents, while selfish parents favor their lifetime fecundity at the expense of their offspring's fitness(*1*). Offspring are often tacitly regarded as passive recipients of parental care, but in reality they actively demand care and influence their parents' behavior and reproduction(*2*, *3*). Evolutionary theory predicts a tension between selfishness and altruism and genetic conflict between parents and offspring due to their incomplete relatedness(*4*). Natural selection should favor coadapted and well coordinated parents and offspring, balanced altruistic and selfish genetic tendencies and resolved genetic conflicts. To date, studies on coadaptation and conflict have focused on phenotypes rather than genes(*2*, *3*), and studies on the molecular basis of parenting focused on genes expressed merely in parents and lacked causal evidence(*5*–*8*).

Including active offspring at the gene level, a combination of these former approaches is required to explicitly consider the interplay between altruistic and selfish genes in parent-offspring interactions. We hypothesized that candidate genes underlying coadaptation should have the following signatures: i) gene expression changes both in the parent and offspring when they behaviorally interact, as coadaptation theory predicts that co-regulated expression may facilitate well-coordinated parenting by enhancing the phenotypic match between parent and offspring(*4*); ii) a change in expression level of the candidate gene in the parent or offspring should influence behavior or fitness of self and the other, via direct and indirect genetic effects (DGE and IGE), respectively(*9*, *10*); iii) when expressed, a gene with selfish function should be beneficial to self and potentially harmful to the other. Conversely, a gene with altruistic function, when expressed, should be beneficial to the other and costly to self. Here we found two genes fulfilling these criteria in the European earwig, *F. auricularia*, one with reciprocally selfish and the other with reciprocally altruistic function.

F. auricularia is a non-model insect species with facultative maternal care which enables experimental manipulations with and without mother-offspring contact. Females produce one or two clutches over their lifetime and provide food (see Movie S1) and protection to their young nymphs(*11*, *12*). Mothers influence the behavior, development and survival of their nymphs and the nymphs, in turn, influence the behavior and future reproduction of their mothers, for instance by chemical communication(*13*, *1*, *14*).

To transcriptomically screen for candidate genes, we conducted a RNA-Seq experiment with manipulated maternal-care treatments. 90 females with their clutches were randomly assigned to one of three groups: no-care (NC), egg-care (EC) and full-care (FC) (Fig. 1). To detect tissue-specific expression patterns, the head, antennae, abdomen and ovaries of females and the whole body of nymphs were sequenced separately. A total of 138 Gb Illumina High-Seq data were generated from 84

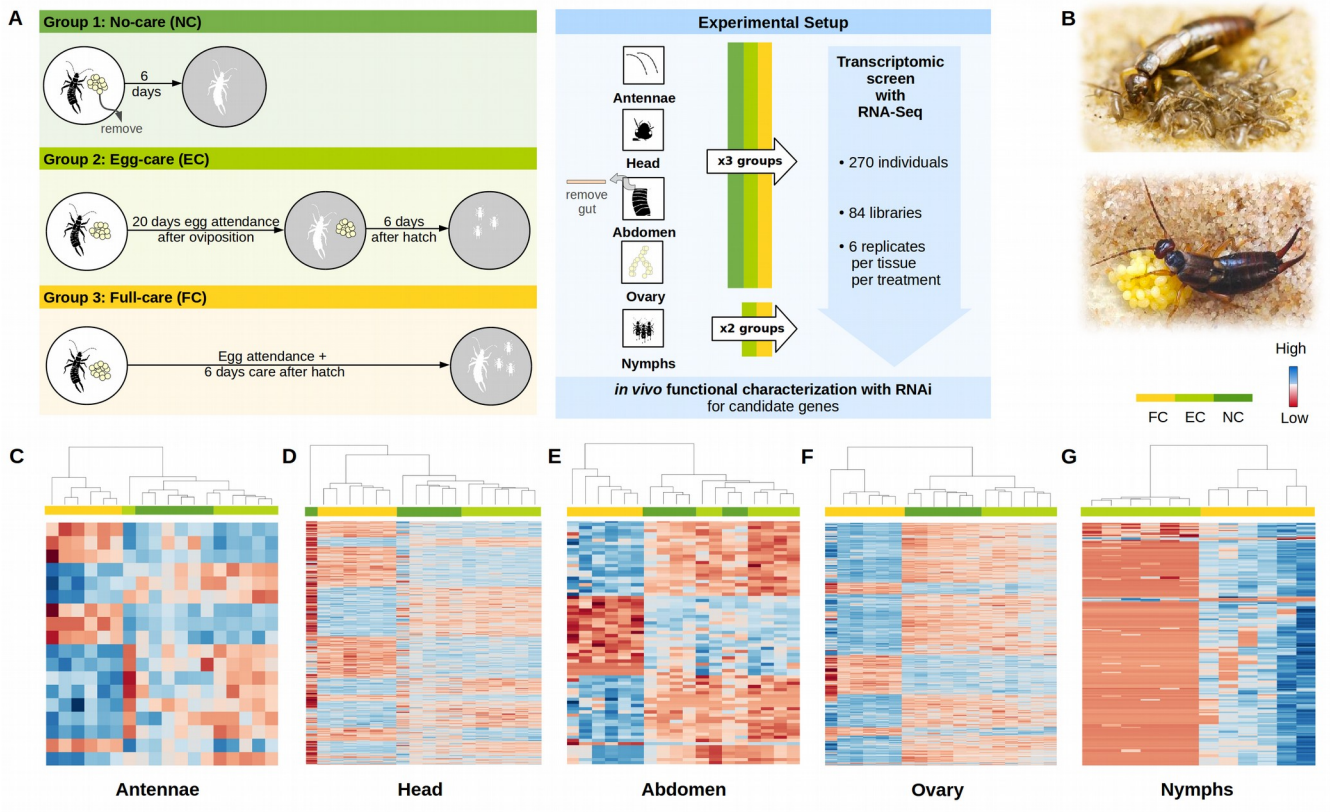

Fig. 1. Experimental design and expression heatmap. (A) Transcriptomic screen for candidate genes underlying parent-offspring coadaptation was based on experimentally manipulated earwigs in the following treatments: No-care (NC) - eggs were removed upon the completion of oviposition. No nymphs were sampled due to insufficient hatching success of untended eggs. Egg-care (EC) - mothers were allowed to tend their eggs and then sampled shortly before eggs hatched(*1*). Nymphs were kept and sampled after hatching without tending females. Full-care (FC) - the mothers tended their eggs and were kept with their nymphs. Antennae, head, abdomen and ovaries from mothers and the whole body of nymphs were sampled in each treatment. Based on this screen, the expression of two identified candidate genes was manipulated using *in vivo* RNAi to assess their causal effects on behavioral and components of evolutionary fitness; (B) Picture of an earwig mothers tending their eggs and hatched nymphs; (C)-(G) Heatmap of differentially expressed genes in different tissue from mothers and the nymphs. 1547 genes in mothers and 114 genes in nymphs showed significant expression differences between FC and EC treatments (P_{FDR}<0.01). Rows are genes, columns are samples. Samples were clustered according to expression patterns and color coded indicating their treatment in the horizontal bar above each panel. All FC samples were well clustered while EC and NC samples exhibit more similar expression patterns to each other.

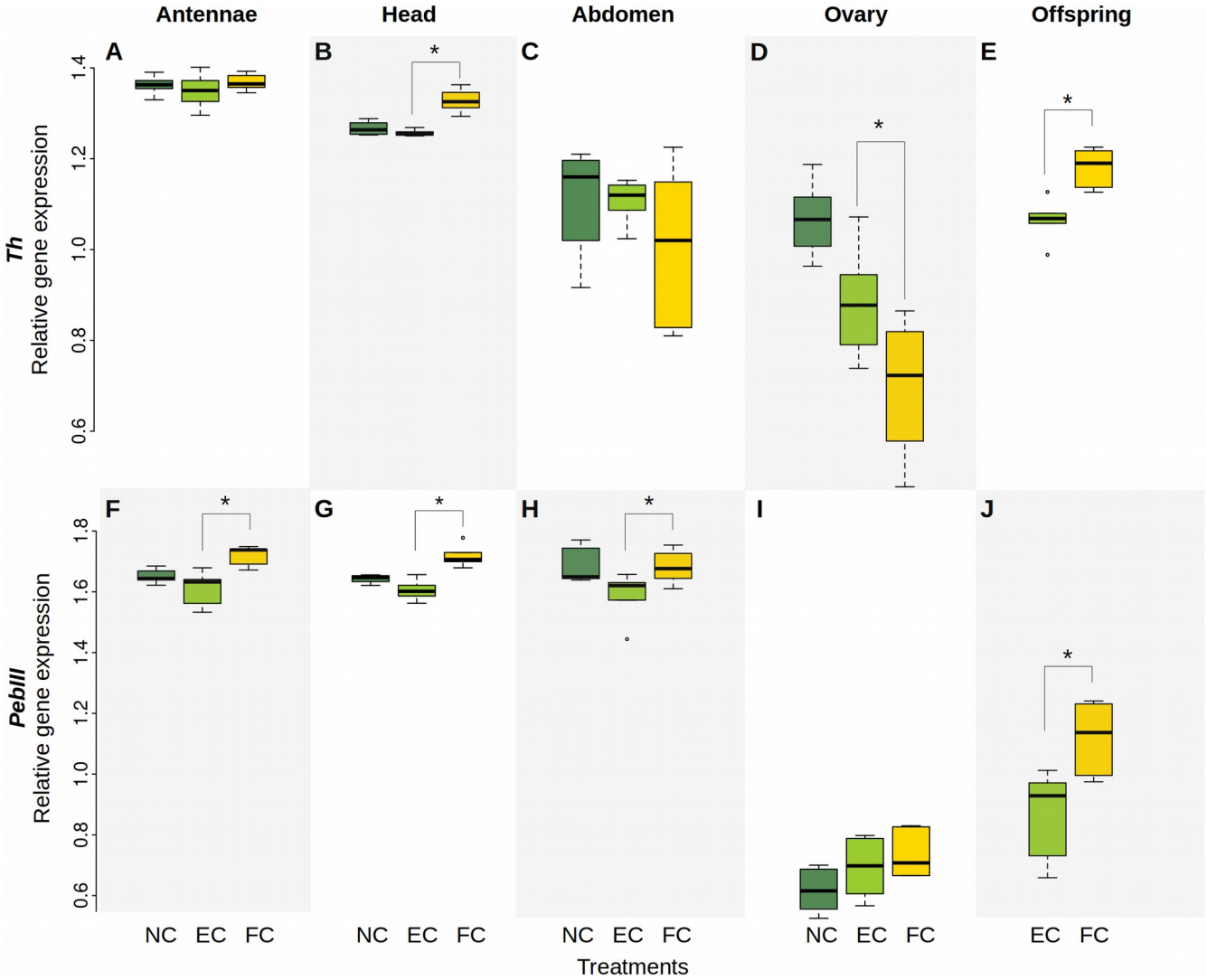

Fig. 2. Expression of *Th* and *PebIII* in maternal tissues and in the offspring. (A)-(E) *Th* expression was significantly higher in FC mothers' head and offspring but lower in mother's ovaries than in the EC treatment. (F)-(J) Expression of *PebIII* was higher in FC mothers' antennae, head, abdomen and in offspring than in the EC treatment. Statistical analyses are presented in Table S1. Treatment: NC: no-care, EC: egg-care, FC: full-care. The box-plots are shown with medians, interquartile range (box), and 1.5x interquartile range (whiskers), P_{FDR}<0.01*.

libraries and mapped to a previously published earwig transcriptome(*15*). 1547 genes in at least one maternal tissue and 114 genes in nymphs were differentially expressed between the FC and EC treatments (P_{FDR}<0.01) (Data S1-5). All of the FC samples were well clustered based on the expressionof these genes while samples from NC and EC treatments were more similar to each other (Fig. 1). This pattern was true for all maternal tissues as well as the nymphs, indicating consistently and broadly altered gene expression when mothers and their offspring behaviorally interacted.

Two genes showed evidence for coordinated differential expression between FC and EC in at least one maternal tissue and in nymphs. One (Contig 4258 in the published earwig transcriptome(15)) is homologous to the D. melanogaster *Th* gene. The other (Contig 29301) is homologous to the *PebIII* gene. *Th* encodes for tyrosine hydroxylase, the first and rate-limiting enzyme in the dopamine synthesis pathway(16) and *PebIII* for ejaculatory bulb protein III, an odorant-binding protein (OBP)(17).

Th expression was elevated in the FC mother's head (P_{FDR}<0.01, t-test) and in her nymphs (P_{FDR}<0.01, t-test; Fig. 3), but its expression in ovaries was reduced (P_{FDR}=0.028, t-test), showing a tissue-specific treatment response in females (Fig 3; Table S1, LMM: parent-offspring interaction x tissue, P=0.001). We also found up-regulation of DDC (Contig 5494), another enzyme involved in dopamine synthesis, in the head; and NAT (Contig 14038), an enzyme for dopamine degradation in the ovaries (Fig. S1; P_{FDR}<0.01 for both genes). These results further corroborated the enhanced dopamine activity in head and decreased activity in ovaries during full care. Dopamine is a well-studied neurohormone with a conserved neuropeptide function in the reward system and for associative learning from insects to mammals(*18*, *19*). Dopamine also function as gonadotropin in various insect species including fruit flies, bees and ants(*20–22*). Thus the fact that earwig mothers suppress their reproduction during parenting(*4*) may be partly regulated by the antagonistic expression of *Th* and dopamine in head and ovaries. The synergistic up-regulation of *Th* in mother's heads and in her nymphs suggested a role of the dopaminergic mutual reward in maintaining the social bonding between the parent and offspring.

PebIII expression in mothers was enhanced in the FC mother's antennae (P_{FDR}<0.01, t-test), head (P_{FDR}<0.01, t-test) and abdomen (P_{FDR}=0.04, t-test), as well as in her nymphs (P_{FDR}=0.034; Fig. 3). Given the putative function of *PebIII* as OBP, its involvement in parent-offspring communication and the perception of chemical cues such as cuticular hydrocarbons (CHC)(*14*) is conceivable. Its up-regulation in the FC treatment may refer to enhanced olfactory sensitivities when mothers and nymphs interact. A link to chemical communication was further supported by the up-regulation of genes homologous to Acyl-CoA desaturase involved in CHC synthesis(*23*) in FC mother's head, abdomen, ovaries and in her nymphs (Fig. S2; Contig 8369 and Contig 10162; nymphs: Contig 3433, P_{FDR}<0.01 for all genes).

To characterize the social function of these two candidate genes *in vivo*, we knocked-down the

expression of *Th* or *PebIII* in mothers (M-/O+), in the offspring (M+/O-) or in both (M-/O-) using RNA interference. The third treatment (M-/O-) served as a control to test the effect of enhanced expression of a gene in mothers when compared with M+/O- or in offspring when compared with M-/O+. The specificity of knock-down was initially validated by RT-qPCR *(*Fig. S3, Wilcoxon test, P<0.05). Treatment effects on behavior and fitness traits were assessed in comparison to a corresponding *YFP (yellow fluorescent protein)* experiment to control for confounding side-effects of ds-RNA injection. A specific influence of a target-gene in mothers or offspring was statistically demonstrated by significant crossed effects between gene identity (target-gene vs. *YFP*) and maternal treatment or offspring treatment, respectively.

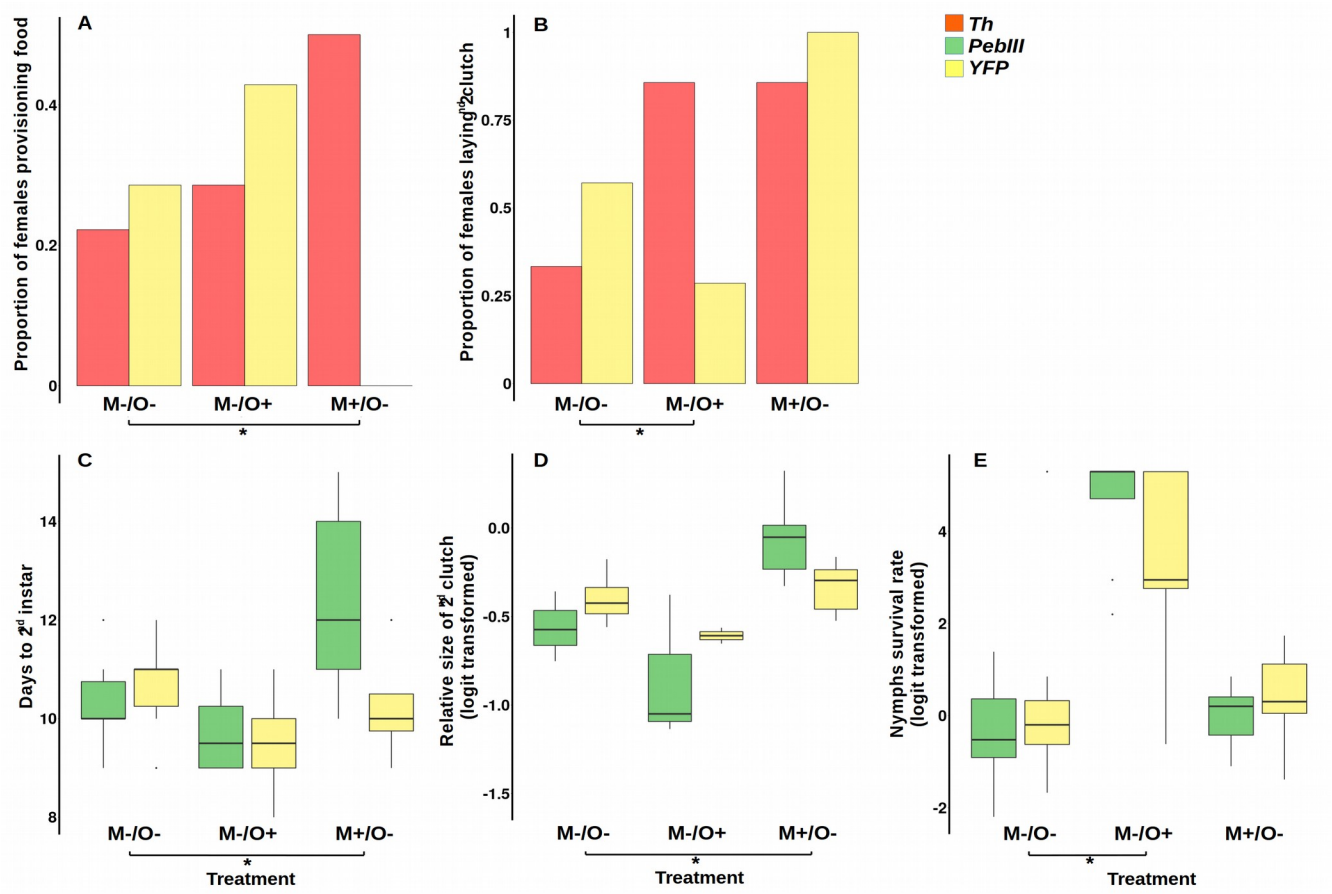

Fig. 3. Experimental effects of *Th* and *PebIII* expression on behavior and components of evolutionary fitness in earwig mothers and nymphs. Target genes were knocked-down in three treatments: only in mothers (M-/O+), only in offspring (M+/O-), and in both (M-/O-). YFP was used as a control for injection of exogenous dsRNA. Statistically, effects of candidate gene expression were assessed relative to the YFP control (Table S6). In (D), the relative size of the 2nd clutch was calculated based on first and second clutch egg-numbers; In (A) and (B) frequencies are shown, and in (C)-(E) box-plots with median, interquartile range (box), and 1.5x interquartile range (whiskers) are shown, P<0.05*.

Mothers expressing comparatively higher *Th* (M+/O-) provided more food to their offspring (Fig. 4a; Table S2, GLM: gene x maternal-treatment, P=0.0097) than controls (M-/O-). For offspring with higher *Th* expression (M-/O+), their mothers were more likely to produce a second clutch (Table S2, GLM: gene x offspring-treatment, P=0.010) (Fig. 4b) than controls. Enhanced food provisioning is a typical parental behavior that elevates the fitness of offspring at a cost to mothers(*11*). Thus elevated expression of *Th* in mothers induced a maternal behavior that is beneficial to offspring and costly for themselves. Increased maternal future reproduction enhances maternal lifetime fecundity, but from the perspective of the current nymphs inducing this effect, it is at a cost of reduced received care, as formerly demonstrated in experimental evolution experiments in this species(*1*). Hence in both mothers and nymphs, higher expression of *Th* enhanced the fitness of the other at a potential expense of self, which is consistent with our prediction of an altruistic gene.

Mothers expressing relatively higher *PebIII* (M+/O-) invested more in their future reproduction with larger relative size of their second clutch (Fig. 4c; Table S2, GLM: gene x maternal-treatment, P=0.027) and the developmental rate of their offspring were slower (Fig. 4d; Table S2, GLM: gene x maternal-treatment, P=0.0036). Higher *PebIII* expression in nymphs (M-/O+) led to better survival than among controls (Fig. 4e; Table S2, GLM: gene x offspring-treatment, P=0.039). In both cases, individuals that expressed *PebIII* gained benefits for themselves, but partially harmed the fitness of the other. These results are consistent with our prediction for a selfish gene.

A long-standing question in the literature is whether genes expressed in parents or offspring control reproductive investment(*24*). Our results in earwigs provide evidence for a compromise with partial control by genes expressed in both parent and offspring. Whether or not a female produced a second clutch was under offspring-control and modulated by *Th* expression in nymphs. However, how much the females invested in her future clutch, relative to her current clutch, was under maternal control and influenced by *PebIII* expression in females.

The mechanism for how *PebIII* affected reproduction could be due to the known link between *PebIII* protein and the yolk protein vitellogenin (Vg) and juvenile hormone (JH) via lipophorin(*25*). In support of this hypothesis, we found evidence for suppressed JH and elevated Vg in FC mothers inferred from the differential expression of genes related to the JH metabolism and Vg (Fig 4). This is

in agreement with previous study showing that low JH titer is associated with maternal care for nymphs in earwigs(*26*). Gonadotropic functions of JH and Vg are well-known in insects and were shown to relate to parental care in the burying beetle (*N. vespilloides*)(*27*) and caste determination and division of labor in eusocial species such as the honey bee (*A. mellifera*)(*28*). The antagonistic regulation between JH and Vg once thought to be unique in honey bee was found in subsocial earwigs as well as burying beetles, suggesting its role in post-hatching parental care and social evolution. In addition, insulin signaling interplays with JH and Vg and has been found to associate with reproductive asymmetry between castes in eusocial ants(*29*). In FC earwigs we found down-regulated *FOXO* transcription factor which is known to suppress insulin signaling(*30*), indicating the social regulation of insulin signaling in subsocial species. Hence, our results also provide indirect evidence for an evolutionary link between parenting genes and genes shaping eusociality regarding the regulation of insulin signaling, JH and Vg.

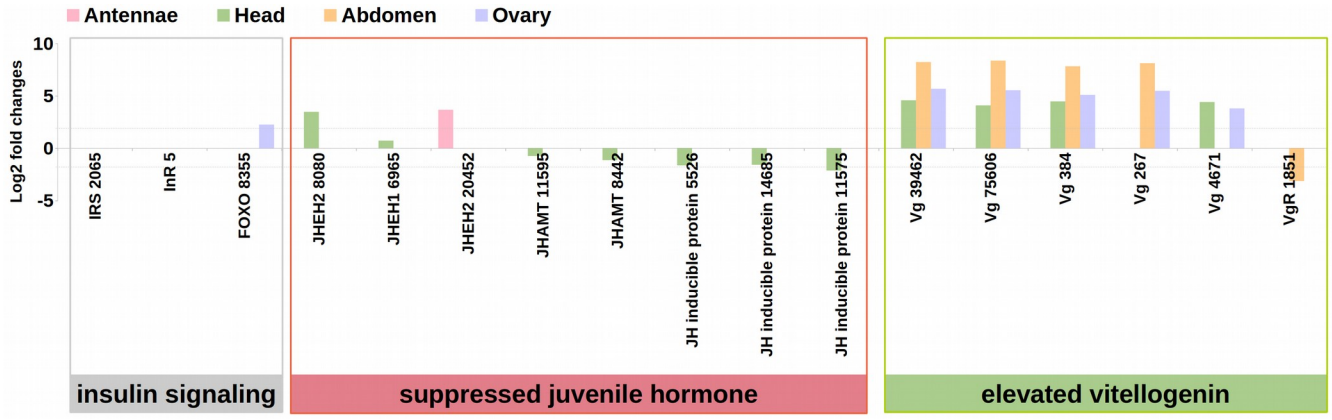

Fig. 4. Differential expression of genes related to the insulin signaling-juvenile hormone-vitellogenin regulation. Bars above zero indicate up-regulation while bars below zero indicate down-regulation in full-care (FC) mothers compared to egg-care (EC) with $P_{FDR}<0.01$. Dotted lines indicate thresholds for 2 and -2 log2 fold changes. The number after each gene name refers to contig ID in the published earwig transcriptome(*15*).

Our study adds to the view that the dopamine signaling pathway may be evolutionarily conserved from insects to primates and human in the context of parent-offspring interactions. In vervet monkeys and humans, variable number tandem repeats (VNTR) in exon III of the dopamine receptor *DRD4* gene were found to be associated with parent-offspring interaction(*31–34*). It is likely that the behavioral difference in vervet monkey and human mothers and offspring were due to differential expression of various alleles or different receptor sensitivity to dopamine resulting from the allelic polymorphism of

DRD4. That this pathway appears to also be crucial to the simpler and non-obligate mother-offspring interaction in earwigs suggests an evolutionarily conserved and ancestral function of dopamine in the evolution of the social bond between parents and offspring.

Unlike the conserved dopamine, the function *PebIII* seems to be co-opted along the trajectory of social evolution from solitary to sub-social and to eusociality. It altered from direct control of offspring development, to social regulation of development and additional control of maternal reproduction, and to reproductive caste differentiation. In the solitary Drosophila, larval development was associated with *PebIII* expression in larvae through DGE(*35*). In earwigs, we found that nymphal development was influenced by *PebIII* expression in mothers through IGE. Although *PebIII* consistently influences offspring development in solitary and sub-social insects, the regulation seemingly shifted from direct control by the offspring to indirect control by the parent. In addition, maternal expression of *PebIII* govern female reproduction in earwigs via DGE. In the eusocial termite *Reticulitermes flavipes*, two versions of this gene are expressed caste-specifically between sterile soldiers and reproductive alate(*36*). This suggests a consistent function on female reproduction between sub-social and eusocial species, but diverged from single-gene determination to two versions of the same gene with potential neofunctionalization or subfunctionalization. Thus, our results on *PebIII* might have captured an intermediate functional state of this gene between solitary and eusocial species.

It is a general expectation that the social bond between parents and their offspring is shaped by both altruistic and selfish behavior. However, evolutionary theory ultimately relies on genetic or genomic support to demonstrate these tendencies. Here we used predictions of coadaptation theory and identified two genes, *Th* and *PebIII*, with such distinct social functions in the interaction between earwig mothers and their nymphs. Neither *Th* nor *PebIII* were altruistic or selfish in a classical sense because both genes had a comparable function when expressed in mothers and offspring. Such reciprocally altruistic and reciprocally selfish gene functions are peculiar because the gene's fitness loss or gain during one life-stage may at least partly be offset by its gain or loss during the other life-stage. We envision that reciprocally altruistic or selfish gene is a signature of parent-offspring coadaptation. They should be typical among genes underlying the social bond between parents and offspring and possibly also in other social systems.

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wrote the manuscript. **Competing Interests:** Authors declare no competing interests. **Data and Materials Availability:** Raw transcriptomic data will be deposited and publicly available on the European Nucleotide Archive upon acceptance of the paper.

Supplementary Materials:

Materials and Methods

Figures S1 to S3

Tables S1 to S3

Movie S1

External Databases S1 to S5

Reference (37-43)

SUPPLEMENTARY MATERIALS

Materials and Methods

Behavioral manipulation The earwigs were maintained in the laboratory as previously described(*1*). A total of 90 randomly picked mated females from the breeding stocks were randomly assigned to three experimental treatments. Females assigned to the no-care (NC) treatment were isolated from their first clutch one day after egg laying which is typically the time when the clutch is complete. These females were then kept in a new petri dish for 6 days without food, as is natural during the period of egg-care, and then sacrificed for RNA extraction. Females assigned to the egg-care (EC) treatment tended their eggs for 20 days to ensure maximal duration of egg care while avoiding contact with hatched offspring (nymphs). Hatching typically occurs after 21-30 days(*1*). The eggs were allowed to hatch and the nymphs were kept with food for six days before 3 nymphs per clutch were sampled for RNA extraction. Females in the full-care (FC) treatment tended their eggs until hatching and then interacted with the nymphs for 6 days before the females and three nymphs per clutch were sampled. 6 days was chosen because mother-offspring interaction reach a peak at this time(*37*).

NC-females were in a state where no maternal care could be expressed except for a maximum one-day contact with eggs during oviposition. EC-females could express care exclusively towards eggs, and only FC-females could behaviorally interact with hatched offspring. With regard to offspring, the EC-nymphs experienced no interactions with their mother, while the FC-nymphs had such interactions for six days. NC-nymphs were not used because the hatching success of eggs without maternal care is too low(*38*). Thus, differential gene expression between the FC and EC treatments in females and nymphs are assumed to be largely due to parent-offspring interactions. Differential expression between the EC and NC treatment in females are assumed to be largely due to egg attendance.

RNA-Seq sequencing The insects were sacrificed by exposure to high concentrations of petroleum ether before dissection. From females the antennae, head, abdomen (without gut to avoid microbial contamination) and ovaries were sampled separately for RNA extraction to investigate tissue-specific variation in gene expression. In nymphs, this was not possible and RNA was extracted from the whole body, using 3 nymphs per family to obtain sufficient RNA quantities. For each of the four maternal

tissues and for the nymphs, the tissues of 5 families were pooled to obtain sufficient RNA and thus used as one biological replicate in the RNA-Seq analysis. All samples were stored in RNAlater (Qiagen) at -80°C.

A total of 84 samples (from 4 female tissues and from nymphs across three treatments) were processed for RNA extraction using the TRIzol protocol (Invitrogen), resulting in 6 replicates per tissue and treatment. The cDNA library was prepared and sequenced as paired-end 100-nt reads on Illumina HiSeq. The samples were indexed using Illumina TruSeq kit and evenly distributed among 4 multiplex lanes.

Bioinformatic analysis An average of 18-million RNA-Seq reads per sample were generated. The reads of each sample were mapped to a previously published earwig transcriptome(*15*) using the BWA-MEM algorithm in BWA version 0.7.8-r455(*39*). Samtools version 0.1.18(*40*) was used to process sam files to bam format and count mapped reads for each contig. Mapped reads with a mapping quality higher than MQ40 were processed for further analysis.

The initial statistical analysis of gene expression differences between experimental treatments was carried out using the egdeR package(*41*) in R. The RNA-Seq data were TMM normalized. Pairwise comparisons of each gene between FC and EC treatments were performed for each female tissue and the nymph samples using exact negative binomial tests. To take into account multiple testing, we used a false discovery rate (FDR) correction as implemented in edgeR. Corresponding P-values are denoted as P_{FDR}.

In maternal tissues, candidate genes were required to show differential expression in at least one tissue with $P_{FDR} < 0.01$. We chose the significance threshold to be less stringent for nymphs, because only whole-body samples were available which could potential mask tissue-specific expression resulting in lower precision of the expression data. In nymphs we set our selection criteria for candidate genes such that $P_{FDR} < 0.1$ and, to confirm insect origin, that the genes had to be present in at least one of five insect genomes (*Apis mellifera, Acromyrmex echinatior, Drosophila melanogaster, Tribolium castaneum,* and *Nasonia vitripennis*) to ensure their insect origin.

Treatment effects on gene expression was then analyzed using linear mixed models (LMM) in R (package lme4). For females, based on the three experimental treatments, the fixed factors "egg-attendance" (Yes/No) and "parent-offspring interaction" (Yes/No) were established. These factors as well as their crossed effects with tissue were entered as fixed factors, and sample ID as a random factor. For nymphs, Welch t-tests were used to test effects of parent-offspring interaction (Yes/No) on expression levels. The expression levels were normalized using the delta-delta Ct method(*42*), calculated relative to the average of the housekeeping genes Rpl32 and Actin, and to the average of all samples. Normalized read counts were used for the calculation of relative expression level.

Gene Ontology and KEGG analysis were performed for differentially expressed genes with Blast2Go version 2.7.2(*43*). A cut-off value was set at 10e-6 for the blastx search against the NCBI non-redundant nucleotide database and the Swissprot database, using the NCBI Blast service.

Experimental design for RNAi To test effects of expression of the two candidate genes on females and nymphs, we carried out an RNA interference experiment where the expression of each target gene, Th or PebIII, was knocked-down in separate treatments: knock-down in mothers (M-/O+), nymphs (M+/O-) and both (M-/O-). A control experiment with the same treatments used YFP (yellow fluorescence protein) dsRNA as control for the injection of exogenous double-stranded RNA. A total of 70 randomly mated female earwigs were randomly assigned to the experimental groups (Table 1). Clutch size was standardized to 20 nymphs in each family one day after hatching.

dsRNA synthesis The amplified sequences of each gene with a T7 promoter overhang at 5' and 3' respectively were cloned from earwig cDNA to plasmids for storage and large scale yields (Table S3). Cloning was confirmed by sequencing the PCR product of each target gene from the plasmids. Double-stranded RNA was synthesized using RiboMAX large scale RNA synthesis system-T7 (Promega). The final concentration of dsRNA used for injection was 6 μg/μl for each gene. The mothers were injected with 2μl and 20 nymphs from each family were injected with a total of 2μl dsRNA on day 4 after the nymphs hatched. For RNAi injection, we used a cellTram air microinjector (Eppendorf) and borosilicate capillaries (Harvard) processed with a P-1000 Micropipette Puller (Sutter Instruments). Prior to injection the earwigs were exposed to low concentrations of petroleum ether (Sigma-Aldrich #77379) vapor for sedation.

Validation of RNAi The knock-down effects were initially validated with RT-qPCR after injection, using three replicates per gene for both mothers and nymphs. Untreated families were used as wild-type control. Mothers of offspring injected with the same volumes of water was used as additional control. Each female sample is one head. Each offspring sample was a pool of three nymphs from the same family. RNA was extracted using the TRIzol-LS reagent (Ambion). The cDNA libraries were synthesized using the GoScript Reverse Transcriptase system (Promega). The qPCR was run in triplicates on the Applied Biosystems 7500 Fast platform, using an EvaGreen 2x qPCR Mastermix reagent (Biotium). Expression levels were calculated using the delta-delta Ct as described before. The calibration was done separately for mothers and offspring (see Fig S3 for results).

Behavior and fitness assay The developmental and reproductive variables were quantified following the standard protocol used in previous study in this species(*1*). The rate of offspring development was quantified as the number of days from hatching until the first nymph of a family reached the second juvenile instar and nymph survival as the proportion of surviving nymphs three days after injection divided by the number of nymphs hatched. The future reproduction of females was assessed by 1) noting if a second clutch was produced within 60 days of hatching of the 1st clutch and by 2) counting the relative size of second clutches as the proportion of eggs in the second clutch over the sum of eggs in the first and second clutch. Food provisioning was recorded during observation sessions under red light (earwigs are nocturnal) of one hour each across three consecutive days. Observations were started 15 minutes after setup to calm down the animals. The occurrence of mouth-to-mouth contact between the female and one of her nymphs was treated as a binomial trait (yes/no). The observer was blind to experimental treatments. For quantitative traits, the average values across the three consecutive observation sessions were used in the analysis. For binary traits, at least one event across the three sessions counted as "Yes".

Statistical analysis for RNAi The effects of the RNAi treatments on these measurements were tested using generalized linear models (GLM) with female treatment, offspring treatment, gene identity (candidate gene vs. *YFP*) and the crossed effects between the treatments and the factor "gene" as fixed effects. Laying date was added as a covariate. Continuous dependent variables were modeled using a gaussian error distribution. Discrete or proportional dependent variables (food provisioning yes/no, 2nd clutch yes/no, relative size of 2nd clutch) were analyzed using a binomial error distribution and a logit-

link. Effects of the candidate gene are detectable as deviation from the *YFP*-side-effects as significant interactions between the factor "gene" and one of the treatment factors. Statistical analyses were carried out using R version 3.1.1, tests were two-tailed with a significance threshold $\alpha = 0.05$.

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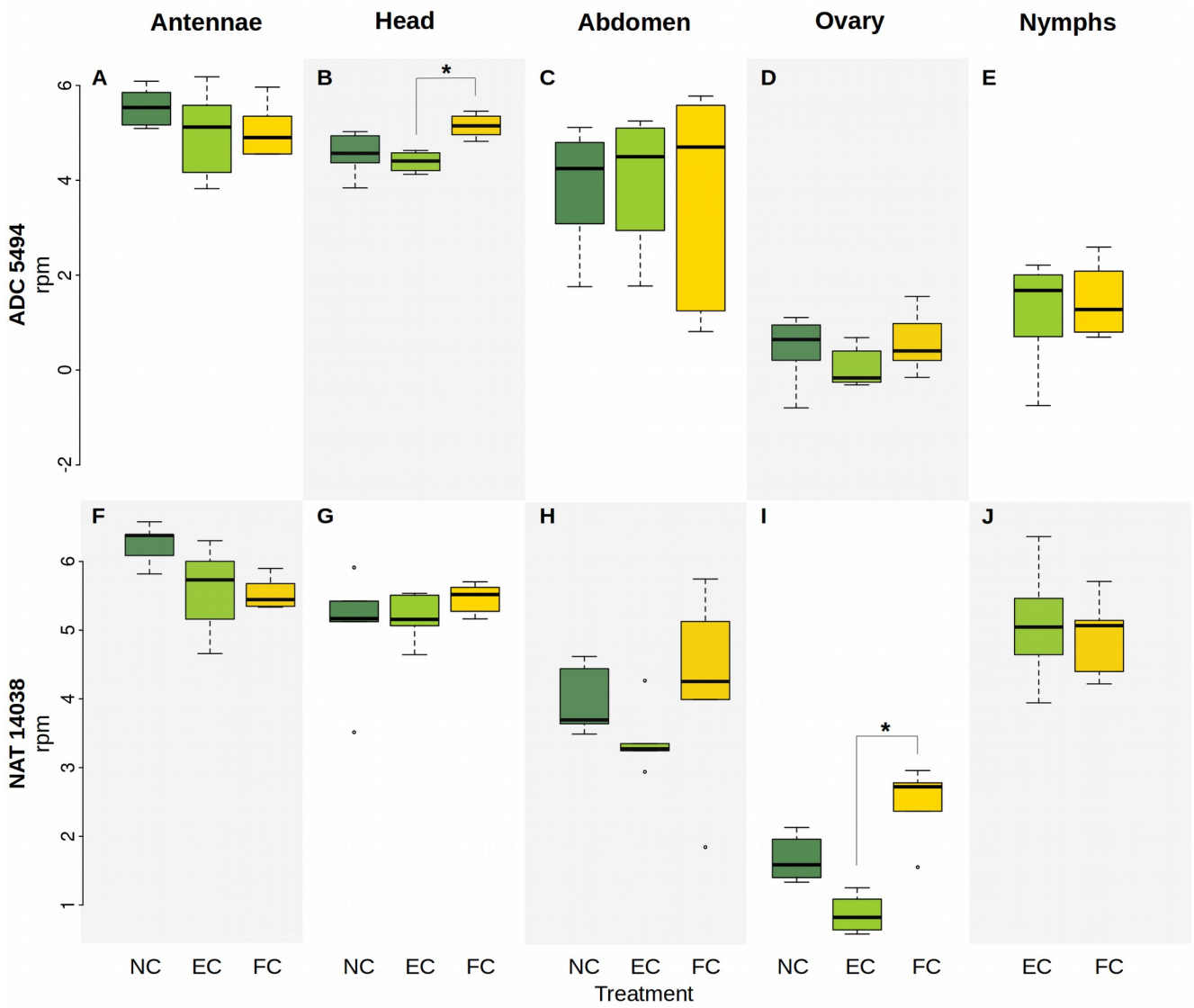
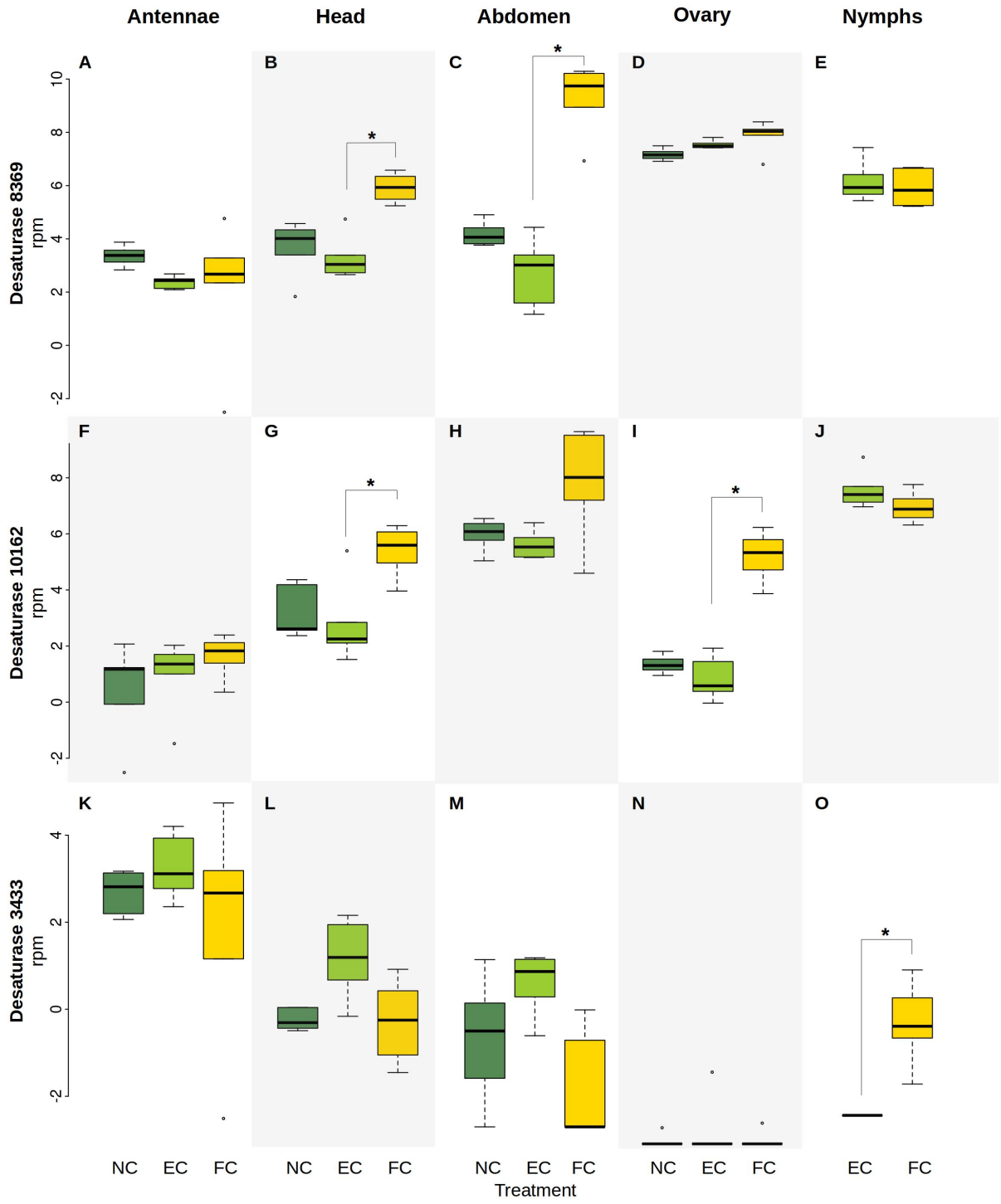
Fig. S1.



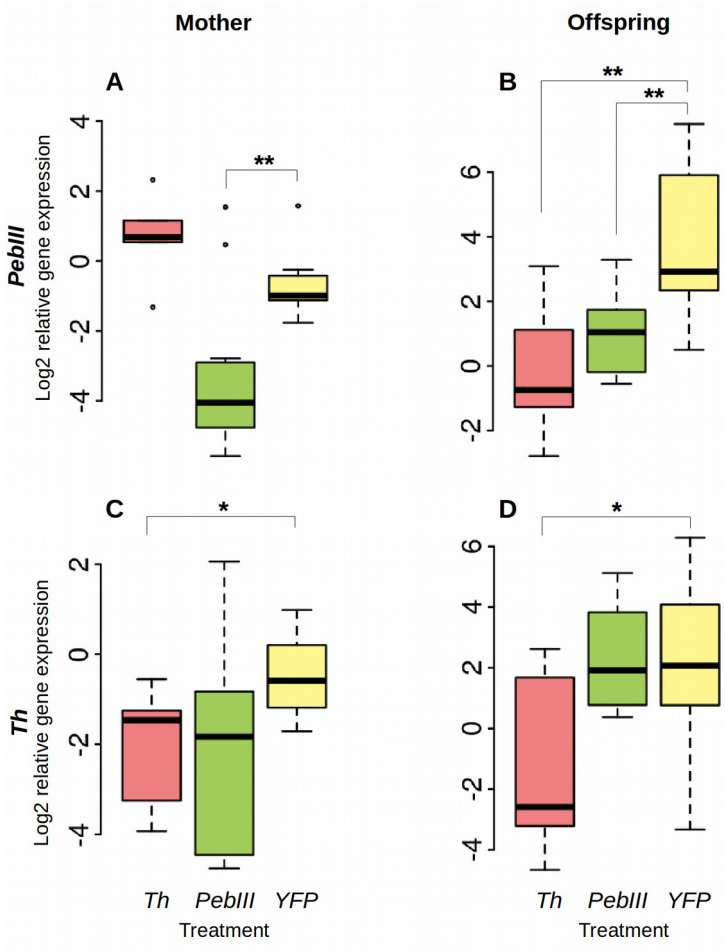
Expression of dopamine related genes. Significant differential expression between EC and FC is labeled with asterisk (P_{FDR}<0.01). Contig ID is labeled next to gene name. Y-axis is normalized read counts.

Fig. S2.



Expression of Acyl-CoA-desaturase genes. Significant differential expression between EC and FC is labeled with asterisk (P_{FDR}<0.01). Contig ID is labeled next to gene name. Y-axis is normalized read counts.

Fig. S3.



RT-qPCR validation for *Th* and *PebIII* knock-down. Comparing to controls with *YFP* ds-RNA injection, *PebIII* expression was down-regulated in mothers and in the offspring with *PebIII* ds-RNA injection (wilcoxon test, $P_{mothers}$=0.0097, $P_{offspring}$=0.0091). *Th* expression in mothers and in the offspring with *Th* ds-RNA injection was lower than *YFP* ds-RNA injected controls (wilcoxon test, $P_{mothers}$=0.035, $P_{offspring}$=0.038). *PebIII* expression was also down-regulated in the offspring with *Th* ds-RNA injection (wilcoxon test, P=0.0059). P<0.05*, P<0.01**.

Table S1.

***Th* expression in mothers**							
	Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr(>F)	
Parent-offspring interaction	0.01	0.01	1	14.515	2.30	0.151	
Egg attendance	0.02	0.02	1	14.176	2.50	0.136	
Tissue	1.69	0.56	3	43.751	89.96	<2.20E-016	***
Parent-offspring interaction:Tissue	0.12	0.04	3	43.751	6.16	0.00138	**
Egg attendance:Tissue	0.07	0.02	3	43.426	3.70	0.0187	*

***PebIII* expression in mothers**							
	Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr(>F)	
Parent-offspring interaction	0.05	0.05	1	13.287	10.37	0.00654	**
Egg attendance	0.0047	0.00	1	12.856	0.98	0.341	
Tissue	9.44	3.15	3	42.541	660.16	<2.00E-016	***
Parent-offspring interaction:Tissue	0.02	0.01	3	42.541	1.74	0.173	
Egg attandence:Tissue	0.04	0.01	3	42.071	3.13	0.0355	*

LMM analysis for the expression of *Th* and *PebIII* genes in mothers based on RNASeq results.

P<0.05*, P<0.01**, P<0.001*** .

Table S2.

Behavior and fitness consequences of *Th*

Maternal food provision (*binary trait: Yes/No, binomial distribution, logit link)*

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)	
NULL	45	54.777				
Gene	1	0.6419	44	54.135	0.42302	
Female treatment	1	0.1143	43	54.021	0.735287	
Gene:Female treatment	1	6.6863	42	47.334	0.009715	**

Likelihood of maternal future reproduction (*binary trait: Yes/No, binomial distribution, logit link*)

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)	
NULL	43	57.682				
Oviposition	1	5.6118	42	52.071	0.01784	*
Gene	1	0.3198	41	51.751	0.571706	
Offspring treatment	1	1.2317	40	50.519	0.267084	
Female treatment	1	6.9373	39	43.582	0.008442	**
Gene:Offspring treatment	1	6.5819	38	37	0.010302	*

Behavior and fitness consequences of *PebIII*

Offspring development *(gaussion distribution, identity link)*

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)	
NULL	42	92.605					
Oviposition	1	4.0018	41	88.603	3.0889	0.087099	.
Gene	1	6.1995	40	82.403	4.7852	0.035099	*
Offspring treatment	1	19.3602	39	63.043	14.9436	0.000432	***
Female treatment	1	2.5437	38	60.499	1.9634	0.169481	.
Gene:Female treatment	1	12.564	37	47.935	9.6978	0.003552	**

Relative investment in maternal future reproduction *(overdispersed binomial distribution, logit link*)

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)	
NULL	24	34.366				
Oviposition	1	0.0028	23	34.364	0.958067	
Gene	1	0.4993	22	33.864	0.479815	
Offspring treatment	1	6.6968	21	27.168	0.009658	**
Female treatment	1	2.2592	20	24.908	0.132819	*
Gene:Female treatment	1	4.9162	19	19.992	0.026607	*

Offspring survival rate *(overedispersed binomial distribution, logit link*)

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)	
NULL	45	106.572				
Oviposition	1	3.927	44	102.645	0.04753	*
Gene	1	0.134	43	102.511	7.15E-001	***
Offspring treatment	1	54.559	42	47.952	1.51E-013	***
Female treatment	1	1.386	41	46.566	0.23909	
Gene:Offspring treatment	1	4.276	40	42.29	0.03865	*

GLM analysis for behavior and fitness consequences of *Th* and *PebIII* in RNAi experiment. To account for *YFP* treatment as the control for each corresponding treatment of target genes, only crossed effects between gene and treatment were regarded as the genetic effects of each target gene. P<0.05*, P<0.01**, P<0.001*** .

Table S3.

Gene	RNA Strand	Name	Primer sequence	Amplicon length
Th	1	TH_F	5'-CTG GGA CAC ATG CCA CTT CT-3	774
		TH_RT	5'-AAA GCG GCC GCT AAT ACG ACT CAC TAT AGG TCG TCA GTT TCC AGC TCC AC-3	
	2	TH_FT	5'-AAA GCG GCC GCT AAT ACG ACT CAC TAT AGG CTG GGA CAC ATG CCA CTT CT-3	774
		TH_R	5'-TCG TCA GTT TCC AGC TCC AC-3	
PebIII	1	PEB_F	5'-TTG GTT CTC TTC GCT GAG GC-3	333
		PEB_RT	5'-AAA GCG GCC GCT AAT ACG ACT CAC TAT AGG TCC AGT TGG GTC GTA TTT CTC TT-3	
	2	PEB_FT	5'-AAA GCG GCC GCT AAT ACG ACT CAC TAT AGG TTG GTT CTC TTC GCT GAG GC-3	333
		PEB_R	5'-TCC AGT TGG GTC GTA TTT CTC TT-3	
YFP	1	YFP_F	5'-TTC AGT GTT TCG CGC GTT ATC-3'	530
		YFP_RT	5'-AAA GCG GCC GCT AAT ACG ACT CAC TAT AGG TTC AGT GTT TCG CGC GTT ATC-3'	
	2	YFP_FT	5'-AAA GCG GCC GCT AAT ACG ACT CAC TAT AGG CAT ACC CAG GGT AAT ACC GGC-3'	530
		YFP_R	5'-CAT ACC CAG GGT AAT ACC GGC-3'	

Primers for double-stranded RNA synthesis.

Movie S1.

Food provisioning in earwigs. An earwig mother surrounded by her offspring is feeding them mouth-to-mouth.

Data S1. (separate file)

List of genes responsive to parent-offspring interaction in of earwig mothers' antennae. Differentially expressed genes between FC and EC treatments in the antennae of earwig mothers. Genes with negative log fold changes were up-regulated in FC samples.

Data S2. (separate file)

List of genes responsive to parent-offspring interaction in earwig mothers' head. Differentially expressed genes between FC and EC treatments in the head of earwig mothers. Genes with negative log fold changes were up-regulated in FC samples.

Data S3. (separate file)**List of genes responsive to parent-offspring interaction in earwig mothers' abdomen.** Differentially expressed genes between FC and EC treatments in the abdomen of earwig mothers. Genes with negative log fold changes were up-regulated in FC samples.

Data S4. (separate file)

List of genes responsive to parent-offspring interaction in earwig mothers' ovaries. Differentially expressed genes between FC and EC treatments in the ovaries of earwig mothers. Genes with negative log fold changes were up-regulated in FC samples.

Data S5. (separate file)

List of genes responsive to parent-offspring interaction in earwig offspring. Differentially expressed genes between FC and EC treatments in earwig nymphs. Genes with negative log fold changes were up-regulated in FC samples.

CHAPTER 3

Transgenerational Effects of Maternal Care on Parental-Offspring Coadaptation

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Abstract

Parental care is common in nature. Parents and offspring typically reciprocally influence the behavior and fitness of each other. Parent-offspring coadaptation was predicted to resolve the conflict in parents and the young over the interests of their own fitness. The transgenerational co-evolution is a key mechanism maintaining parent care where the care and the effects of care are genetically correlated. Offspring who receive adequate maternal care are expected to be genetically predisposed towards the same mothering style in adulthood. In the European earwig (*Forficula auricularia*), an insect species with facultative uni-parental female care, two parent-offspring coadapted genes *PebIII* and *Th* were recently identified (Wu et al, in prep; Chapter 2). Both genes are co-regulated in mothers and offspring and influence their behavior and fitness in mothers and offspring. In this study, we manipulated the interaction between earwig mothers and offspring over two generations and found transgenerational effects on the expression of these two coadapted genes and on the fitness in mothers and offspring. Our results indicate an epigenetic regulation of genes underlying parent-offspring coadaptation.

Introduction

Parental care is common in nature. Parents and offspring typically reciprocally influence the behavior and fitness of each other. Parents provide care including food provisioning, anti-predator and anti-parasite defense to the offspring, at the cost of their own fitness such as future reproductive success and survival [1],[2]. Offspring that tend to aggregate with their parents on one hand are more likely to benefit form parental care and convert it into their own fitness, for instance, higher survival and growth rate [3], [4]. On the other hand, offspring can also affect parental care through behavioral or chemical signals demanding for resource [5]. To resolve such conflict in family living, parental-offspring coadaptation was predicted selecting for the combined optimization of fitness in both parent and offspring [6]. A key mechanism maintaining parent care and driving parent-offspring coadaptation is the transgenerational co-evolution between genes expressed in mothers and in offspring, where the care and the effects of care are genetically correlated [7]. The theory predicts that offspring who receive adequate maternal care are genetically predisposed towards the same mothering style in adulthood [8], [9]. In rodents, post-natal maternal care influence the expression of estrogen receptor-α gene, DNA methylation in the promotor of this gene and maternal behavior of female offspring [16], [17]. Such maternal effect on DNA methylation and maternal behavior could be transmitted over two generations [18].

The European earwig (*Forficula auricularia*) is an insect species with facultative uni-parental female care. Heritable component of maternal care was confirmed in earwigs [10], [11]. And it was shown that maternally deprived offspring become poor mothers themselves [11]. These results suggested a role of genomic imprinting partly underlying parent-offspring coadaptation in earwigs. However, the molecular basis of this transgenerational effect is still unknown. Sociogenomic studies in *F. auricularia* previously identified two genes involved in parent-offspring co-adaption [12]. Both genes are differentially expressed in both earwig mothers and offspring during the phase of active post-hatching parenting compared to without such interaction and thus are co-regulated in mothers and offspring. One gene is *PebIII* which when expressed in offspring increases offspring survival, when expressed in mothers delays offspring development and enhances the relative maternal investment in future reproduction. The other gene is *Th* which when expressed in mothers enhances her food provisioning and when expressed in offspring increased the likelihood of maternal future reproduction.

In this study, we manipulated the prenatal and postnatal interaction between earwig mothers and offspring over two generations and investigated the transgenerational effect on the expression of the two previously identified parent-offspring coadapted genes. We also quantified the transgenerational fitness consequences of the presence/absence of maternal care in females and their offspring in the third generation. If there are epigenetic effects, we expect differential gene expressions in both mothers and offspring in response to the experimentally manipulated grandparents experience and the fitness difference should be explained by the differential expression of these genes.

Material and Methods

Manipulation of parental care over generations. The starting population (P_0 generation) of earwigs in this experiment were originally from Docedo, Italy, July 2008 and it is the 7th generation breed in the lab. Females and unrelated males were housed in groups of approx. 40 individuals, in plastic containers *(24 x 14 x 16 cm)* with humid sand as substrate, plastic tubes / egg cardboard as shelters, and at 20˚C and a light-dark cycle of 14h/10h in climate chambers. They were fed twice a week with standard laboratory food [13]. After the first female in a group started laying eggs, all females were isolated in individual petri dishes (10 x 2 cm, containing humid sand as substrate) for egg laying, initially transferred to complete darkness at 10˚C for two weeks to stimulate oviposition and then to complete darkness and 15˚C until hatching. Earwig females fast during egg attendance and no food was provided to females after oviposition until hatching. One day after hatching, the clutches were transferred back to a light-dark cycle of 14h/10h at 20˚C for brood care and food was provided twice a week.

A total of 90 mated P_0 females were randomly assigned to three groups manipulating their maternal behaviors: no care group (NC), where females were isolated from their eggs one day after oviposition; egg care group (EC), where females were attending the eggs for 20 days and isolated from their eggs shortly before the nymphs hatch to avoid mother-offspring interactions; full care group (FC), where females were caring for both eggs and nymphs until 6 days after hatching (Fig 1.). The hatched nymphs are called F_1 generation. Every 40 unrelated nymphs from the same treatment were set up in a plastic container and were mated within the container when they reached adulthood. There were 3 containers for each treatment as replicates in control for mating condition.

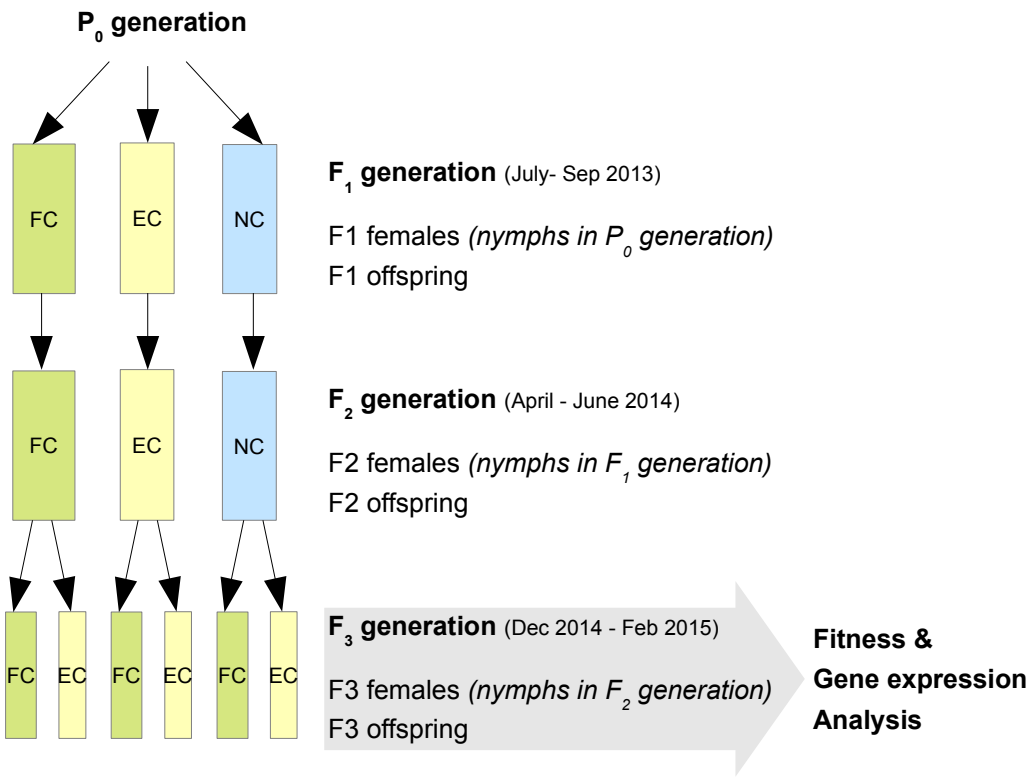

Figure 1 | Experiment design. Manipulation of parental-care over generations. FC: full-care treatment, EC: egg-care treatment, NC: no-care treatment.

Once the first F_1 female started oviposition, females were individually isolated in petri dishes, following their parental treatments, except in FC treatment the females were caring for the nymphs until 14 days after hatching. The offspring of F_1 females are called F_2 generation. Once the first F_2 female started oviposition, a total of 203 females were individually isolated in petri dishes, following their parental treatments. The offspring of F_2 females are called F_3 generation. Every 20 adult F_3 males and 20 adult F_3 females were set up in plastic containers for mating. Males and females were unrelated. There were again 3 mating containers for each of the three groups. When the first F_3 female started oviposition, a total of 177 F_3 females were individually isolated in petri dishes and randomly assigned to two groups EC or FC. The gene expression and fitness differences among the three treatment lines were quantified with F_3 females and her offspring.

Quantifying gene expression. To quantify the expression of two parent-offspring coadapted genes *PebIII* and *Th*, females and nymphs from each treatment group were sampled six days after hatching.

A total of 90 randomly selected F_3 females and her nymphs were sampled and stored with RNAlater (Qiagen) in -80°C freezer before RNA extraction. Each female sample is a pool of head tissue from three females. Each nymph sample is a pool of nine nymphs from three families (three per family). The animals were exposed to petroleum ether (Sigma- Aldrich #77379) vapor before dissection. RNA was extracted using TRIzol-LS reagent (Ambion). The cDNA libraries were synthesized using GoScript Reverse Transcriptase system (Promega). The qPCR was run in triplicates on Applied Biosystems 7500 Fast platform, using EvaGreen 2x qPCR Mastermix reagent (Biotium). The expression of target gene was calculated using the delta-delta Ct method relative to the reference gene *Actin*, calibrated by the average of untreated samples [14]. The calibration was done separately for females and nymphs.

Fitness measurement. Standard fitness trait of earwig mothers and offspring were measured according to previous studies [13]. Hatching success were measure as the ratio of hatched nymphs over number of eggs. Egg developmental rate were measured as the number of days from oviposition to hatching. The nymphs were weighted one day and 14 days after hatching. Growth rate of nymphs were measured as the averaged weight gain per day per individual in the first 14 days after hatching. The females were set up in a new petri dish for second clutch egg laying 14 days after the nymphs hatched in the first clutch. Maternal investment in future reproduction were measured with two traits, one was whether or not the females lay a second clutch, the other was the relative size of second clutch (the proportion for the number eggs produced in the second clutch over the total number of eggs produced in two clutches) if the female do lay a second clutch. Latency of maternal future reproduction were measured as the number of days between ovipositions in the first and the second clutch. Growth rate, maternal future reproduction and latency of maternal future reproduction were quantified for families that were not dissected for gene expression analysis.

Statistical analysis. transgenerational treatment effects, effects of treatment in the current generation effect as well as their interaction on fitness traits and gene expression were statistically tested as fixed factor for gene expression and fitness consequences using generalized liner model (GLM). Oviposition date as covariates for the fitness of females and nymphs respectively. Number of eggs was included as covariates for egg development and hatching success. Number of nymphs was included as covariates for relative maternal investment in future reproduction, offspring growth, latency of future reproductionand the likelihood of future reproduction. We used a backward model simplification

procedure, removing non-significant terms from the model. Continuous dependent variables (egg developmental rate, nymphs growth rate, latency to future reproduction and gene expression) were modeled using a gaussian distribution and identity link function. Discrete or proportional dependent variables (survival rate, the likelihood of second clutch production, relative size of 2nd clutch) were analyzed using a binomial distribution and a logit-link function. All statistics were done in R version 3.1.1 [15].

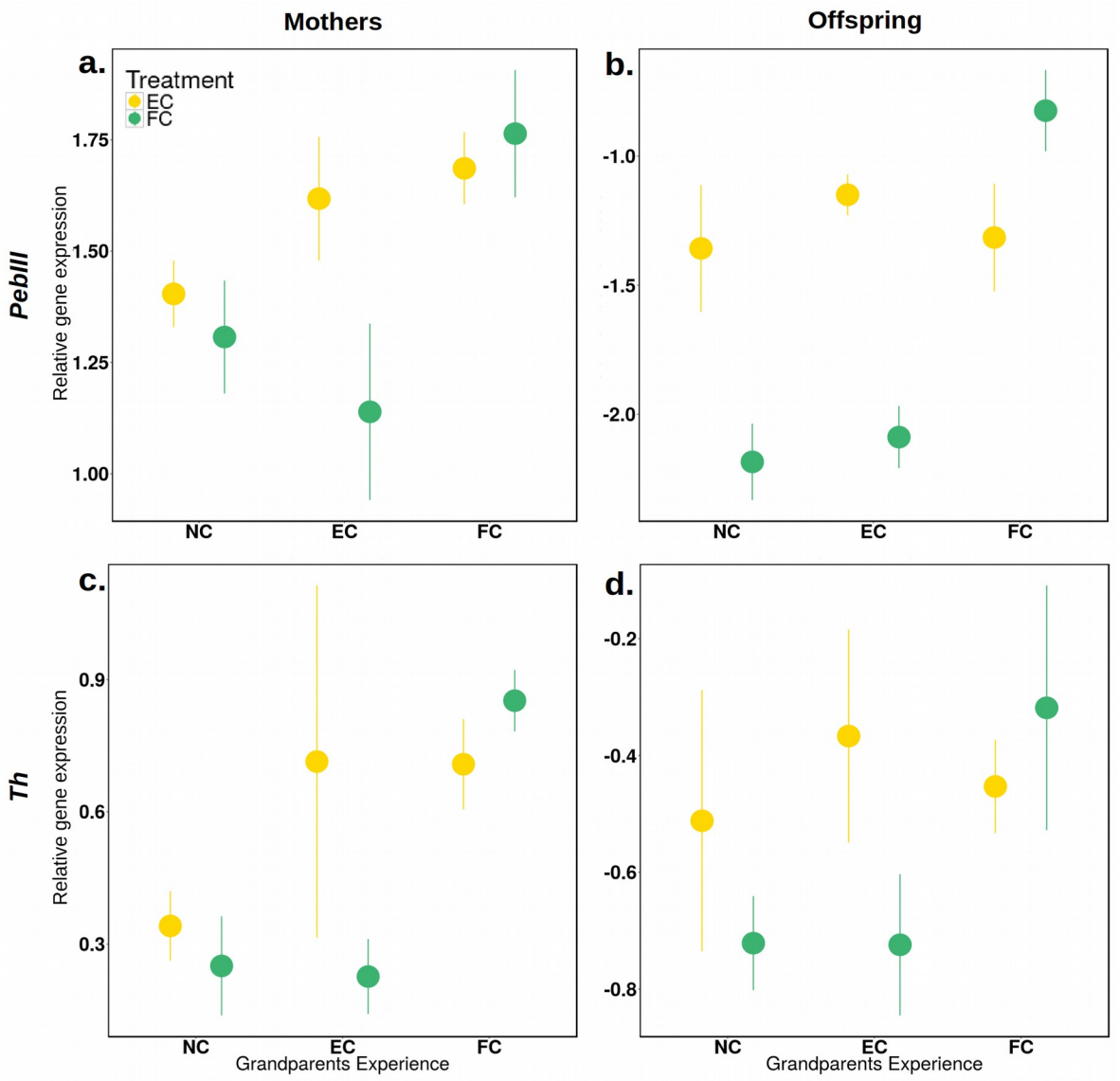

Figure 2 | transgenerational effects on the expression of parent-offspring coadapted genes. Relative expression of *PebIII* (a and b) and *Th* (c and d) in the head of mothers (a and c) and whole body of offspring (b and d). Current generation treatments are color coded: full-care (FC) treatment in green, egg-care (EC) treatment in yellow for both genes. The same abbreviations were used for grandparents experience on the x-axis, NC: no-care treatment.

Results

Transgenerational effect on the expression of parent-offspring coadapted genes

Significant transgenerational effects were found for the expression of *PebIII* in both the head of mothers (Fig 2a; GLM: P=0.018, Table S1) and whole body of offspring (Fig 2b; GLM: P=0.00090, Table S1) and for the expression of *Th* in mothers (Fig 2c; GLM: P=0.043, Table S1). The expression of *PebIII* in the offspring showed significant effects of current generation treatment (Fig 2b; GLM: P=0.0052) and current generation by transgeneration treatment interaction (GLM: P=0.00039, Table S1).

PebIII and *Th* in mothers showed the highest expression in females from full-cared genetic background and lower expressed in no-care and egg-care genetic background females. The highest expression of *PebIII* were found in offspring from full-cared background and lower expressed in offspring from no-care and egg-care background. Moreover, the expression of *PebIII* is higher in full-care compared to egg-care regarding current generation treatment, when the grandparents experienced full-care treatment. However, the *PebIII* expression is higher in egg-care compared to full-care regarding current generation treatment, when the grandparents experienced egg-care or no-care treatment.

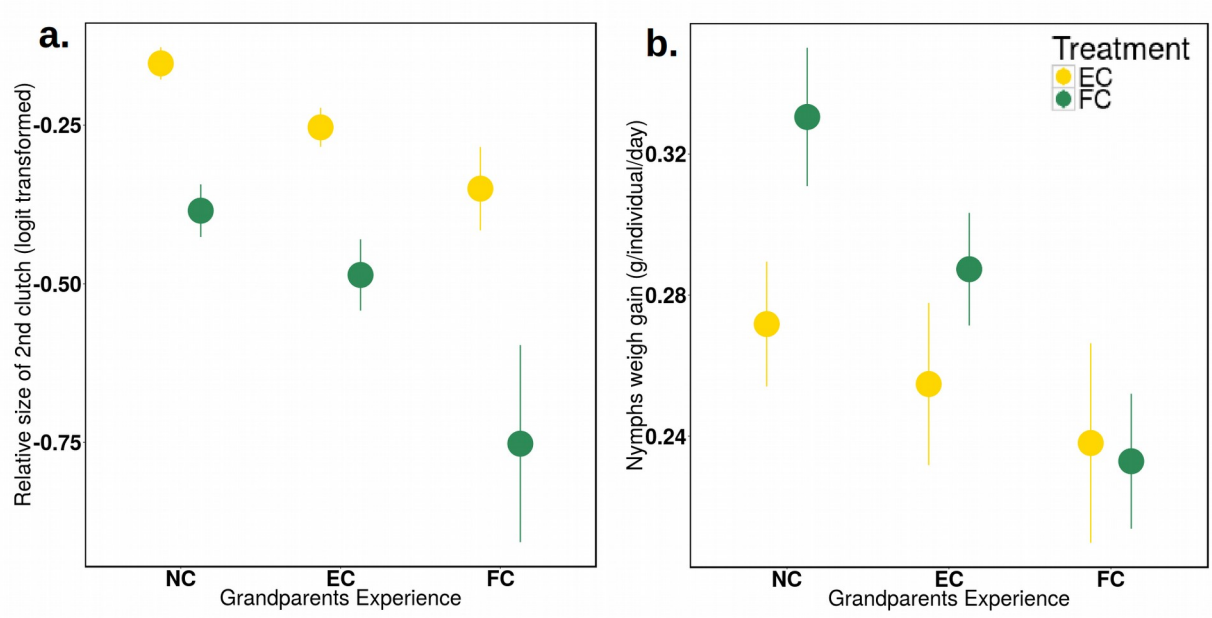

Figure 3 | transgenerational effects on the fitness of mothers and offspring. a) Relative maternal investment in future reproduction; b) offspring growth rate. Current generation treatments are color coded: full-care (FC) treatment in green and egg-care (EC) treatment in yellow. The same abbreviations were used for grandparents experience on the x-axis, NC: no-care treatment.

Transgenerational effect on the fitness of mothers and offspring.

Significant transgenerational effects were found for relative maternal investment in future reproduction measured by the over all portion of eggs in 2nd clutch (Fig 3a; GLM: P=0.040, Table S2) and offspring growth measure by the weight gain in 14 days after hatching per individual per day (Fig 3b; GLM: P=0.0011, Table S2). Maternal future reproduction (Fig 3a; GLM: P=0.00027, Table S2) and latency for maternal future reproduction measured by days between two ovipositions (Fig S1; GLM: P=0.018, Table S2) showed significant effects of current generation parental care treatment. No effect was found for hatching success, egg development rate and the likelihood of maternal future reproduction.

Females from no-care genetic background invest the most in her future reproduction, females from full-care genetic background invest least and females from egg-care background invest intermediate in her future reproduction. Offspring weight gain is the highest in families with no-care genetic background, lowest in families lines with full-care genetic background and intermediate for egg-care genetic background. Regarding current generation treatment, females took less time and invest more in her future reproduction in egg-care treatment compared to full-care treatment.

Discussion

Our results showed transgenerational effects on the expression of parent-offspring coadapted genes and the fitness in mothers and offspring. *PebIII* and *Th* in mothers exhibited a similar pattern of transgenerational effects, with the highest expression in females from full-cared genetic background and lower expressed in no-care and egg-care genetic background females. transgenerational effects, current generation treatment effects and transgeneration by current generation treatment interaction for *PebIII* were found in the offspring. The transgenerational effect in offspring showed the same pattern as in mothers, with the highest expression of *PebIII* in offspring from full-cared background and lower expressed in offspring from no-care and egg-care background. Moreover, the expression of *PebIII* is higher in full-care compared to egg-care regarding current generation treatment, when the grandparents experienced full-care treatment. However, the *PebIII* expression is higher in egg-care compared to full-care regarding current generation treatment, when the grandparents experienced egg-care or no-care treatment. Notably, there is absolutely no physical interaction between mothers and offspring in the no-care treatment. Thus, the lowered expression of *PebIII* could only be passed on to the next generation through epigenetic signals carried in germ lines prior to oviposition. Possible epigenetic modifications

regulating gene expression could be DNA methylation or histone acetylation, methylation, phosphorylation or ubiquitination for related genomic region [9]. In rodents, low levels of post-natal maternal licking and grooming (LG) reduced the expression of estrogen receptor-α gene and maternal behavior of female offspring [16]. Elevated DNA methylation in the promotor of this gene is associated with exposure to low levels of LG [17]. Such maternal effect on DNA methylation and maternal behavior could be transmitted over two generations [18].

We also found transgenerational effects on the fitness in mothers and offspring. The relative maternal investment in future reproduction and offspring growth showed transgenerational effects. Current generation treatment effects were found for relative maternal investment in future reproduction and latency for future reproduction. The current generation treatment effects on the two maternal traits are expected as the full-care females have to allocate more resource and energy attending current clutch of nymphs and therefore caused the trade-off in her future reproduction. The transgenerational effect on the relative maternal investment in future reproduction revealed that females from no-care genetic background invest the most in her future reproduction, females from full-care genetic background invest least and females from egg-care background invest intermediate in her future reproduction. It suggested that higher future reproduction in females originated from less cared families may resulted from less care provided to her own offspring. Indeed, previous study in earwigs showed that maternally deprived females abandon their clutches for longer and provide less food to the nymphs compared to maternally tended females [11].

A cross-fostering experiment demonstrated that the relative investment of caring mother in her second clutch is shaped by the genetic mother of nymphs [10]. The maternal fitness consequence of transgeneration treatment could be due to decreased *PebIII* and *Th* expression in mothers and/or offspring from less-cared genetic background which might result in for instance reduced efficiency of parent-offspring communication mediated by the odorant binding protein *PebIII* [19] and inactive dopaminergic rewarding system restricted by *Th* [20]. In earwigs, *PebIII* has been demonstrated to directly influence the relative maternal investment in future reproduction and *Th* has been shown to directly influence the maternal food provisioning in an RNAi experiment [12].

In addition, transgenerational effect was found for offspring growth rate. The pattern of this trait

paralleled maternal future reproduction. Offspring weight gain is the highest in families with no-care genetic background, lowest in families lines with full-care genetic background and intermediate for egg-care genetic background. The accelerated growth rate found in nymphs with poor parental-care genetic background is opposite to a previous selection study in earwigs that nymphs mass gain is higher is selection lines where females invest less in her future reproduction and possibly invest more in attending current clutch [13]. Our results could be explained by fast growing offspring being selected in absence of parental care, probably through a bottle-neck for the first generation without parental care followed with enhanced sibling competition and induced self-foraging in later generations. The hatching success was significantly lower in no-care treatment compared to egg-care and full-care treatment for the offspring in P_0 generation (Fig. S2), indicating it is extremely costly for the nymphs in no-care condition. It is likely that only fast growing and stronger nymphs could survive during this bottle-neck. This could also explain why there is no difference for offspring survival and development in tested generation. The fact that maternal loss yield larger body and forceps size were shown in previous study in earwigs [11]. It was not necessarily a short-term benefit for the nymphs as the author argued, but rather that only nymphs with higher fitness could survive without parental care. In *C. elegans,* the progeny and grandprogeny of starved larvae of are more resistant to starvation. Such inheritance was exclusively from the most-severely-affected first generation by starvation [21]. Increased adiposity was found in the grandprogeny of female mice undergo low-calorie diet than control group [22]. Loss of maternal care could be stressful for earwig nymphs as offspring survival, development and behavior are influenced by their mother [23]–[27]. Thus the elevated growth rate in earwigs could also be explained by a compensation for stressed condition.

In conclusion, our study provide clear evidence for transgenerational effects of maternal care on the expression of parent-offspring coadapted genes and on the fitness of earwig mothers and offspring. Future study about the epigenetic mechanisms involved in regulating gene expression would help understanding how parent-offspring coadaptation is maintained over generations by epigenetic effects.

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Author Contributions

Conceived the study: M.W; designated the experiment: M.W., M.K.; Performed the experiment:M.W., Analyzed the data; M.W., M.K; Wrote the manuscript draft: M.W.

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Table S1. GLM analysis for the expression of parent-offspring coadapted genes.

Expression of *PebIII* gene *(gaussian distribution, identity link)*							
Mother							
	Df	Deviance Resid.	Df	Resid. Dev	F	Pr(>F)	
NULL	29	3.6105					
Current generation treatment	1	0.20634	28	3.4042	2.3117	0.14147	
Trans-generation effect	2	0.85659	26	2.5476	4.7985	0.01766	*
Current generation : Trans-generation effect	2	0.40542	24	2.1422	2.2711	0.12494	
Offspring							
	Df	Deviance Resid.	Df	Resid. Dev	F	Pr(>F)	
NULL	29	10.6569					
Current generation treatment	1	1.3517	28	9.3052	9.4747	0.0051509	**
Trans-generation effect	2	2.721	26	6.5842	9.5365	0.0008955	***
Current generation : Trans-generation effect	2	3.1602	24	3.424	11.0756	0.0003912	***
Expression of *Th* gene *(gaussian distribution, identity link)*							
Mother							
	Df	Deviance Resid.	Df	Resid. Dev	F	Pr(>F)	
NULL	29	5.8904					
Current generation treatment	1	0.15709	28	5.7333	0.9379	0.34247	
Trans-generation effect	2	1.20354	26	4.5298	3.5929	0.04316	*
Current generation : Trans-generation effect	2	0.51	24	4.0198	1.5225	0.23851	

Table S2. GLM analysis for behavior and fitness traits.

Relative maternal investment in future reproduction *(binomial distribution, logit link)*						
	Df	Deviance Resid.	Df	Resid. Dev	Pr(>Chi)	
NULL	68	68.576				
Trans-generation effect	2	6.4373	66	62.139	0.0400086	*
Current generation treatment	1	13.238	65	48.901	0.0002743	***
Oviposition date	1	2.0146	64	46.886	0.1557936	

Offspring growth rate *(gaussian distribution, identity link)*							
	Df	Deviance Resid.	Df	Resid.Dev	F	Pr(>F)	
NULL	79	0.58568					
Trans-generation effect	2	0.059735	77	0.52595	4.7835	0.01112	*
Current generation treatment	1	0.01652	76	0.50943	2.6458	0.10808	
Oviposition date	1	0.021967	75	0.48746	3.5182	0.06464	.
Clutch size (Nymphs)	1	0.025414	74	0.46204	4.0703	0.04727	*

Latency for maternal future reproduction *(gaussian distribution, identity link)*							
	Df	Deviance Resid.	Df	Resid.Dev	F	Pr(>F)	
NULL	68	3520.6					
Current generation treatment	1	238.04	67	3282.5	5.9025	0.017892	*
Oviposition date	1	445.25	66	2837.3	11.0405	0.001467	**
Clutch size (Nymphs)	1	215.91	65	2621.4	5.3539	0.023848	*

Likelihood of maternal future reproduction *(binomial distribution, logit link)*						
	Df	Deviance Resid.	Df	Resid. Dev	Pr(>Chi)	
NULL	86	91.326				
Trans-generation effect	2	0.9529	84	90.373	0.620972	
Current generation treatment	1	0.0078	83	90.366	0.929503	
Oviposition date	1	10.5716	82	79.794	0.001148	**
Clutch size (Nymphs)	1	4.0079	81	75.786	0.045287	*

Egg development *(gaussian distribution, identity link)*							
	Df	Deviance Resid.	Df	Resid.Dev	F	Pr(>F)	
NULL	170	780.95					
Trans-generation effect	2	16.4449	168	764.5	1.8217	0.16503	
Current generation treatment	1	1.2164	167	763.29	0.2695	0.60438	
Oviposition date	1	0.1339	166	763.15	0.0297	0.86346	
Clutch size (Egg)	1	15.1648	165	747.99	3.3597	0.06863	.
Trans-generation:current generation	2	12.2609	163	735.73	1.3582	0.26002	

Hatching success *(binomial distribution, logit link)*						
	Df	Deviance Resid.	Df	Resid. Dev	Pr(>Chi)	
NULL	172	193.44				
Trans-generation effect	2	2.5473	170	190.89	0.2798	
Current generation treatment	1	0.9414	169	189.95	0.3319	
Oviposition	1	23.9378	168	166.01	9.95E-007	***

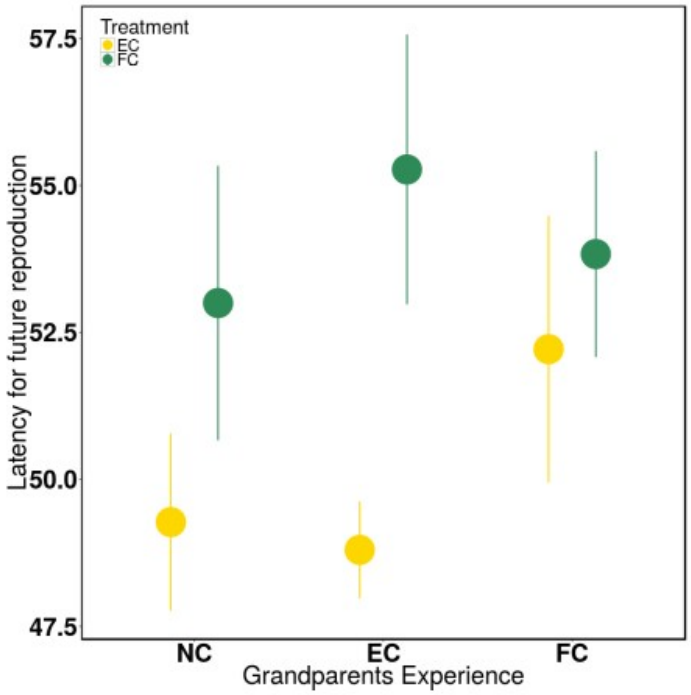

Figure S1 | Current generation treatment effect on latency of maternal future reproduction. Current generation treatments are color coded: full-care in green, egg-care in yellow.

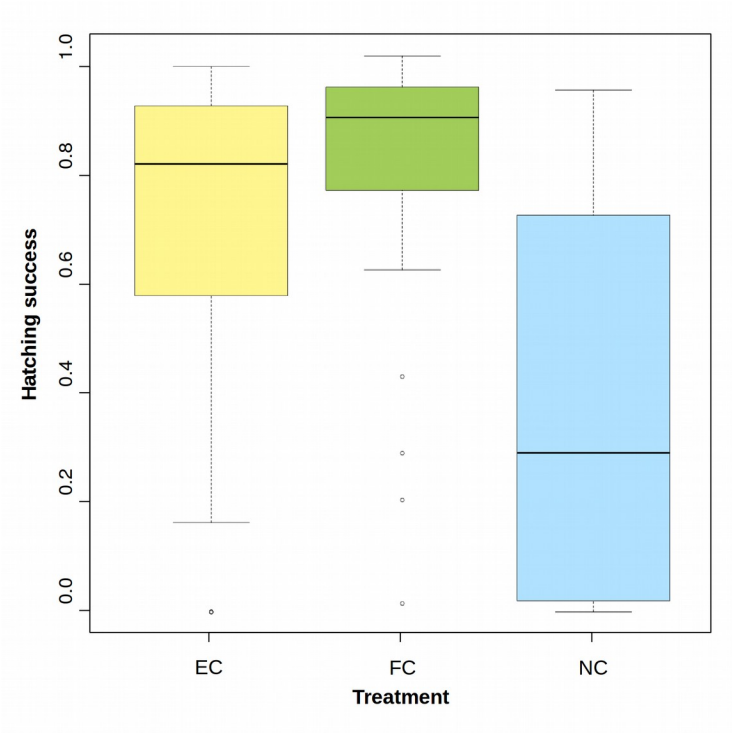

Figure S2 | Hatching success for the offspring in P_0 generation. Treatments are color coded: full-care (FC) in green, egg-care (EC) in yellow, no-care (NC) in blue. NC is significantly lower than EC and FC (ANOVA: $F_{2,117}$=26.6, P=3.01e-10).

CHAPTER 4

Preprogrammed Expression of Parent-Offspring Coadapted Genes in Earwig Mothers

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Abstract

Conflict and cooperation are ubiquitous in nature and animal families where parents and offspring reciprocally influence each other's behavior and fitness. Evolutionary models predict selection for parent-offspring coadaptation. Two genes, *PebIII* and *Th* were previously identified underlying parent-offspring coadaptation in the European earwig, *Forficula auricularia*. Using Fluidigm gene expression array, we confirmed their differential expression in mothers during parent-offspring interaction in an independent replicate experiment. With an additional time control treatment, we further revealed that their expression in mothers was due to maternal reproductive stage instead of social interactions. This is consistent with the age effect in division of labor in eusocial honey bee workers, suggesting the preprogrammed expression of parent-offspring coadapted genes in sub-social species might be the first step of social evolution.

Introduction

Animal sociality is characterized by a continuum of social complexity ranging from eusociality to simpler forms of family living where parents themselves provide care for their dependent offspring (Costa 2006). Current evidence is consistent with the hypothesis that eusociality originally evolved from such simpler forms family living (Keller and Chapuisat 2002; Keller L. and Chapuisat M. 2014). This hypothesis posits that genes involved in the regulation of parental care were evolutionarily coopted, and ultimately form the genomic building blocks of complex animal sociality (Page and Amdam 2007). If true, genes underlying caste differentiation in eusocial systems should be conserved and have their original function in the regulation of parental care; genes mediating the social interactions between parents and their offspring would be the core genes of social evolution.

Taking intimate reciprocal interactions between parent and their offspring explicitly into account, evolutionary models predict the coadaptation of parent and offspring genes ((Kölliker, Royle, and Smiseth 2012)). Such coadaptation is reflected by their co-regulation in parents and offspring either through physical linkage in the genome or coopted regulatory network (Kölliker, Royle, and Smiseth 2012; Kölliker and Johnstone 2016). coadaptation must strike balance between parents pursuing self-fitness versus offspring demanding parental investment. Ultimately, it facilitates well-coordinated parenting and optimized cooperation with their offspring in the face of sexual reproduction and genetic recombination (Kölliker and Johnstone 2016; Kölliker, Royle, and Smiseth 2012) which cause genetic conflict (Trivers 1974; Kilner and Hinde 2012). Two genes, *Th* and *PebIII*, in the European earwig, *Forficula auricularia*, was found underlying parent-offspring coadaptation (see Chapter2).

F. auricularia is a sub-social insect with facultative uniparental female care. Earwig mothers protection their eggs and nymphs against parasites and predators and provide food to the offspring. Maternal care in earwigs influence survival, development and behavior of the offspring (Vancassel et al. 1984; Boos et al. 2014; Kölliker and Vancassel 2007; Wong, Lucas, and Kölliker 2014; Kölliker 2007). In turn the offspring influence the behavior and future reproduction of their mothers (Mas, Haynes, and Kölliker 2009; Meunier and Kölliker 2012). These reciprocal interactions are at least partly mediated by chemical communication (Mas, Haynes, and Kölliker 2009; Wong, Lucas, and Kölliker 2014).

Evidence of co-regulation in mothers and nymphs was found for both *Th* and *PebIII* in a transcriptomic

screen (see Chapter 2). Their social function and effects on behavior and fitness was characterized by *in vivo* RNA interference. *Th* is reciprocally altruistic, with direct genetic effects on mothers' food provision and indirect genetic effects on the likelihood of maternal future reproduction. *PebIII* is reciprocally selfish, with direct genetic effects on mothers' relative investment on her second clutch and nymphs' survival and indirect genetic effects on offspring development. In this study, we investigated whether the expression of genes underlying parent-offspring coadaptation is dependent on the social cues or it is rather preprogrammed in the parents.

Material and Methods

Experimental design. To validate differential expression of 37 candidate genes between the EC and FC treatment identified in previous RNASeq experiment, we carried out an independent and fully replicated experiment using 48x48 fluidigm gene expression dynamic arrays (Fig. S4). The experimental design was identical to the RNASeq experiment with the exception of an added fourth treatment (referred to as TC) to disentangle if differences in female gene expression between the EC and the FC treatment were due to the interactions with nymphs, as we assumed, or rather due to a more advanced reproductive stage of the female, a difference in temperature, photoperiod schedule and/or food intake. Females of this treatment were isolated 20 days after oviposition (when EC females were sacrificed) and kept in a new petri dish with food for anotheruntil six days after the nymphs hatched (i.e., they were sacrificed at a similar stage and under the same temperature, food- and photoperiod conditions as FC females, but without interactions with nymphs).

As in the RNASeq experiment, four female tissues (antennae, head, abdomen without gut and ovary) of the mothers and the whole body of nymphs (3 nymphs per family) were processed separately for Fluidigm analysis, from a total of 24 families. Samples were stored in RNAlater (Qiagen) at -80˚ before extraction. RNA was extracted using TRIzol (Invitrogen) as before and cDNA libraries were prepared using the GoScript Reverse Transcriptase system (Promega). No pooling of samples from different families was required and each family represented an independent biological replicate in the statistical analysis.

Fluidigm dynamic arrays. Primers were designed based on earwig transcriptome sequences (Roulin et al. 2014) using Primer3 (Untergasser et al. 2012). The PCR efficiencies of primer pairs were between

1.74 to 1.91 as calculated in qBasePlus (Hellemans et al. 2007). Each sample was triplicated for all genes on Fluidigm runs. Replicates with difference of Cq values >0.5 were excluded for further analysis. As before relative expression levels were calculated using the delta-delta Ct method , relative to the average of reference genes *Actin* and *Rpl32* (Roulin et al. 2014), and scaled by the average of all samples.

Statistical analysis. We focused our statistical analysis on the two main candidate genes showing evidence for co-regulation. For comparison of all 41 genes with differential expression in the RNASeq and the Fluidigm experiment, we present heat-maps of both data sets in Fig. S1. Initially, and to confirm the results of the RNASeq experiment for females, a similar linear mixed model analysis was conducted with "tissue", "egg-attendance" (Yes/No) and "parent-offspring interaction" (Yes/No), as well as their interactions with tissue, as fixed effects, and with female ID as random effect. Subsequently, to test and disentangle effects of parent-offspring interactions from effects due to female stage, temperature, photoperiod experience or food intake the factor "reproductive stage", which was based on the new treatment EC2, and its interaction with tissue was added to the model. Effects on parent-offspring interactions on offspring gene expression were tested using Welch t-tests as before.

Results

Validation of RNA-Seq with Fluidigm gene expression dynamic array

To validate the results of RNA-Seq, we conducted a replicate experiment with Fluidigm gene expression arrays. We analyzed the expression of 37 genes including *Th, PebIII* and genes from potentially related pathways (Table 1, Fig. S1). The results were overall similar to those of the RNA-Seq (Fig. 1), but also showed some notable differences. The tissue-specific differential expression of *Th* in mothers was confirmed (Fig. 1, Table 1: Fluidigm-3G, LMM: parent-offspring interaction x tissue, $P<0.0001$). The main effect of parent-offspring interaction of *PebIII* in mothers was confirmed (Fig. 1, Table 1: Fluidigm-3G, LMM: parent-offspring interaction as main effect, $P=0.05$), with additional significant tissue-specificity in this effect and the strongest up-regulation in the antennae (Fig. 1, Table 1: Fluidigm-3G, LMM: parent-offspring interaction x tissue, $P<0.0001$).

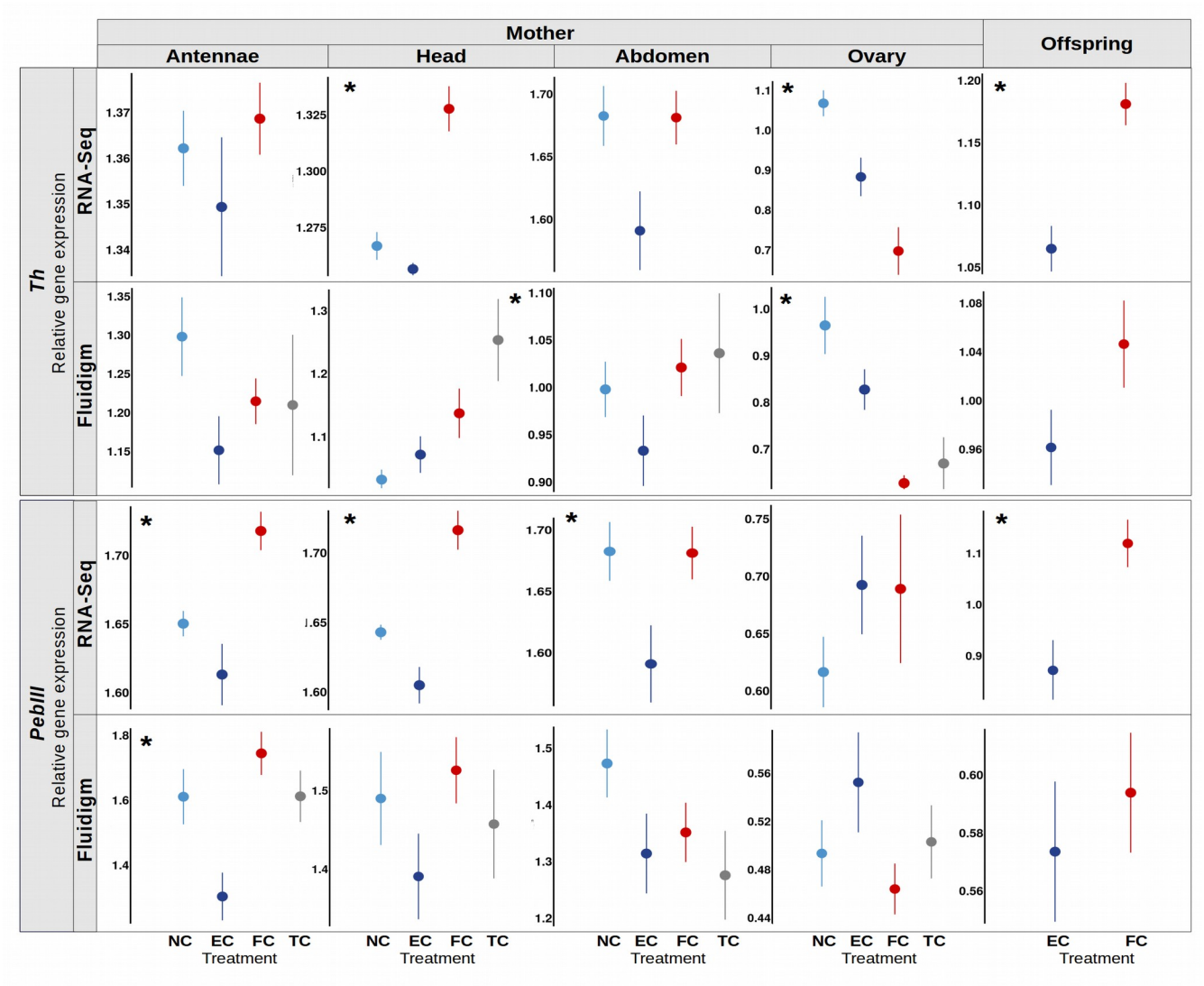

Figure 1. Differential expression of *Th* and *PebIII* in maternal tissues and the offspring. Results are based on the RNA-Seq experiment (top panel) and the Fluidigm gene expression array experiment (bottom panel) for each gene. Significant differential expression between egg-care (EC) and full-care (FC) samples is marked by asterisks on the top left of each plot ($p < 0.05$ in *t*-test). A time-control treatment (TC) was included in the Fluidigm experiment to disentangle effects of parent-offspring interactions from effects of maternal reproductive stage. Significant differential expression between EC and TC samples is marked by asterisks on the top right of the plot ($p < 0.05$ in *t*-test). Means and standard errors are shown (6 replicates per tissue per treatment), see Table S1for details of statistical models. Error bars are standard error.

In nymphs, although both methods show a similar pattern the expression difference measured with Fluidigm between EC1 and FC was not statistically significant for *PebIII* ($t_{7.27}$=-0.03, P=0.979) andshowed only a statistical trend for *Th* ($t_{10.0}$=-1.80, P=0.103). A global two-way ANOVA combining expression data from the RNA-Seq and Fluidigm experiment was used to assess if the difference between the two experiments was due to lack of statistical power or due to an actual difference of results. The global model confirmed significant overall treatment effects (*PebIII*: P=0.00002; *Th*: P=0.00128, Table 1). The treatment effect was similar in the two experiments for *Th* (experiment by treatment interaction: P=0.76, Table 1), but significantly different for *PebIII* (experiment by treatment interaction: P=0.00116, Table 1). Thus, the difference between experiments are most likely due to a lack of power for *Th* expression, but due to a real albeit unknown difference for *PebIII* expression.

Preprogrammed expression of Th and PebIII

The Fluidigm experiment contained an additional treatment (referred to as time control or TC in Fig. 1) to assess whether the observed expression differences between the FC and EC treatment were due to physical mother-nymph contact or a simple time effect. The EC-TC treatment difference was similar to the EC-FC treatment. For both genes, the variations of gene expression among the four treatment groups were tissue specific and was explained by maternal reproductive stage instead of parental interaction (Fig. 1, Table 1: Fluidigm-4G, LMM: female state x tissue, *PebIII*: P=0.0002, *Th*: P=0.0004).

Discussion

The differential expression of *Th* and *PebIII* found by RNA-Seq (see Chapter 2) in mothers and nymphs during parent-offspring interaction was confirmed in an independent experiment with qPCR (see Chapter3). In this experiment, differential expression of both genes was confirmed in mothers. That the expression of *Th* in nymphs showed the same trend as in previous studies, but lack of statistical power was likely due to less biological samples in Fluidigm experiment. Although the number of replicates per tissue per treatment was the same for Fluidigm and RNA-Seq experiments, there was no pooling of maternal tissue for Fluidigm samples. Both experiments pooled nymphs, but

each Fluidigm sample consisted of only 3 individuals from the clutch of one mother while RNA-Seq sampled clutches from 5 mothers with 3 nymphs per clutch. The number was 5 times more for each RNA-Seq sample. Thus, the actual biological sample size in Fluidigm was not comparable to RNA-Seq experiment. Another possible reason for no detected expression difference in Fluidigm experiment was long-term sample storage. Although at -80°C, RNAlater solution is stable for RNA storage as long as 8 months ((Gorokhova 2005). The Fluidigm samples were stored in RNAlater at -80°C for over one year. The difference between RNA-Seq and Fluidigm could be purely due to degradation of RNA in dissected samples. A third possible reason is the technical difference between RNA-Seq and Fludigm gene expression array. The former one was based on number of reads mapped to a high-quality transcriptome, while the later one was based on an array of quantitative PCR.

Th expression in mothers														
	Sum Sq		Mean Sq		NumDF		DenDF		F.value		Pr(>F)			
	3G	4G	3G	4G	3G	4G	3G	4G	3G	4G	3G		4G	
Parent-offspring interaction	0.0001	0.01	0.0001	0.01	1	1	15	20	0.02	0.77	0.904		0.390	
Egg attendance	0.04	0.02	0.04	0.02	1	1	15	20	5.32	2.59	0.0358	*	0.123	
Tissue	1.31	1.31	0.44	0.44	3	3	45	60	63.27	47.76	3.33E-016	***	6.66E-016	***
Parent-offspring interaction:Tissue	0.17	0.03	0.06	0.01	3	3	45	60	8.13	0.92	0.000197	***	0.435	
Egg attendance:Tissue	0.07	0.07	0.02	0.02	3	3	45	60	3.22	2.43	0.0313	*	0.0737	.
Female state	/	0.01	/	0.01	/	1	/	20	/	0.93	/		0.346	
Female state:Tissue	/	0.19	/	0.06	/	3	/	60	/	6.95	/		0.000433	***
PebIII expression in mothers														
	Sum Sq		Mean Sq		NumDF		DenDF		F.value		Pr(>F)			
	3G	4G	3G	4G	3G	4G	3G	4G	3G	4G	3G		4G	
Parent-offspring interaction	0.05	0.01	0.05	0.01	1	1	15	20	4.62	0.78	0.0484	*	0.389	
Egg attendance	0.05	0.04	0.05	0.04	1	1	15	20	4.30	3.53	0.0558	.	0.0747	.
Tissue	10.43	10.43	3.48	3.48	3	3	45	60	304.54	326.78	<2.20E-016	***	<2.20E-016	***
Parent-offspring interaction:Tissue	0.46	0.05	0.15	0.02	3	3	45	60	13.44	1.45	0.00000214	***	0.24	
Egg attendance:Tissue	0.21	0.21	0.07	0.07	3	3	45	60	6.05	6.49	0.00150	**	0.000708	***
Female state	/	0.01	/	0.01	/	1	/	20	/	1.14	/		0.298	
Female state:Tissue	/	0.25	/	0.08	/	3	/	60	/	7.82	/		0.000173	***

Table 1. Linear mixed model analysis for the expression of *Th* and *PebIII* in mothers. Tow different analysis were conducted for the Fluidigm gene expression array experiment, 3G: data including 3 treatment groups for comparison with the RNA-Seq experiment; 4G: data including 4 treatment groups, with an additional treatment controling for female reproductive state; P<0.05*, P<0.01**, P<0.001*** .

The fourth treatment TC introduced in the Fluidigm experiment suggested that female gene expression patterns were not flexibly induced by interactions with the nymphs. The expression patterns found in

mothers did not require interactions with the offspring *per se*, but rather reflected a pre-programmed adjustment of gene expression according to reproductive stage when caring for the offspring. Such a pattern is to be expected for basic processes triggering maternal instincts and prevent the loss of maternal care when nymphs temporarily separate from their mothers. This finding is consistent with division of labor in honey bees, where the transition from nursing to foraging, or between pollen and nectar foraging, are primarily driven by the age of the worker (Corona et al. 2007). Therefore, the preprogrammed expression of parent-offspring coadaptated genes in the earwig mothers might be the first step in the evolution trajectory from sub-social parental care to eusociality.

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Author Contributions

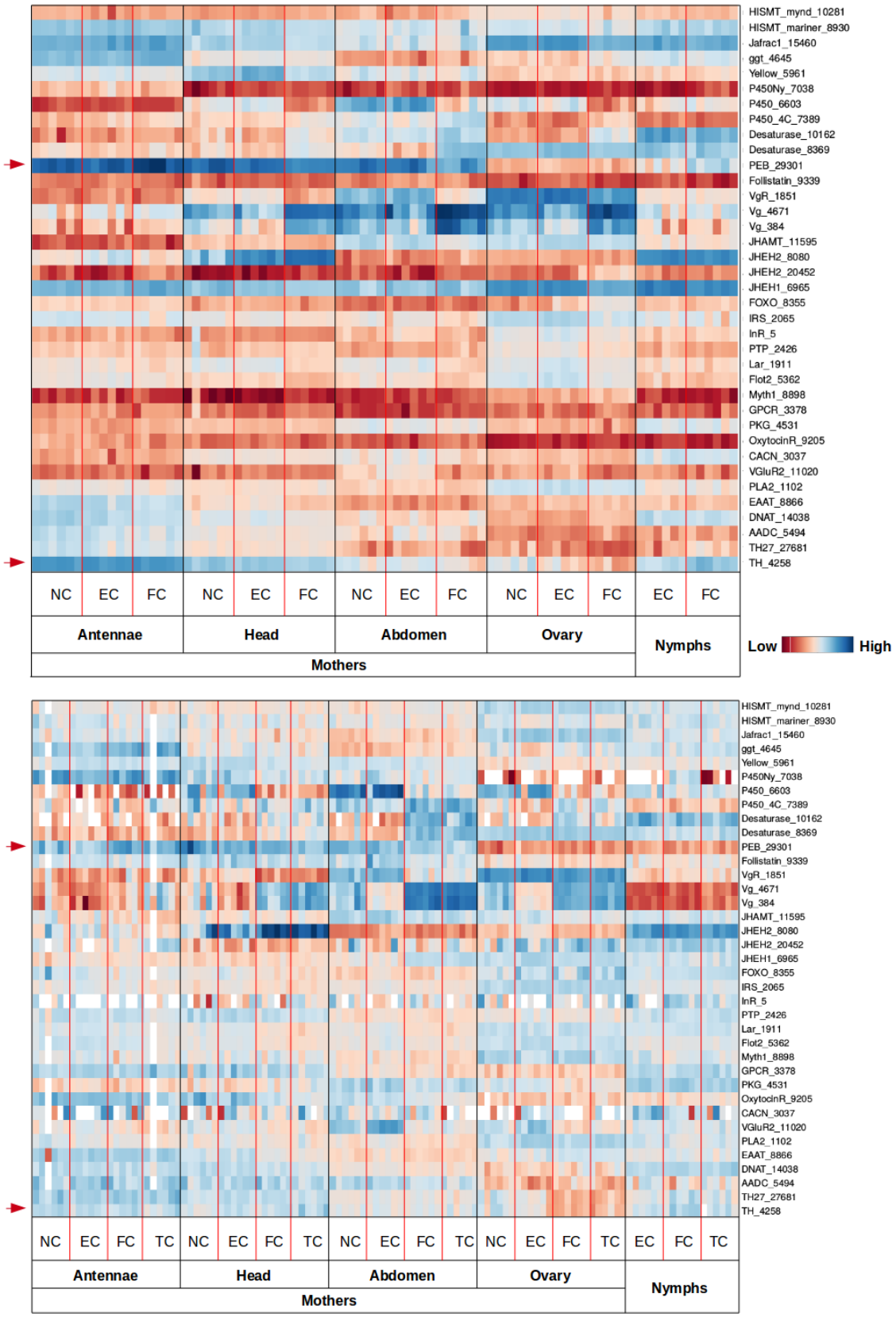
Conceived the study: M.K. and J-C.W; design the experiment: M.K., J-C.W and M.W.; Performed the experiment: M.W., Analyzed the data; M.W., M.K; Write the manuscript: M.W, M.K, J-C.W.

SUPPLEMENTARY MATERIALS

Table. S1. Primers for Fluidigm gene expression array.

	Gene	Contig ID	Forward primer	Reverse primer	Amplicon lengths
1	*Th*	4258	CCAGCCTCGCCTTCCGCATC	CGGATGCACCCAAAGAGGCCA	177
2	*Th*	27681	TCTACCGGCTGCGCTCTCGT	CTGCGGGATCGACGGGGTTG	188
3	*AADC*	5494	ATCGGGAAGCTGTCAAAGAA	TCTGGTTAAAATGGGCGAAC	158
4	*OctopaminR*	9205	CCACGACCATGAACGCCCCC	GGTCGGCGACAGCGAGTGAC	198
5	*OxytocinR*	3378	AGGCCCAGTGAAGAAGGCAGA	AGCCATCGCATTTAAGCCCGC	167
6	*PLA2*	1102	TTCGGGAGGGACCACTGCCT	TGTGGGGCCACGTTTGCTTGA	155
7	*CACN*	3037	AGGGCTTGGGCGAAACGGTG	CCCGATCGTGCGTTCTGGCA	190
8	*vGlut2*	11020	GGCGAAATGGGCTCCGCCTT	TACGCCTGCTCCACCCGTGA	157
9	*EAAT*	8866	TGGGGAATTGCCGCTGCTCC	GTCGAcccCCGTGTTGTTCGT	191
10	*myth1*	8898	TGGTTCTACGGTGACATGGA	GCCCATGACCACAAGAAGTT	195
11	*PKG*	4531	CGCCAGGCTACCGTTACTGCC	TCCACTCGGCCAAAACCGCC	227
12	*DNAT*	14038	TCCCAGGCCTCTCATCGTGC	CCCCGCGTTCTATTGGCAGA	191
13	*Flot2*	5362	GGCTTCCCGAATGCCTGCGT	GGAGGTCGCCGCACCTGATG	181
14	*Lar*	1911	CGCGCTGGGACTTCCGTCTG	GGCCGAACCGGGTGCTTCAT	197
15	*PTP*	2426	TCGTGCAAGGGCTGCAAGGG	GTCCGGGCAACGGAGTGTCG	167
16	*InR*	5	CAAATGGTAAAGTGCGAAAG	GATGTGCCTCCAAACAATAA	202
17	*IRS*	2065	ACAGGCGAACCTGGGCTGTT	ACCTCCCAAGCCGCCTCCTT	177
18	*FOXO*	8355	GACGCGCTACTGGACGAGGC	CTGCAGAACCTCCGCAGCCC	184
19	*JHEH1*	6965	TCGGCCAGCCTTTCTTGCGG	CGGTGGCGGTGTCAATGGCT	166
20	*JHEH2*	20452	ACCTGCTGGGCTTGCTGCTT	CAGGTTGAGCGCCATTTCGGC	211
21	*JHEH2*	8080	GCAGCCAGACCTCCAGGGGA	TGAGCTTATGGTCGCTGGGGCT	186
22	*JHAMT*	11595	AACGCCTGTTCCAAACGGCT	GCGACTGTGGGTCAGTGGCT	171
23	*VgR*	1851	AGCGACCTGCCCAGGCCATA	TTTCGGCCCCTCGTCACTGC	175
24	*Vg*	4671	TGCAAAAGCAGCGGTGGCAA	TGGTCTGCGTAACGCCCAAGC	175
25	*Vg*	384	CGGGCGATGGCTGGGTTTGT	GGCGAAGCCCACCCTGTGAG	185
26	*yellow*	5961	AGACCAGGCACCGGGAAACCA	TGTCCTTGCAGCAGACACCGT	184
27	*Follistatin*	9339	GTGTGTGGAACTGATGGAAG	CAGTGAGGACTCAGGTTTTG	179
28	*PebIII*	29301	GCTTGTCTCCGTCCTCGCGT	AGTTCGGCCCCATCAGGGCT	174
29	*Acyl-CoA desaturatse*	8369	ACTCGGTGATCCCGTGCCCA	TCAAAGCACGCTGGCTCACCC	196
30	Acyl-CoA *desaturatse*	10162	GATGAGACCATAATCGATGC	ACACCACAGCTCCAATTTAC	225
31	*CytochromeP450*	7389	TTTGCCGCCACCATTCGGGA	TGATGCGAGCGACACGACCC	167
32	*CytochromeP450*	6603	GACGGGCCACAAAGCCGACA	TCGATCTTCGGGAACGAAATCTCCA	170
33	*CytochromeP450*	7038	CAGCTGCCTCGTGATGCCCA	AGACCACTGCGCACGCCATC	175
34	*ggt*	4645	TGTGCTGTTGGCGTAGCCCG	CGGGTCAGCAGTGGACGCAG	169
35	*Jafrac1*	15460	ACCCCACGCAAACAAGGCGG	CGACGTTACGGCCAACAGGCA	193
36	*HISMT_mynd*	8930	CGTGGGTGGGCAGCGGATTC	CGGGCTCTTGAGCCAATGCCA	157
37	*HISMT_mariner*	10281	GCGATGAAAAATGGGTTGTT	AGAATTGATCGTTCGGTTGG	185

Figure S1. Heatmap comparing the expression of 37 genes in Fluidigm and RNASeq.



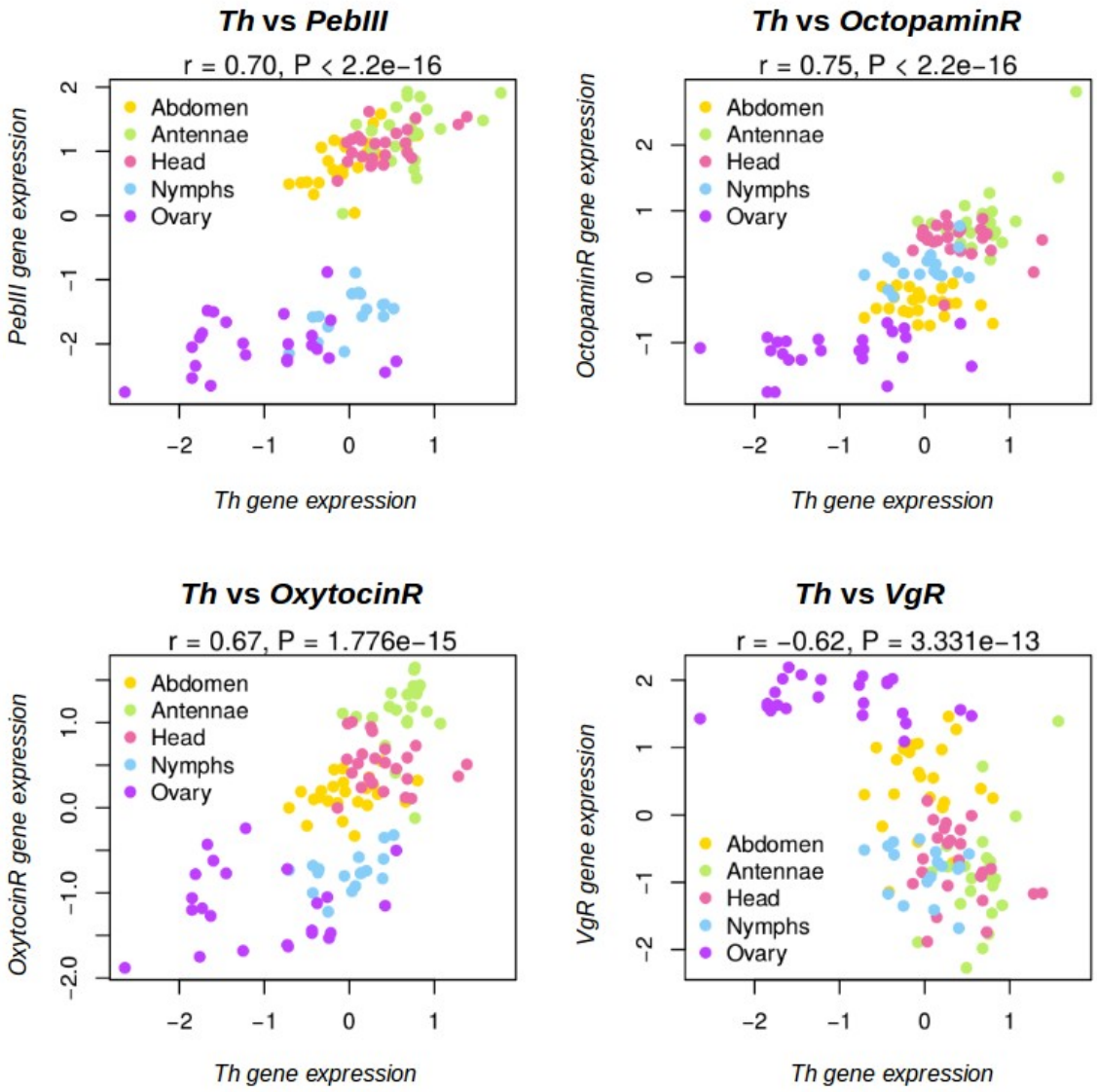


Figure S2. Correlation of gene expressions. The expression of *Th* is possitively correlated with *PebIII*, o*ctopamine receptor*, *oxytocin receptor* and negatively correlated with *vitellogenin receptor* genes. It suggested the functional association of these genes in the earwigs. Dopamine and octopamine has been found involved in layered reward signaling in Drosophila. Oxytocin has been reported relating to maternal-care behavior in mice and parent-infant contact in human.

CONCLUDING REMARKS

The present work studied the sociogenomics of maternal care and parent-offspring coadaptation in the European earwigs (*Forficula auricularia*). The three chapters of my thesis were a series of continuous work. We first published *de novo* hybrid assembled transcriptome of the earwigs. I validated the transcriptome with gene expression studies for candidate sex-biased gene. To understand the genomic basis of parent-offspring coadaptation, I identified two genes that are co-regulated in mothers and offspring during active post-hatching parenting and confirmed their expression in an independent experiment. Functional study of these two genes revealed their influence on maternal and offspring fitness and behavior. transgenerational effect of maternal care on the expression of these genes suggesting epigenetic mechanisms underlying parent-offspring coadaptation.

In the first chapter, we obtained a comprehensive transcriptome of the European earwig from various tissues and developmental stages and sexes. Possible microbial contamination and repeated elements were screened for *de novo* assembled data. Comparison to the eukaryotic core gene dataset revealed that the hybrid assembly yield a transcriptome with high completeness and low level of fragmentation. More than 8,800 contigs of the hybrid assembly show significant similarity to insect-specific proteins. Finally, I validated the transcriptome with qPCR and confirmed sex specific expression of five earwig homologs that are known sex-biased in the honeybee. This experiment also revealed differential expression of these genes between the brain and antenna tissues. The transcriptome presented here offers new opportunities to study the molecular bases and evolution of parental care and sociality in arthropods.

In the second chapter, I identified two genes, *PebIII* and *Th,* underlying parent-offspring coadaptation in the European earwig, using comparative transcriptomics from experimentally manipulated mother-offspring interactions. *In vivo* RNAi revealed the social function of these two genes with causal effects on behaviour and fitness in both mothers and offspring. *PebIII* is reciprocally selfish. It enhances offspring survival, mothers' relative investment in future reproduction through direct genetic effects (DGE) and delays offspring development through indirect genetic effect (IGE); *Th* is reciprocally altruistic. It enhanced food provisioning in mothers through DGE, but its expression in offspring enhanced the likelihood of maternal future reproduction through IGE. Metabolic pathway analysis

suggested the role of *Th*-restricted dopaminergic reward, *PebIII* mediated chemical perception, regulations between insulin signaling, juvenile hormone and vitellogenin in parent-offspring coadaption and social evolution.

In the third chapter, I demonstrated transgenerational effects of maternal care on the expression of the two parent-offspring coadapted genes *PebIII* and *Th* found in chapter 2. Significant transgenerational effects were found for the expression of *PebIII* and *Th* in the head of mothers. The expression of *PebIII* in the whole body of offspring showed significant effects of transgeneration treatment, current generation treatment and current generation by transgeneration treatments interaction. Significant transgenerational effect was found for relative maternal investment in future reproduction and offspring growth rate. Maternal future reproduction and latency for maternal future reproduction showed significant effects of current generation parental care treatment. Our results indicates an epigenetic regulation of gene expressions underlying parent-offspring coadaptation.

In the last chapter, the differential expression of *Th* and *PebIII* in earwig mothers during parent-offspring interaction detected by RNA-Seq in chapter 2 were confirmed in an independent replicate experiment using Fluidigdm gene expression dynamic array. With an additional time-control treatment, we found their expression were not due to the social interaction *per se,* but rather reflected the reproductive stage of mothers. This result suggested the preprogrammed expression of parent-offspring coadapted genes in the sub-social earwigs might be ancestral to eusocial division of labor which is also age-dependent.

Outlook

There are several aspects worth further investigation based on the results of this work. First of all, we identified two parent-offspring coadapted genes *PebIII* and *Th. PebIII* is an odorant-binding protein [1], *Th* is the rate-limiting enzyme for dopamine synthesis [2]. It is likely that *PebIII* regulateg the interaction between earwig mothers and offspring through chemical signals such as cuticular hydrocarbons (CHC). Significantly increased expression of *desaturases* genes that involved in CHC synthesis during active parenting were found in RNA-Seq experiment supports the idea of differentially expressed CHC in earwigs due to maternal care and parent-offspring interaction. Therefore, it would be fruitful if future studies focus on CHC difference between earwig mothers and nymphs with

manipulated parent-offspring interaction. For the other gene *Th,* the effects of dopamine on behavior and fitness in earwig mother would be interesting as well.

The three different combinations of tissue-specific expression for the five earwig *Vg* transcripts raise the question whether it was due to gene duplications or splicing variance. Subfunctionalization/ neofunctionalization of duplicated *Vg* gene was found in harvester ants (*Pogonomyrmex barbatus*) and showed differential expression regarding reproductive and non- reproductive castes [3]. Novel function of *Vg* independent of *Vg receptor* could explain the mismatched expression pattern of these two genes in earwig mothers. Hence the molecular evolution and function of various earwig *Vg* transcripts deserve further studies.

In rodents, post-natal maternal care influence the expression of *estrogen receptor-α* gene, DNA methylation in the promoter of this gene and maternal behavior of female offspring [4], [5]. Such maternal effect on DNA methylation and maternal behavior could be transmitted over two generations [6]. Given the transgenerational effect of maternal care on the expression *PebIII* and *Th* genes, the epigenetic mechanism regulating gene expressions is definitely the next step towards understanding the molecular mechanisms underlying parent-offspring coadaption.

Final Conclusion

Present work established several technical platforms for studying the molecular mechanisms in earwigs and provided significant insights into the genomic basis of parent-offspring coadaptation. First, qPCR method was established to validate the *de novo* hybrid assembled transcriptome of the European earwig. Sex-biased and tissue biased expression of five candidate genes in earwigs were revealed applying this method. Based on this comprehensive earwig transcriptome, two genes, *PebIII* and *Th,* were identified using RNA-Seq data from manipulated parent-offspring interaction. Both were synergistically up-regulated in mothers' head and offspring during active host-hatching parental care. Their expression pattern was confirmed in independent experiment with Fludigm gene exrpession dynamic array and qPCR experiments. To manipulate gene expressions and study the social functions of these two genes, I established *in vivo* RNAi techonology for earwigs and revealed causal effects of *PebIII* and *Th* on the behavior and fitness of earwig mothers and nymphs through direct and indirect genetic effects. I further unveiled the transgenerational effects of maternal care on the expression of

PebIII and *Th,* and opened the door for future studies of the epigenetic mechanisms regulating gene expression over generations and maintaining parent-offspring coadaptation in earwigs.

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APPENDIX I

Parent–Offspring Conflict and the Genetic Trade-offs Shaping Parental Investment

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Abstract

The genetic conflict between parents and their offspring is a cornerstone of kin selection theory and the gene-centred view of evolution, but whether it actually occurs in natural systems remains an open question. Conflict operates only if parenting is driven by genetic trade-offs between offspring performance and the parent's ability to raise additional offspring, and its expression critically depends on the shape of these trade-offs. Here we investigate the occurrence and nature of genetic conflict in an insect with maternal care, the earwig *Forficula auricularia*. Specifically, we test for a direct response to experimental selection on female future reproduction and correlated responses in current offspring survival, developmental rate and growth. The results demonstrate genetic trade-offs that differ in shape before and after hatching. Our study not only provides direct evidence for parent–offspring conflict but also highlights that conflict is not inevitable and critically depends on the genetic trade-offs shaping parental investment.

Introduction

Parenting takes time, resources and energy, and ultimately reduces the parent's ability to produce additional offspring. It only pays off evolutionarily because it enhances the fitness of offspring to which the parent is genetically related[1]. But parenting is not necessarily harmonious altruism. Sexual reproduction is thought to introduce genetic conflict between family members. Each offspring should demand more investment than parents are selected to provide because it is more related to itself than to any of its siblings, whereas parents are equally related to all of their offspring[2]. Although the premise of parent– offspring conflict was conceptually quickly confirmed and accepted after Trivers' original formulation in 1974[3–5], almost two decades later the lack of empirical tests was striking and the topic considered a 'case of arrested development'[6]. Godfray[7] identified the lack of testable predictions of the theory as the main problem and proposed a major shift in the research programme away from the conflict as such (that is, the 'conflict battleground'[7]) to how parents and offspring should behave to resolve conflict[7–9]. This approach triggered a great amount of experimental research on behavioural parent–offspring interactions that provided evidence broadly consistent with conflict (reviewed in refs [5,10–14]). However, the downside of this approach was that it sidestepped the fundamental question whether genetic parent–offspring conflict actually occurs and, thus, whether its assumed prominent role as driver of parenting and family life is justified.

There are three main predictions that empirical tests of a Triversian parent–offspring conflict battleground have to address. First, the conflict is over parental investment (PI) and not over parenting behaviour. Thus, it is essential to quantify PI according to its ultimate definition, that is, to measure any investment by a parent that enhances offspring fitness at the expense of the parent's expectation for additional offspring (Fig. 1)[1,2,4,15]. Second, the conflict is among genes, not traits or behaviours, and therefore only occurs if PI is shaped by genetic rather than phenotypic trade-offs between parents and offspring. Hence, empirical tests should demonstrate that genotypes with enhanced performance as offspring exhibit reduced ability to raise many offspring as parents (due to higher PI), and vice versa for genotypes with reduced performance as offspring[16,17]. Finally, while genetic trade-offs provide evidence for antagonistic parent– offspring co-evolution, they per se are not sufficient evidence for parent–offspring conflict over the amount of PI. This conflict occurs when PI fitness optima differ for parent and offspring[2,7,14], a condition requiring sexual reproduction and depending on the shape of the genetic trade-offs. It is only occasionally reached when offspring fitness gains show constant or

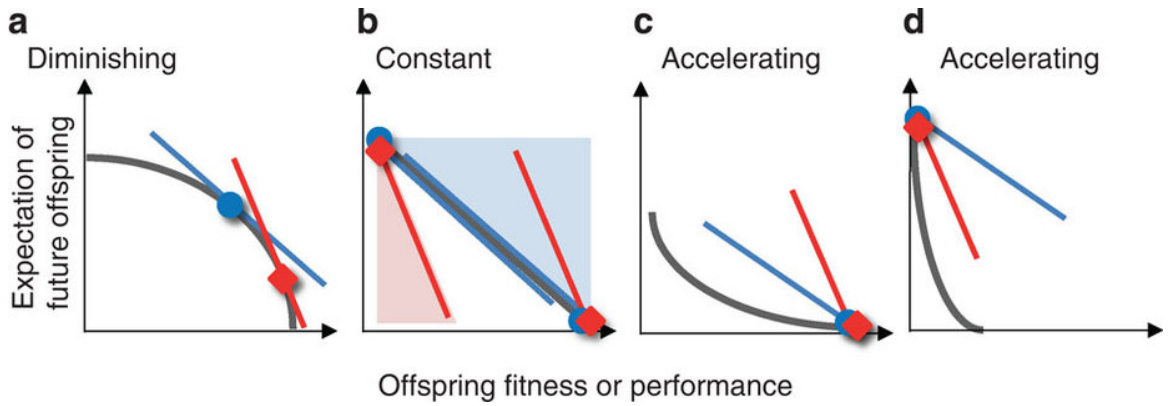

Figure 1: Theoretical plots depicting how the shape of genetic trade-offs affect the parent–offspring conflict battleground. (**a**) Curved trade-off with diminishing returns (grey line). The intersection of the fitness isoclines (tangent lines) to this curve are optima and their slope is steeper for the offspring (red line) than for the parent (blue line) because each offspring is at least twice as related to itself than to its sibling, whereas the parent is equally related to all its offspring (slope for parent=−1; slope for offspring=−2 in case of full siblings[4]). The parent and offspring optima (blue circle and red diamond, respectively) differ and, thus, there is parent–offspring conflict over the amount of PI in current offspring (modified from ref. [4]). (**b**) Linear trade-off with constant returns. When the trade-off lines have slopes that lay in the blue area, parent and offspring agree that the parent should not produce future offspring. Conversely when the trade-off lines have slopes that lay in the red area, parent and offspring agree that the parent should terminate PI and produce additional offspring. When the trade-off lines have slopes equivalent to the fitness isoclines, no optima occur and all combinations of parent and offspring values are equivalent. Only in the white area there is conflict; not over the quantitative partitioning of PI among offspring, but over whether or not future offspring should be produced. (**c**,**d**) Curved trade-off with accelerating returns. (**c**) When current offspring stand to gain substantially from PI, the parent should invest all its resources in current offspring, produce no future clutch and there is no conflict. (**d**) When current offspring do not gain much from further PI, the parent should terminate its investment, produce a second clutch and there is no conflict. Conflict can only occur for trade-off curves intermediate to (**c**) and (**d**); not over the quantitative partitioning of PI among offspring, but over whether or not future offspring should be produced.

accelerating returns, but always met under diminishing returns, that is, when offspring stand to gain less from an additional unit of investment when they are already in good than when they are in poor condition (Fig. 1)[2,4,5,18]. Hence, experimental tests should investigate the presence and shape of the genetic trade-offs, with evidence for conflict being most compelling under diminishing returns.

Theoretically, PI contains on the one hand the trade-off between investment in current offspring and the parent's expectation of future offspring (potentially leading to between- clutch conflict), and on the

other hand the reallocation of investment among offspring within clutches (potentially leading to within-clutch conflict)[5,19–21]. In this study, we focused on the former and tested the three above predictions using a large scale and replicated selection experiment in an insect with extended maternal care, the earwig *Forficula auricularia*. The genetic trade- offs shaping PI were investigated by exerting selection on the mothers and quantifying the correlated responses in offspring. *F. auricularia* is an ideal system for this study: the species reproduces sexually (a prerequisite for parent–offspring conflict), females care for eggs and hatched nymphs, and they produce up to two clutches in their lifetime[22–24]. From the viewpoint of earwig females, first-clutch offspring are current offspring, the relative size of the second clutch is an estimate of the female's expectation for future offspring, and the relationship between the size of the second clutch and the performance of first-clutch offspring quantifies the trade-offs shaping PI. Finally, multiple paternity is common in earwigs[25], leading to variation in genetic relatedness within and between first and second clutches that can further mediate scope for conflict.

We selected females with low expectation of future offspring (that is, Small relative size of (or no) second clutch; S-lines), high expectation of future offspring (that is, Large relative size of second clutch; L-lines) and intermediate expectation of future offspring (that is, Control; C-lines) in ten independent experimental populations over the course of six generations. The experiment included a total of 2,720 females with their offspring (287,636 eggs and 214,815 nymphs of first and second clutches). We predicted a correlated response to selection in offspring performance that was antagonistic to the direct response in females, with increased performance in S-lines and decreased performance in L-lines. Offspring performance was followed by covering the periods of maternal care before and after hatching and including measures of developmental rate, growth and survival. Finally, we explored the shape of the genetic trade-offs emerging between selection lines in the last generation. Overall, our results demonstrate (1) the occurrence of genetic trade-offs between the mother's expectation of future offspring and several offspring performance traits expressed before and after hatching; and (2) diminishing returns for offspring performance before hatching, but constant returns after hatching when mothers and offspring interact. Our study provides clear evidence for a parent–offspring conflict battleground during the egg stage, and highlights that its occurrence and nature critically depends on the genetic trade-offs shaping PI.

Results

Direct response to selection in mothers. S-line females evolved towards a lower relative second-clutch size as compared with L-line females (Fig. 2a), as expected. Per generation, the S- and L-lines diverged by 0.106 s.d. units (Fig. 2b) resulting in a mean difference of 0.637 s.d. in generation six (Fig. 2a). This response was due to significant changes in the size of the second clutch, while the size of the first clutch did not change significantly (Table 1). Furthermore, S-line females gained significantly less mass within 14 days after hatching of their first clutch (Table 1), a morphological proxy predicting second-clutch production[24]. These findings together confirm that S-line females evolved lower expectation for future offspring production than L-line females.

Correlated responses to selection in offspring. Four performance traits of first-clutch offspring showed the antagonistic correlated responses to selection expected under a genetic trade-off. During the egg stage, hatching success and the rate of embryonic development increased in the S-line compared with the L-line (Fig. 2c,d; Table 1). The effect on hatching success was partly mediated by filial cannibalism, as L-line females showed an increasing tendency for egg cannibalism compared with C- or S-line females (Table 1). After hatching, early nymph survival and their relative mass gain until day 14 showed the expected correlated responses, increasing in the S-lines relative to L-lines (Fig. 2e,i). The correlated responses in early and late nymph developmental rate were not significant (Fig. 2f,g) and nymph body mass at hatching decreased, rather than increased, in S-lines (Fig. 2h).

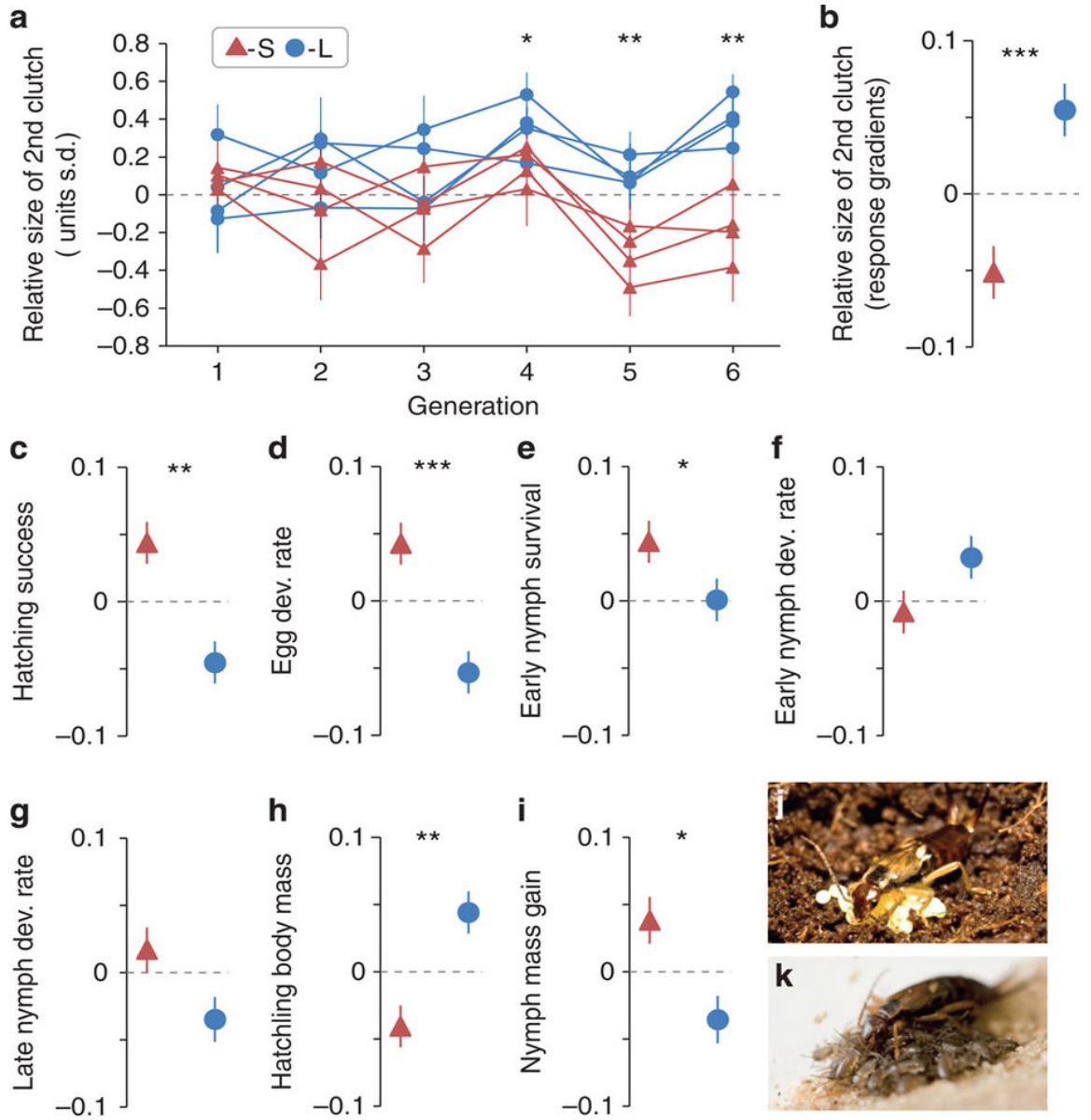

Figure 2: Direct and correlated responses to selection. N=4 S-lines (red symbols and lines), N=2 C-lines and N=4 L-lines (blue symbols and lines) throughout. Direct response to selection as (a) time course of the mean (±s.e.m.) trait values per replicate selection line (population pair), computed as deviation from the mean of the two control (C) lines and (b) as linear response gradients (estimated using linear mixed models (LMMs); see 'Statistical analysis' in Methods section and Table 1; n=2,289 females with offspring). The correlated responses to selection in first-clutch offspring are displayed as linear response gradients: (c) proportion of hatched eggs (n=2,628); (d) egg developmental (dev.) rate between oviposition and hatching (n=2,519); (e) proportion of nymphs surviving from hatching until day 14 (n=2,474); (f) early nymph developmental rate from hatching to molt to second instar (n=2,438); (g) late nymph developmental rate from second instar to adult emergence (n=2,228); (h) mean nymph body mass 1 day after hatching (n=2,507); and (i) proportional nymph mass gain from hatching until day 14 (n=1,415). The scales on the y-axes are in units of s.d. *P<0.05, **P<0.01, ***P<0.001; LMM. (j) Picture of an earwig female tending her eggs, and (k) of a female tending her nymphs. Picture credits: J.M.

Shape of the genetic trade-offs. The shape of the trade-off curves was inferred from the relationships between the population means for the size of second clutches and the offspring performance traits across the three selection treatments (Fig. 3). Only the data from the last generation were used because the likelihood to detect diminishing returns, if present, is highest when mean trait values have diverged most. Qualitative evidence for a con- cave curved genetic trade-off and, thus, for diminishing returns and conflict was found for the egg stage in relation to hatching success and embryonic developmental rate (Fig. 3a,b). In contrast, the trade-offs after hatching with mass gain and nymph survival were approximately linear and indicated constant rather than diminishing returns (Fig. 3c,d). The slope with nymph mass gain was less steep than -1 (slope= -0.63), but steeper than -1 (while also less clearly linear) with regard to nymph survival (slope= -1.37).

Discussion

Behaviours in families are generally thought to be the outcome of a genetic conflict over parental investment. This conflict is a cornerstone of kin selection theory and the gene-centred view of evolution[2,7,26]. However, an empirical demonstration of the conflict battleground[7] has remained an unsolved difficulty to this day, partly due to intrinsic limitation of behavioural or phenotypic studies to demonstrate genetic conflict[6,12,14], and partly due to experimental difficulties of quantifying PI27 and demonstrating different fitness optima for parents and offspring[7,14].

In this study, we addressed these open questions using a selection experiment in the earwig *F. auricularia* and show empirical evidence for genetic conflict between parent and offspring over PI, at least during the egg stage. More specifically, we show that experimentally selecting on the females' expectation for future offspring (that is, the relative size of their second clutch) resulted in a direct response in terms of second-clutch size and correlated antagonistic responses to selection in offspring performance traits. These results demonstrate genetic trade-offs shaping PI, which is an essential (albeit not sufficient; see introduction) precondition for conflict to occur. The direct and correlated responses to selection were consistent among replicate lines with small and nonsignificant variation between population pairs due to drift. Furthermore, different fitness optima for earwig mothers and offspring were inferred by examining the shape of the genetic trade-offs in the last generation. They showed diminishing returns during the egg stage revealing scope for parent–offspring conflict over hatching success and egg develop- mental rate. After hatching, the trade-offs were linear implying constant

returns and a probably minor role for conflict over nymph survival and growth (see below).

The correlated responses to selection in offspring were in the direction predicted by genetic trade-offs with regard to four offspring performance traits. As compared with L-line offspring, S-line offspring evolved towards enhanced hatching success, faster egg development, higher nymph survival and mass gain. The trade-off with hatching success was partly due to L-line females evolving a higher tendency to cannibalize their eggs, which fits the expectation that females with higher expectation for future reproduction should prioritize somatic maintenance (that is, food intake by egg recycling) over parenting and current offspring survival1[15]. The responses in egg developmental rate may be due to changes in maternally transferred hormones or resources in the eggs, which are common maternal effect mechanisms across taxa[28–30], or in maternal egg care behaviour[31]. The correlated responses of nymph survival and growth indicate enhanced post-hatching maternal care in S-line females, for example, through food provisioning[23,32] and/or maternal modulation of siblicide among nymphs. In earwigs, nymph mortality is partly due to siblicide[33] and, thus, the enhanced survival of nymphs in the S-line could also indicate a reduced siblicidal tendency of S-line nymphs. Compared with these four traits, the correlated response to selection in nymph body mass at hatching is less straightforward to interpret leaving room for two alternative interpretations. It could either reflect more maternal care during the egg stage by S-line females because attended eggs are known to develop into lighter hatchlings than orphaned eggs[31], possibly due to the selective survival of heavier hatchlings under low levels of egg care. In this case, the observed response would be according to the predictions of a trade-off. Alternatively, because hatchlings from smaller eggs tend to be lighter[34], S-line females may produce smaller eggs, which would be opposite to prediction. Given the straightforward interpretation of the first four offspring performance traits as components of the genetic trade-offs shaping PI, we focused on these in our examination for diminishing returns and scope for conflict.

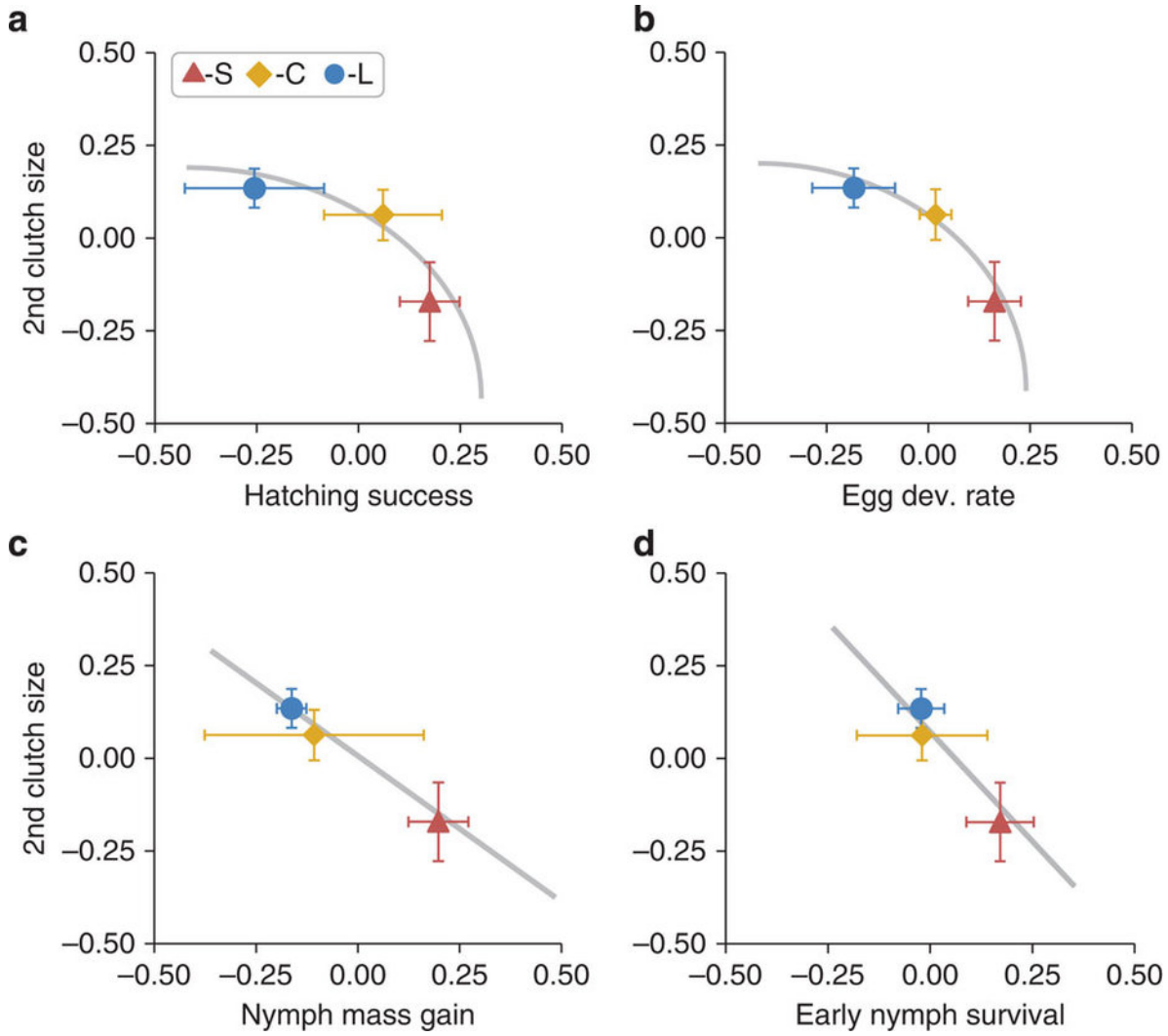

Figure 3: Shape of genetic trade-offs between second (2nd) clutch size and offspring performance. Shown are the trait means (±s.e.m.) from the last generation (generation six) across the S-lines (red symbols; *N*=4 lines, *n*=134 families), the C-lines (yellow symbols; *N*=2 lines, *n*=73 families) and the L-lines (blue symbols; *N*=4 lines, *n*=145 families). Curved trade-offs with diminishing returns before hatching for (a) hatching success and (b) egg developmental (dev.) rate. Linear trade-offs with constant returns after hatching for (c) nymph mass gain (slope (±s.e.)=−0.63 (0.01)) and (d) nymph survival (slope (±s.e.)=−1.37 (0.31)).

The shape of the genetic trade-offs was inferred by comparing the evolutionarily diverged offspring performance traits and relative size of the females' second clutches between the three selection treatments. The curved genetic trade-offs during the egg stage indicate diminishing returns providing evidence for conflict over hatching success and egg developmental rate. Specifically, the increase in hatching success/developmental rate per unit decrease in the size of the female's second clutch was less

between the C- and S-lines (high offspring performance) than between the C- and L-lines (low offspring performance). At first view, conflict during the egg stage may be thought to have little evolutionary consequence because the eggs are developmentally constrained in their ability to influence PI, and part of the conflict was due to female filial cannibalism that eggs cannot prevent. However, embryos are known to respond developmentally to other, more subtle forms of maternal influences (for example, maternal hormones in the eggs), and conflict can operate on these mechanisms[29,30]. The potential occurrence, scope and function of such maternal effect mechanisms remain to be investigated in *F. auricularia*.

Despite genetic trade-offs, the evidence for conflict was weak after hatching when earwig mothers provide food to their young and nymphs signal their condition by solicitation pheromones[32]. The trade-off curves with nymph mass gain and survival were approximately linear indicating constant returns. Under constant returns, scope for conflict is limited and, if it is predicted, it is not over the partitioning of the amount of PI, but over whether or not the mother produces a second clutch (Fig. 1). The slope of the trade-off line was less steep than -1 for mass gain, which implies that with regard to effects on this offspring trait, earwig mothers and nymphs agree that females should not produce a second clutch (which could explain why a fraction of earwig females produces only one clutch in their lifetime[24]). For nymph survival the slope was steeper possibly in the range of mother–offspring conflict over second-clutch production. Indeed, our former research demonstrated that nymphs can influence whether or not caring females produce a second clutch, mediated by a paternally inherited effect[35]. Thus, whether or not earwig females produce a second clutch may have partly evolved due to the genetic trade-offs with nymph growth and survival. Our result that scope for conflict is more limited after than before hatching is somewhat surprising because parental feeding and offspring begging is the classical context used to model how parents and offspring should behave to resolve conflict[5,7,8,12,13], where diminishing returns are commonly assumed, and thus the one where conflict over the amount of PI is a priori most expected.

By selecting on the relative size of second clutches, we focused on genetic trade-offs operating between clutches, which can drive conflict over PI among successive breeding attempts as originally envisaged by Trivers[2]. S-line nymphs evolved towards a higher mean offspring performance, without a significant change in the size of first clutches, which confirms the prominent role of the between-clutch trade-off

for PI and scope for this form of conflict in *F. auricularia*.

Our findings highlight that the nature of conflict depends on genetic trade-offs and that conflict is not inevitable. Parent and offspring behaviours may also be driven by antagonistic mother– offspring co-evolution with no or minor influences of conflict. Such a process should result in coadapted[17,36] and well-coordinated parenting with low-cost honest begging[6]. Thereby, the genetic link between parental reproduction and offspring performance allows PI to quickly evolve and adapt in a changing environment.

From a life-history perspective, the constant returns and weak evidence for conflict after hatching, as compared with the egg stage, may at least partly reflect the partial independence of earwig nymphs from their mother's care during this stage[23]. Partial independence may limit conflict as compared with systems where offspring are fully dependent on their parents, such as altricial birds or mammals. Under partial independence, constant returns may be more likely because low levels of care have less devastating effects on offspring performance than under full offspring dependence and obligate care. If correct, this hypothesis would imply that parent–offspring conflict had limited impact in the early evolution of parenting when offspring did not fully depend on their parents and that, if present, conflict was mainly over whether or not parents should reproduce again (that is, their parity). More generally, the biological importance of genetic conflict should depend on factors determining the curvature of the genetic trade-offs shaping PI such as the life history and possibly also ecology of a population/species.

In conclusion, our study shows clear evidence for a genetic conflict between parents and offspring over PI. It thereby solves a long-standing problem that was previously conceived prohibitively difficult to address and, thus, fills a major gap in our empirical proof of concepts in the evolution of behaviours in families. Furthermore, and contrary to former thought, our results also reveal that conflict may not globally and a priori be assumed to be the major driver of parenting and family life. The nature and scope for conflict critically depends on the shape of the genetic trade-offs underlying PI, which needs empirical testing, and PI may also evolve by conflict-free antagonistic parent–offspring co-evolution enabling PI to evolve as coadapted and well-coordinated parenting and family life.

Methods

Laboratory breeding. The animals forming the base population of this selection experiment were caught from a wild population in early June 2009 in Dolcedo, Liguria/Italy (7° 56'55" E, 43° 54'14" N, altitude 78m a.s.l.). It consisted of B1,200 predominately fourth juvenile instars and recently emerged adults. After transfer to the laboratory, the field-caught individuals were assigned randomly to 20 mating groups of 60 individuals each (30 females and 30 males) and kept separately in plastic containers for mating (see ref. 24 for a detailed description of the base population). The artificial selection experiment was initiated based on the progeny of these field-caught animals, that is, the first laboratory-born generation of adults (F1). Upon emergence as adults, the F1 males and females were randomly assigned to 20 mating groups of 48 individuals each (24 females and 24 males). The mating groups were held in plastic containers (dimensions: 37x22x25 cm) with humid sand as substrate and with egg cardboard and plastic tubes as shelters. The containers were lined with fluon and covered with nylon thighs to prevent escape of the animals. They were fed with our standard laboratory food (a food jelly made from 20 g egg yolk, 60 g wheat germ, 120 g carrots, 60 g bird food, 60 g dry cat food, 60 g flower pollen, 40 g Agar, 1,800 ml water, 2 g ascorbic acid and 2 g sorbic acid) with adequately sized pieces twice a week (see also ref. 24).

The mating groups were held in climate chambers at a light:dark photoperiod schedule of 14:10 h and at a constant temperature of 20 °C (to which we refer as ‘summer conditions’) with relative humidity kept between 60 and 80%. As soon as at least two females from two different mating groups laid eggs, all females from all mating groups were set-up individually in Petri dishes (10x2 cm). The dishes contained humid sand as a substrate and a plastic tube as shelter. All females were kept for 7 days at 10 °C (no light) and then at 15 °C (no light) for oviposition and for the duration of egg care until hatching. Such ‘winter conditions’ are required to terminate the diapause of the eggs and trigger embryonic development[23,24]. Each female was provided food twice a week until oviposition, and no food was provided during egg care until hatching23. On day 1 after hatching, we set-up the hatched nymphs with their mother in a new Petri dish (10x2 cm) and returned them to ‘summer conditions’ (see above). During the first 2 weeks after hatching (that is, from day 1 until day 14), food was provided every other day. On day 14, females were separated from their nymphs and set-up in a new Petri dish (10x2 cm) for production of the second clutch (if any). Also on day 14, a total of 20 of her nymphs (or fewer in case of smaller nymph numbers) were chosen haphazardly Mating and set-up in larger Petri dishes (14.5x2

cm) where they were reared as family groups until adulthood. After day 14, both females and nymphs were fed twice a week.

If a female produced no second clutch within 60 days after hatching of the first clutch, the female was considered to produce only one clutch in her lifetime24.If the female produced a second clutch, we took the performance measures of second- clutch offspring up until hatching (see section 'Trait measurements' below). We did not rear any second-clutch offspring into adulthood. These basic procedures were applied to all generations of the selection experiment. The selection experiment was carried out over the course of six generations between spring 2010 and fall 2013.

Experimental design. A graphical illustration of the experimental design can be found in Fig. 4. The selection experiment was initiated after one generation of laboratory breeding without selection to reduce a potential impact of environ- mental variation modifying the response to selection through maternal effects37.Of each brood produced by the 24 F1 females of each of the 20 mating groups, a female and a male were randomly selected to form the new 20 mating groups. The number of individuals per mating group was 24 females and 24 males across all generations of the selection experiment. To avoid brother–sister mating and minimize potential effects of inbreeding depression due to sib-mating, the 20 mating groups were randomly assigned into paired populations among which the females and males were exchanged each generation to form the mating groups of the next generation. For example, the female progeny of former population A were set-up with the male progeny of former population B to form the new population A (and vice versa for the new population B). The assignment of mating groups into population pairs was established at set-up of the field-caught individuals (F0) and was maintained over the whole course of the selection experiment. In this selection design, the unit of replication (that is, the selection line) is the paired population as it defines the independent gene pools that may evolve in response to selection.

From the total of 10 population pairs (that is, replicate selection lines), four were selected for a relatively small second clutch ('S-lines'), four for a relatively large second clutch ('L-lines') and two for an intermediate relative size of the second clutch (control 'C-lines'). The relative size of the second clutch was computed as the number of eggs in the second clutch divided by the sum of eggs in the first and second clutches (the sum corresponding to the lifetime number of eggs in F. auricularia[24]). In the S-

lines, we selected the bottom 50% (including females producing a single clutch), in the L-lines the top 50% and in the C-lines the intermediate 50% of the distribution in the relative size of second clutches among females of each mating group.

Although the relative size of the second clutch is a maternal trait with sex-limited expression, we applied selection through both sexes by using sons and daughters of the selected females/families (Fig. 4). We aimed at selecting two sons and two daughters of each selected female/family to keep mating groups of constant size (that is, 24 females and 24 males). This was not always possible due to cases of juvenile mortality, hatching failure or insufficient individuals from both sexes upon adult emergence in some of the families. In these cases, the number of selected individuals per brood/sex was adjusted by balancing stronger selection (using more individuals from mothers with the best fit to the selection criterion) against maintenance of genetic variability (using individuals from as many families as possible). The mean (±s.d.) numbers of females and males per family used over the six generations were 2.46 (0.86) and 2.50 (0.91), respectively. Only progeny from first clutches were used for breeding.

Trait measurements. We took various measures of offspring performance including estimates of survival (separate for eggs/embryos and nymphs), estimates of developmental rate (separate for eggs/embryos, early nymphs (hatching—second instar) and late nymphs (second instar—adulthood)) and estimates of growth (separate for body mass at hatching and body mass gain during the first 14 days after hatching, as measure of growth after hatching). Survival is a direct component of fitness, and mass gain and fast development gives nymphs a headstart in competitive/cannibalistic interactions[38,39]. In addition, a range of reproductive parameters was recorded. The oviposition and hatching dates for first and second clutches were taken upon observation of the first eggs of a female and corresponded to the date of first observation of egg laying or hatching in a given clutch, respectively. Clutch sizes were determined by counting the number of eggs of the first and second clutches for each female 1 day after the first observation of the start of oviposition. Similarly, the number of hatched nymphs was counted 1 day after observation of the first hatched nymph in a clutch. Because hatching is sometimes asynchronous, the unhatched eggs were kept for another day to count further hatched nymphs (if any) on the subsequent day, and the number of unhatched eggs was also counted. The total number of hatched nymphs over the 2 days as proportion of clutch size was used to quantify hatching success.

Earwig females sometimes cannibalize some of their eggs during the period of egg care[34]. To obtain a quantity of egg cannibalism, the sum of the hatched nymphs and remaining unhatched eggs at hatching was compared with the original clutch size. Any reduction in the number of eggs between oviposition and hatching is most likely due to maternal egg cannibalism, and the difference in progeny number between oviposition and hatching was used as a measure of filial egg cannibalism in the analysis.

The body mass of nymphs was measured twice, 1 day after hatching and on day 14 after hatching. For each clutch, ten haphazardly chosen nymphs were jointly added to an Eppendorf tube and the tube was weighed with and without the nymphs. The difference divided by ten was taken as the average nymph body mass of a given clutch. Hatchling body mass was taken in all generations. Body mass at day 14 was only available for generations F1, F2, F3 and F6. The relative mass gain of nymphs over the course of the first 2 weeks after hatching was calculated as the proportional increase in mass relative to the body mass at hatching. We also took two measurement of female body mass, once at hatching and once 14 days after hatching. The weight gain of females from hatching of the first clutch until day 14 is a predictor for the likelihood and size of the second clutch[24]. All mass measurement were done to the nearest 0.01mg using a Mettler-Toledo MT5 Micro-balance (Mettler, Roche, Basel). For measures of developmental rate we calculated the number of days between egg laying and hatching (egg developmental rate), the number of days between hatching and the first nymph in a clutch molting into second instar (early nymph development), and the number of days between second instar to the first adult emergence in a clutch (late nymph development).

Statistical analysis. All variables were standardized to a mean of zero and unit variance within each generation for homogeneous variances across generations. To test for divergence of maternal and offspring traits between selection lines, we estimated standardized linear response gradients over the course of the six gen- erations using linear mixed models and restricted maximum likelihood estimation. The trait of interest (standardized) was entered as the dependent variable, the selection treatment as fixed factor (H-lines, C-lines and L-line), the generation as continuous variable (linear term), the interaction between the selection treatment and generation as fixed factor and the paired populations (for example, ‘A–B’) as random effect. A linear response to selection is in this model demonstrated by a significant interaction between the selection treatment and generation. The regression coefficients from this interaction term are standardized linear response gradients, that is, the

slopes of the linear trend for the S- and L-lines relative to the control C-line. Standardized response gradients estimate the per-generation change in population mean trait values expressed in units of s.d.. The random effect (the paired population) accounted for the dependencies of individuals from the same selection line (that is, sharing the same gene pool) and for differences between lines within selection treatments arising for reasons other than selection as, for example, genetic drift. Proportional variables (relative size of second clutches, hatching success and nymph survival) were logit-transformed40 before standardization and analysis, and measures of developmental rate were computed by multiplying the standardized values of duration (number of days) by minus one, such that large positive values corresponded to fast development and large negative values to slow development. All reported P values are two tailed with a significance threshold a of 0.05. The statistical analyses were carried out using JMP PRO V11.0 statistical software (SAS Institute, Inc.).

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Author contributions

M.K. conceived the study and analysed the data; M.K. and J.M. designed the experiment and wrote the manuscript; S.B., J.M., J.W.Y.W., L.R. and D.S. managed the selection lines; and all authors collected the data and contributed to manuscript revisions with comments.

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APPENDIX II

Genetics and Developmental Biology of Cooperation

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Abstract

Despite essential progress towards understanding the evolution of cooperative behaviour, we still lack detailed knowledge about its underlying molecular mechanisms, genetic basis, evolutionary dynamics and ontogeny. An international workshop "Genetics and Development of Cooperation," organized by the University of Bern (Switzerland), aimed at discussing the current progress in this research field and suggesting avenues for future research. This review uses the major themes of the meeting as a springboard to synthesize the concepts of genetic and nongenetic inheritance of cooperation, and to

review a quantitative genetic framework that allows for the inclusion of indirect genetic effects. Furthermore, we argue that including nongenetic inheritance, such as transgenerational epigenetic effects, parental effects, ecological and cultural inheritance, provides a more nuanced view of the evolution of cooperation. We summarize those genes and molecular pathways in a range of species that seem promising candidates for mechanisms underlying cooperative behaviours. Concerning the neurobiological substrate of cooperation, we suggest three cognitive skills necessary for the ability to cooperate: (i) event memory, (ii) synchrony with others and (iii) responsiveness to others. Taking a closer look at the developmental trajectories that lead to the expression of cooperative behaviours, we discuss the dichotomy between early morphological specialization in social insects and more flexible behavioural specialization in cooperatively breeding vertebrates. Finally, we provide recommendations for which biological systems and species may be particularly suitable, which specific traits and parameters should be measured, what type of approaches should be followed, and which methods should be employed in studies of cooperation to better understand how cooperation evolves and manifests in nature.

1 INTRODUCTION

The question of how cooperation evolves has been a major conceptual puzzle for biologists for centuries. Despite significant inroads in our understanding of the evolution of cooperation over the past 60 years, it remains one of the major challenges in biology to date. While most research into cooperation has been devoted to the functional significance of cooperation, an increasing number of scientists argue that a more holistic approach incorporating functional and mechanistic aspects of phenotypic traits is necessary to provide a complete picture (Bshary & Oliveira, 2015; Soares et al., 2010; Taborsky & Taborsky, 2015; Weitekamp & Hofmann, 2014). First, by only focussing on the functional significance, one implicitly assumes that cooperation is not constrained by the underlying physiological, neural, molecular and developmental mechanisms. However, behaviours such as cooperation can only evolve by changes in those underlying mechanisms (Fawcett, Hamblin, & Giraldeau, 2013). Second, an integrative approach allows us to address questions of convergent molecular evolution (Aubin-Horth, 2015) which is of particular importance for cooperation as it is thought to have evolved multiple times independently (Maynard-Smith & Szathmary, 1997). Finally, theoretical and empirical research can be mutually informative. Detailed knowledge of the mechanisms underlying cooperation and evolutionary constraints on cooperative traits could lead to the

development of models that better reflect the actual environmental complexity (McNamara & Houston, 2009; Soares et al., 2010).

Our goal in the workshop "Genetics and Development of Cooperation" organized by the University of Bern, held in Bern, Switzerland, in February of 2016, was to explore new horizons in the fields of genetics and developmental mechanisms of cooperation. A list of the guest speakers and the titles of talks, as well as the names of the workshop participants, is provided in the Supporting Information. In the workshop, we focused on cooperative strategies such as reciprocity, mutualism, and coercion between family groups and nonkin for feeding, protection and raising young. We also discussed cooperative parental care, parent–offspring and sibling conflict, and communal nesting. Plenary talks were used as a launching pad for discussion sessions and poster sessions showcased individual participants' research. In the following sections, we relate the content and questions raised by the workshop sessions. Moreover, we provide an outlook and further avenues for research in an effort to synthesize the various key points raised by the workshop.

2 MODES OF INHERITANCE OF COOPERATION

Defining cooperation is notoriously difficult because of the complex interplay of fitness costs and benefits that accrue over different time periods and the varieties of situations under which it occurs (Sachs, Mueller, Wilcox, & Bull, 2004). For the purpose of the workshop, we followed the definition given in Taborsky and Taborsky (2015) stating that "cooperation refers to the simultaneous or consecutive acting together of two or more individuals by same or different behaviours." Cooperative acts are typically costly for the individuals involved, but their net result is a fitness benefit. Cooperation can evolve if it yields immediate or delayed fitness benefits for all partners. Alternatively, if one partner can coerce the other into cooperation, only the receiver gains fitness benefits. Cooperative acts that yield direct fitness benefits for all partners are, for instance, improved prey capture when hunting in small groups in wolves (MacNulty, Smith, Mech, Vucetich, & Packer, 2012), lowered predation risk through flocking behaviour in birds (Beauchamp, 2003), reduced heat loss in huddling penguins (Ancel, Visser, Handrich, Masman, & Le Maho, 1997) and increased energetic benefit during V-formation flight in migrating birds (Voelkl & Fritz, 2017; Voelkl et al., 2015). Altruistic behaviours, however, impose costs on actors without yielding direct benefits and result in a net decrease in the actor's direct fitness while increasing the recipient's fitness (Lehmann & Keller, 2006). Examples of

altruism include sterile castes of social insects that raise a queen's offspring (reviewed in Ratnieks and Wenseleers (2008)), but also the willingness to share food, engage in collective warfare, or to bear costs to punish noncooperators in encounters with unrelated and even unknown individuals in humans (Fehr & Fischbacher, 2003).

An explanation of how such costly altruistic behaviours may evolve is predicated in the theoretical work by Hamilton who suggested that altruistic genes evolve under the scenario of inclusive fitness (Hamilton, 1964a, 1964b). In his seminal paper (Hamilton, 1964b), he stipulates under which conditions altruism should evolve by deriving the famous Hamilton's rule, $rB > C$. Under this scenario, costs to the focal individual (C) are outweighed by the benefits to the receiver (B), weighted by the genetic relatedness (r) between the two individuals. If the costs and benefits are similar, cooperation should arise based on genetic relatedness, which is also known as kin selection. Despite this illuminating theoretical foundation, definite evidence for specific drivers for the evolution of cooperation remains difficult to identify for many species that display cooperative behaviours. For example, the evidence for kin selection as a driver of cooperation is mixed (Riehl, 2013; Taborsky, Frommen, & Riehl, 2016) and costs and benefits can be difficult to assess and compare objectively within and between species (Hatchwell & Komdeur, 2000; Sachs et al., 2004). Knowledge of the genetic, molecular and physiological mechanisms that underlie cooperative behaviours can greatly improve our understanding of the evolution of cooperation. For instance, genetic variation in cooperative behaviours reflects their evolutionary potential, that is, how those traits can respond to natural selection. Evolutionary theory predicts that cooperative behaviour, like other phenotypic traits, should have a heritable basis if they are the product of adaptive evolution (Hofmann et al., 2014; Komdeur, 2006; Tinbergen, 1963). In fact, there is some empirical support for heritable differences in cooperative behaviours (e.g., in western bluebirds *Sialia mexicana* (Charmantier, Keyser, & Promislow, 2007), and in humans (Cesarini et al., 2008). However, an individual's cooperative tendency is likely to be influenced additionally by social and nonsocial environmental conditions to allow for plasticity during development or to fine-tune pay-offs in its current situation (Fischer 2014; Kasper, Kölliker, Postma, & Taborsky, 2017; Koenig, Pitelka, Carmen, Mumme, & Stanback, 1992; Stiver, Dierkes, Taborsky, & Balshine, 2004; Sanderson et al., 2015b). Moreover, nongenetic inheritance of cooperation through social interactions and cultural transmission may add additional layers to the complexity of the evolution of cooperation (Uller & Helanterä, 2017; Avital & Jablonka, 2000; Danchin et al., 2011), but

this field is thus far underdeveloped for cooperation.

2.1 Genetic inheritance and indirect genetic effects

For a cooperative—or any other—trait to be subject to selection, it needs to vary among individuals. This variation should result in differential fitness and should be heritable (Lewontin, 1970). Quantitative genetic models allow researchers to explore the extent to which genetic variation influences phenotypic variation by estimating the proportional contributions of heritable genetic variation and environmental variation to the total phenotypic variation. By combining these estimates with estimates of the fitness consequences of this variation, we can predict how a trait will respond to selection (Lande & Arnold, 1983).

Accounting for the social environment of individuals adds a further dimension to cooperative behaviour because it involves interactions with other individuals, making the behaviour of an individual contingent upon the behaviour and genotype of its social partners. Therefore, the cooperative phenotypes should be considered as being partly influenced by interactions with social partners and the genes they carry, that is, their "interacting phenotype" (Moore, Brodie, & Wolf, 1997). This influence of the social environment sets those traits apart from traits that are solely influenced by heritable genetic and nonsocial environmental components, and therefore requires additional theoretical considerations (Bleakley, Wolf, & Moore, 2010). Especially for cooperative traits, we can expect that the genotypes of interaction partners affect the fitness of an individual in a similar way as the individual's own genes (McGlothlin, Wolf, Brodie, & Moore, 2014). For instance, in species that provide biparental care, parents can negotiate the amount of care each provides which equally affects both parents' fitness in terms of offspring survival (McNamara & Houston, 2005). Another example where social environment may play a key role is cooperative breeding, where helpers might adjust their helping effort based on the contributions of other group members (Adams, Robinson, Mannarelli, & Hatchwell, 2015). Parents can reduce their level of care when helpers are present (Johnstone, 2011; Taborsky, Skubic, & Bruintjes, 2007), or where subordinates are coerced into helping (Clutton‐Brock & Parker, 1995; Fischer, Zöttl, Groenewoud, & Taborsky, 2014).

In his talk, "A social effects perspective on kin selection," Jason Wolf outlined the quantitative genetic version of Hamilton's rule that takes into account the impact of the focal individual's own phenotype on

its fitness ("nonsocial selection gradient," β_N), but also the phenotype of the individual with whom it interacts ("social selection gradient," β_S, Figure 1 (McGlothlin et al., 2014). This model demonstrates that selection will favour altruism when the benefits (β_S), weighted by the phenotypic similarity of the partners, are greater than the costs ($-\beta_N$). In cases where phenotypic similarity solely arises due to genetic relatedness, it is equivalent to Hamilton's relatedness term (McGlothlin et al., 2014; Queller, 1992). However, genetically unrelated individuals can be phenotypically similar. Covariances between the partners can arise due to the influence of genes expressed in another individual, providing an "alternative pathway from genotype to fitness" via indirect genetic effects (IGEs, McGlothlin et al., 2014). Unlike a direct genetic effect (DGE) where an individual's genotype directly affects its phenotype, IGEs are the expression of one individual's genotype influencing the expression of another individual's phenotype. Thus, IGEs need to be scaled by a parameter that reflects the genetic influence of an interaction on the trait expressed in the focal individual. Here, the interaction effect coefficient (ψ) illustrates this relationship and ranges from −1 to 1 (Figure 1). In the absence of genetic relatedness, cooperation should only evolve if benefits scaled by the interaction effect coefficient ($\psi\beta_S$) outweigh costs ($-\beta_N$). This framework provides an extension of the quantitative genetics approach to Hamilton's rule to interactions between unrelated individuals. Mutually beneficial behaviours can evolve even in the absence of relatedness between the interaction partners, because both partners gain direct net fitness benefits immediately or with some delay, and hence, no conflict of interest occurs (Lehmann & Keller, 2006). Many examples of cooperation in birds (Riehl, 2013), fish (Wong & Balshine, 2011), vampire bats (Wilkinson, Carter, Bohn, & Adams, 2016), humans (Jaeggi & Gurven, 2013) and insects (Field & Leadbeater, 2016; Gadagkar, 2016) demonstrate that interaction partners are indeed often unrelated. Therefore, kin selection may not be the primary evolutionary force driving cooperation in these systems (Taborsky et al., 2016), and alternative hypotheses focusing on the IGEs should be considered.

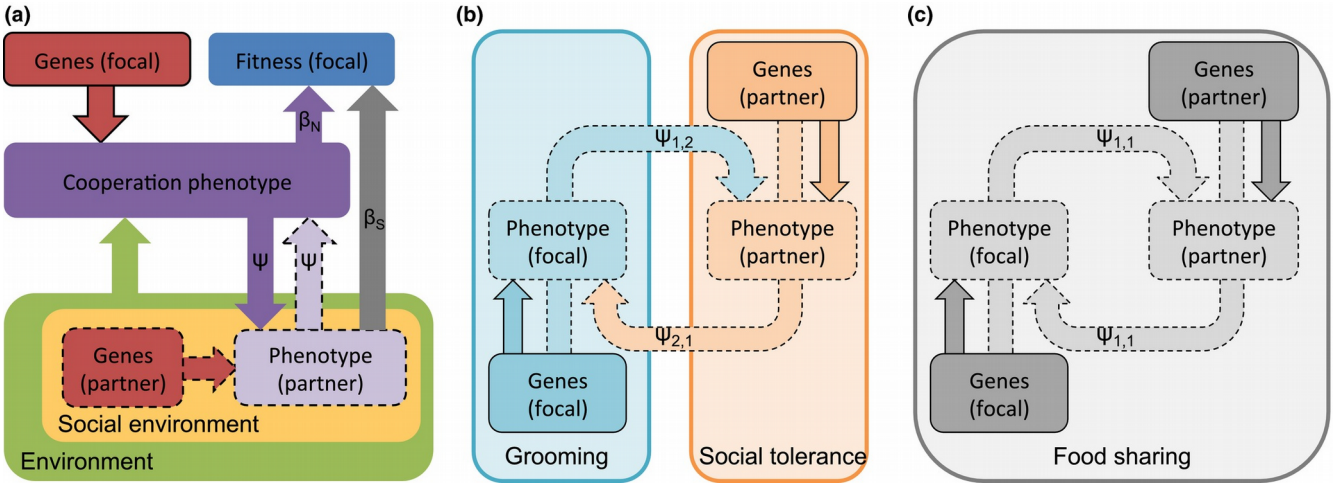

Figure 1. Indirect genetic effects on cooperation. An individual's own genes and its environment jointly influence its cooperation phenotype (direct genetic effect, solid black outline), which influences the focal's fitness ("nonsocial selection gradient," β_N). Cooperative behaviours are expressed in a social setting that constitutes a component of the environment. (a) Nonreciprocal indirect genetic effect: A cooperation partner's genes influence the focal's phenotype indirectly via the partner's phenotype (indirect genetic effect, dashed black outline). The strength of the influence of the genes in the social environment on the focal's phenotype is reflected by the interaction effect coefficient, ψ, and thus, the focal's fitness is affected by genes expressed in other individuals ("social selection gradient," β_S). (b) Two different traits expressed in two individuals influence each other reciprocally. For instance, the focal individual grooms its partner, which leads to an increased tolerance towards the focal ($\psi_{1,2}$), which, in turn, results in more grooming by the focal ($\psi_{2,1}$). (c) The same trait expressed in two different individuals influences itself reciprocally. For instance, the focal's propensity to share food with its partner could increase the partner's propensity to share food and vice versa ($\psi_{1,1}$). Assuming a ψ of 0.75, the feedback loops depicted in (b) and (c) lead to a five- and ninefold increase in the evolutionary rate compared to models without IGEs (Moore et al., 1997)

2.2 Nongenetic inheritance

Heritability is not limited to the transference of genetic information from parent to offspring. Nongenetic information can potentially contribute to the evolution of a cooperative trait if it is transmitted from one generation to the next (Uller & Helanterä, 2017). Distinguishing between different forms of heritability is crucial, because the form of transmission determines who inherits from whom and also how reliable the transmitted information is. In his talk "Nongenetic inheritance, maternal effects, epigenetics, and cultural transmission: where are we now?," Etienne Danchin discussed the concept of inclusive inheritance, which allows not only for the transference of information via genes, but also through mechanisms of nongenetic inheritance (Danchin, Wajnberg, &

Wagner, 2014). Nongenetic inheritance is defined as the transmission of factors other than the DNA sequence from ancestors to offspring that affect the offspring's phenotype (Bonduriansky & Day, 2009). Some of these mechanisms include heritable epigenetic effects, parental effects, ecological (or habitat) inheritance and cultural (or social) inheritance (Danchin et al., 2011).

Narrow-sense epigenetic inheritance occurs when phenotypic variation arises from heritable changes in gene expression, rather than differences in the DNA sequence itself. This variation can occur as a result of structural changes to the genome. For example, the modification of histone proteins or the methylation of cytosine bases in DNA can upregulate, downregulate or silence gene expression (Jenuwein & Allis, 2001; Lee, Smith, & Shilatifard, 2010; Suzuki & Bird, 2008). These epigenetic modifications can be inherited from one generation to the next (Danchin et al., 2011; Jablonka & Raz, 2009). For example, mice that are conditioned to fear an odour for its associated negative stimulus pass on the fear of this odour to their descendants. Hypomethylation of an odour receptor gene (*Olfr151*) is transferred via the gametes, resulting in naïve mice having an innate fear of the odour (Dias & Ressler, 2014). If and how epigenetic inheritance influences cooperative traits and learned social behaviours warrants further investigation.

Parental effects—effects that parents have on the phenotype of their offspring, but not via the inherited genome—can also act as mechanisms for nongenetic inheritance (Mousseau & Fox, 1998). The relevance of parental effects is now widely accepted and considered an additional source of heritability that contributes to parent–offspring resemblance with important evolutionary implications. Parental effects can be genetic, when parental genetic variation is the cause of the environmental component affecting offspring development (Danchin et al., 2011). However, parental effects can also be nongenetic (Danchin et al., 2011). For instance, helping tendencies in cooperative breeders have been shown to be influenced by maternal identity (Kasper et al., 2017). To date, the exact mechanism of transmission is unclear, but candidate mechanisms are maternal allocation of resources towards egg size or composition (Robinson, Fernald, & Clayton, 2008; Russell, Langmore, Cockburn, Astheimer, & Kilner, 2007; Taborsky et al., 2007), or parental care quality (Fischer, 2014; Goodson, Saldanha, Hahn, & Soma, 2005), which may have subsequent bearing on offspring phenotypes. Parental effects can be accounted for in quantitative genetics models by including them as IGEs (see “Genetic inheritance of cooperation”).

Individuals may modify their environments through a process known as "niche construction" that might alter the selective forces they experience (Laland, Matthews, & Feldman, 2016). These modified environments can be passed down to offspring through ecological inheritance, which contributes to inclusive heritability (Danchin et al., 2011). For example, termite mounds are cooperative efforts to modify temperature and humidity and are inherited both within and across generations (Odling‐Smee, Laland, & Feldman, 2003). Within the quantitative genetic framework we developed earlier, this means that phenotypes of others (i.e. the "partners" in Figure 1) modify the environment, which changes the selection gradients affecting the fitness of the focal individual (β_N and possibly β_S in Figure 1) and these environments can be inherited.

Finally, cooperative behaviours can also be transmitted via cultural inheritance (Avital & Jablonka, 2000; Danchin et al., 2011). For cultural information to be conveyed, a trait must be (i) socially learned, (ii) transmitted across generations or from older to younger individuals, (iii) expressed sufficiently to be picked up by younger individuals and (iv) individuals must be able to generalize the social information to use it in new contexts (Danchin & Wagner, 2010). For example, in cooperatively breeding long‐tailed tits, *Aegithalos caudatus*, individuals preferentially help at the nests of related birds. Kin recognition and inclination to help are determined through the similarity of vocalizations, which are learned in early development (Hatchwell, Ross, Fowlie, & McGowan, 2001; Sharp, Mcgowan, Wood, & Hatchwell, 2005). If kin recognition operates only via those vocalizations and individuals are able to recognize kin they have never encountered before based on their dialect, kin recognition depends on culturally inherited differences in song.

An important consideration for all nongenetic inheritance mechanisms is their significance relative to genetic inheritance mechanisms. The contributions of nongenetic inheritance are likely to be highly variable depending on the trait and species in question, and their effect on the pace and direction of evolution and maintenance of traits can be highly significant (Kirkpatrick & Lande, 1989). For instance, nongenetic inheritance could explain the missing heritability—a lack of genetic markers explaining parent–offspring resemblance—in certain traits. Nongenetic inheritance could also play a role in the spread of novel alleles, maladaptive behaviours and major organizational transitions (Danchin et al., 2011). An interesting way to investigate the relative importance of nongenetic inheritance is by incorporating it in quantitative genetic models through the introduction of a double

pedigree: one for genetic and one for nongenetic correlations (Day & Bonduriansky, 2011; Helanterä & Uller, 2010).

Prior to any empirical efforts, it is vital to consider under which conditions nongenetic inheritance is expected to be adaptive. In a group discussion on "Nongenetic inheritance and the evolution of social/cooperative traits" led by Reinder Radersma, we explored such conditions for adaptive nongenetic inheritance. First, the transference of information across generations is beneficial in cases where the environment varies in a repeatable and predictable way over time. Generation time should be shorter than the period of environmental change, leading to a correlation between the parental phenotype and the environment the offspring will encounter. Second, changes in the environment should happen at a rate faster than the genome is able to accommodate (English, Pen, Shea, & Uller, 2015b; Leimar & McNamara, 2015; Figure 2). Third, within-generation phenotypic plasticity should not be too costly, or individuals are physically, developmentally or behaviourally constrained to adequately respond to the changing environment (Uller, 2008). Finally, the benefits of nongenetic inheritance of a trait must outweigh the costs of the inheritance mechanism (Uller, 2008). The reliability and quality of the information offspring or parents are able to gather about the environment is a critical component of the costs and greatly affects the adaptiveness of different inheritance mechanisms (Leimar & McNamara, 2015). Further theoretical development, in tandem with empirical studies, should help to elucidate and quantify nongenetic inheritance of cooperative traits and behaviours in the future.

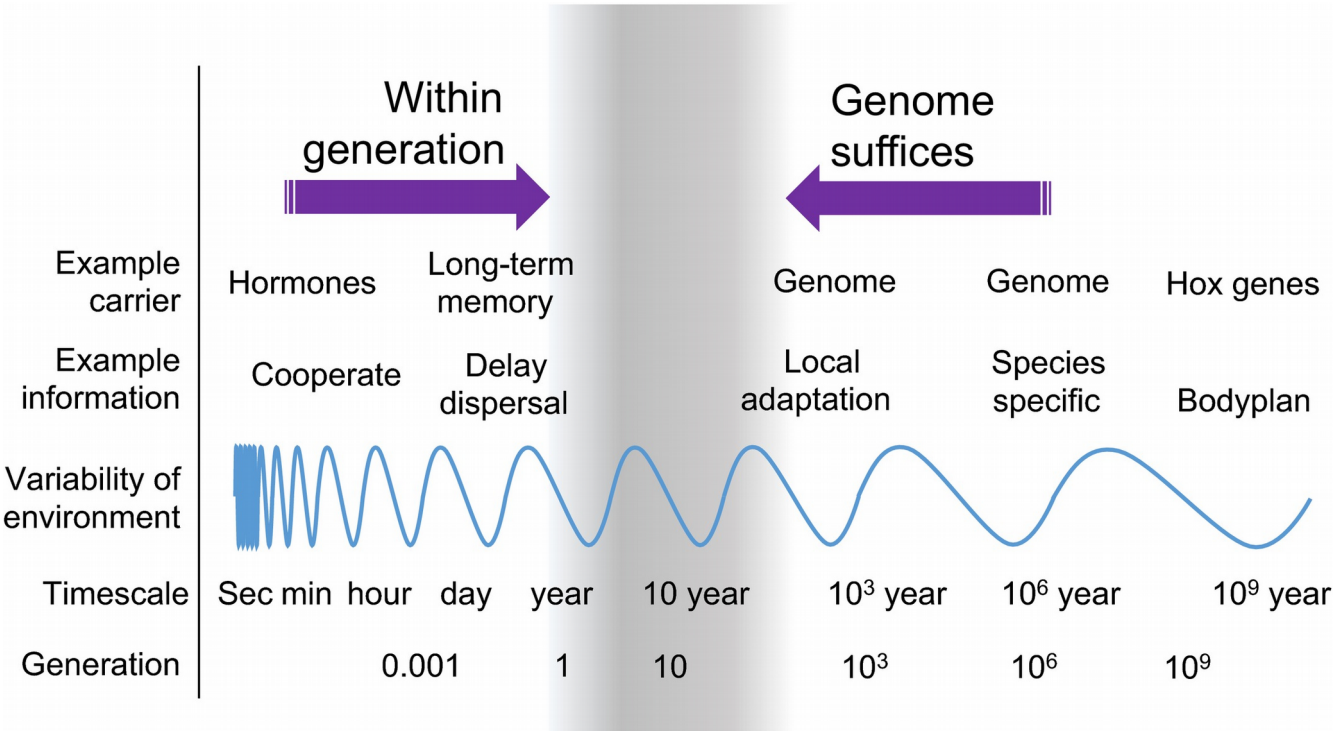

Figure 2. Nongenetic inheritance indicated on the information retention axis (in grey). The information retention axis symbolizes the timescale at which information needs to be retained in a biological system to be adaptive. This adaptiveness depends on the variability of the selective environment. There is scope for nongenetic inheritance when information needs to be transferred over generations (arrow pointing to the right), and the environment is too variable for genes to adapt (arrow to the left). The number of generations, the timescale and the variability of the environment are conceptual examples—roughly at scale—and are study system specific. The types of information and the information carriers are hypothetical examples

2.3 Relevance of IGEs and nongenetic inheritance to understanding the evolution of cooperation

The IGE framework has the potential to improve our understanding of the evolution of cooperation by modelling how social interactions with conspecifics shape the fitness of cooperating individuals. Specifically, IGEs can be thought of as epistatic interactions between the focal trait and genes expressed in conspecifics and are thus part of the genetic architecture of a trait (Meffert, Hicks, & Regan, 2002). By providing the possibility of more realistic models of the nonadditive selective pressures posed by the social environment on cooperative traits, different conclusions about the rate and even the direction of evolution might be drawn than from frameworks that do not explicitly model IGEs. For instance, for social interactions that involve feedback loops between the same or different traits expressed in interacting individuals, the rate of evolution is expected to be 5–9 times faster than in the absence of IGEs, given that ψ is rather high (Figure 1b,c, Moore et al., 1997). Furthermore, by

changing the resemblance of relatives, the presence of IGEs could mask or exaggerate heritable genetic variance (Bijma & Wade, 2008).

The inclusive inheritance framework provides a more nuanced view of the evolution of cooperation by treating inheritance as a multidimensional phenomenon. For instance, failing to incorporate cultural inheritance into models of evolution of human behaviour is demonstrated to lead to substantive discrepancies between predicted and observed evolutionary outcomes (Richerson & Boyd, 1978). Moreover, the phenotype with maximum fitness can differ depending on the mode of inheritance—for example between genetic and cultural inheritance—and thus conflict between these systems can arise. This means that maladaptive behaviours like costly acts of altruism towards unrelated individuals could spread in a population in cases where variance in cultural transmission is higher than variance in genetic transmission. Consequently, positive cultural selection could override negative selection in the genetic domain (Aguilar & Akçay, 2017).

3 GENETIC AND MOLECULAR PATHWAYS UNDERLYING COOPERATION

A cursory review of genetic mechanisms in various systems demonstrates that there are numerous molecular pathways leading to the evolution of cooperative traits (Table S1). Although a variety of molecular mechanisms have been identified, the overwhelming majority of studies indicate that hormonal regulatory pathways seem to hold the key to the evolution of cooperation in many of the examples found in social insects and vertebrates (Table S1).

The changes in how reproductive hormonal signalling systems work can have significant consequences for the emergence of helping behaviour which is often associated with suppressed reproduction. The interplay between insulin-signalling pathway, juvenile hormone (JH) and vitellogenin (Vg) is a fundamental component involved in the evolution of cooperation in insects. Here, both JH and Vg are related to reproduction with JH being a gonadotropin and Vg being a yolk protein (Corona et al., 2007). In many insect species, JH and Vg are synergistically regulated (Comas, Piulachs, & Bellés, 1999; Handler & Postlethwait, 1978; Sheng, Xu, Bai, Zhu, & Palli, 2011). In contrast, the regulation of JH and Vg in eusocial honeybees, *Apis mellifera*, is antagonistic (Corona et al., 2007) and regulates caste differentiation and division of labour in honeybees. The same regulatory pattern in the interplay between JH and Vg was recently discovered in two subsocial species, the European earwig *Forficula*

auricularia and the burying beetle *Nicrophorus vespilloides* (Engel et al., 2016; M. Wu, J.-C. Walser, L. Sun, M. Kölliker, unpublished). These findings suggest that this pathway may be co-opted in posthatching parental care behaviours and in social evolution (Corona et al., 2007).

In vertebrates, oxytocin (OXT), vasopressin (AVP), their nonmammalian homologs mesotocin, isotocin (IT) and vasotocin, and dopamine and serotonin are key endocrine players in cooperative behaviour (Anacker & Beery, 2013; Ebstein, Israel, Chew, Zhong, & Knafo, 2010; Madden & Clutton-Brock, 1995; Soares et al., 2010). These hormones affect social affiliation (Reddon et al., 2015), social recognition and approach (Thompson & Walton, 2004), reward estimates (Messias, Paula, Grutter, Bshary, & Soares, 2016a), social learning (Messias, Santos, Pinto, & Soares, 2016b; Soares, Paula, & Bshary, 2016) and pair bonding (Insel & Shapiro, 1992). For example, in humans, OXT is suggested to favour trust and parochial cooperation (De Dreu, 2012), whereas AVP increased cooperative tendencies in reciprocal interactions (Rilling et al., 2012). Cooperation can also be enhanced or decreased by social stress and its underlying hormones (glucocorticoids, GCs). For example, in many social species, reproductive suppression of subordinate individuals is regulated by behaviours of dominant individuals that elicit higher levels of GCs in subordinates (Creel, Creel, & Monfort, 1996; Sanderson et al., 2015a).

The neuroendocrine pathways regulated by hormones appear critical for the evolution of cooperative behaviours in vertebrates (Donaldson & Young, 2008; Goodson, 2005, 2013; O'Connell & Hofmann, 2011a, 2011b; Soares et al., 2010), but the strength and direction of their regulatory effects depends upon the species, social context and sex. A recent comparison of brain gene expression of IT and AVT and their receptors between different social and nonsocial species pairs of cichlids revealed contrasting patterns (O'Connor, Marsh-Rollo, Ghio, Balshine, & Aubin-Horth, 2015). Furthermore, experimentally increased OXT (or its homolog IT) increased helping behaviours and decreased aggression in cooperatively breeding meerkats, *Suricata suricatta* (Madden & Clutton-Brock, 1995), and the sensitivity to social information in *N. pulcher* (Reddon, O'Connor, Marsh-Rollo, & Balshine, 2012), but it decreased sociability in this species (Reddon, Voisin, O'Connor, & Balshine, 2014). However, the direction of the effect of IT treatment depended on the pretreatment sociability in goldfish (Thompson & Walton, 2004), and OXT had no effect in house mice, *Mus musculus domesticus* (Harrison, Lopes, & König, 2016). In humans, experimentally administered OXT increased cooperation within groups, but

also enhanced competition between groups (De Dreu, 2012). Interestingly, these effects of OXT on social behaviour in humans have been demonstrated to differ between women and men (Gao et al., 2016).

The evolution of sociality from solitary ancestry and the evolution of cooperative from noncooperative behaviours requires the emergence of novel social traits (Taborsky & Taborsky, 2015). Genes present in solitary species could be co-opted towards social evolution. For example, Vg encodes the precursor of yolk protein (Corona et al., 2007). In subsocial European earwigs and burying beetles, Vg expression is associated with parental care (Roy-Zokan, Cunningham, Hebb, McKinney, & Moore, 2015; M. Wu, J.-C. Walser, L. Sun, M. Kölliker, unpublished), but in eusocial honey bee, it regulates division of labour and caste differentiation (Amdam, Norberg, Fondrk, & Page, 2004; Amdam, Norberg, Hagen, & Omholt, 2003). Another example is the *PebIII* gene which had a direct genetic effect on the metamorphosis of the solitary insect *Drosophila melanogaster* (Sabatier et al., 2003). In the subsocial European earwigs, this gene is coregulated and co-adapted between parent and offspring. RNAi knockdown of this gene showed an indirect genetic effect on offspring development and a direct genetic effect on maternal future reproduction in the earwigs (M. Wu, J.-C. Walser, L. Sun, M. Kölliker, unpublished). Potential neofunctionalization or subfunctionalization of this gene was found in the eusocial termite *Reticulitermes flavipes*, with differential expression of two transcripts of *PebIII* between reproductive castes (Steller, Kambhampati, & Caragea, 2010).

4 NEUROBIOLOGICAL MECHANISMS OF COOPERATION

Group-living animals often cooperate, as well as compete, with the same individuals multiple times over their lifespan. To assess the costs and benefits of social interactions, individuals need to continuously process social stimuli and keep track of past interactions. Responding to the multitude of daily social challenges encountered by social species requires behavioural flexibility and social competence (sensu Taborsky & Oliveira, 2012; Bshary & Oliveira, 2015). These complex social decisions require highly developed neuronal networks, which integrate many brain areas and populations of neurons (Platt, Seyfarth, & Cheney, 2016). For example, group size and the corresponding availability of social partners predict structural changes of the thickness of grey matter in multiple brain regions (Sallet et al., 2011). Group size also leads to functional change in terms of different co-activation of two brain regions, the superior temporal sulcus and the rostral prefrontal

cortex (Sallet et al., 2011). To understand how individuals make flexible social decisions while engaged in cooperative or competitive interactions, researchers often focused on species with more complex cognitive abilities such as humans or primates. However, recent work has highlighted that many physiological and neurological mechanisms are conserved across taxonomic groups (O'Connell & Hofmann, 2011b). Further, seemingly cognitively demanding abilities, such as individual recognition or keeping track of past interactions, might be the result of learning processes involving operant-conditioning rather than sophisticated cognitive mechanisms (Bshary, Zuberbühler, & van Schaik, 2016). Hormones and ontogeny can also affect the cognitive skills necessary for the ability to cooperate. We suggest these consist of three aspects: (i) event memory, (ii) synchrony with others and (iii) responsiveness to others. For example, zebra finches failed to sustain cooperation in a prisoner's dilemma task when their stress hormone levels were experimentally raised. These hormones reduce memory capacity required for reciprocity and remove the incentive for cooperation (Larose & Dubois, 2011). In addition, humans and many animals cooperate better when they are more receptive to social stimuli through synchronization in terms of personality, experience or hormonal physiology. For example, shared excitement synchronizes brain activity in humans to enable better cooperation in times of need (Nummenmaa et al., 2012).

It is now well established that two evolutionarily conserved neural circuits are fundamental in regulating social decision-making in vertebrates and are commonly referred to as the social decision-making network (SDMN) (O'Connell & Hofmann, 2011b). The SDMN is comprised of two neural circuits: the mesolimbic reward system, which evaluates the salience of external stimuli to generate an adaptive response, and the social behaviour network, which evaluates external stimuli (Goodson, 2005). Only the interconnected activity of both systems enables animals to regulate and implement adaptive behavioural outputs in response to environmental challenges and opportunities. Many hormones that influence key aspects of cooperative behaviour, such as OXT, AVP, dopamine or serotonin, are part of the SDMN. However, even though the SDMN is doubtlessly an important player in social behaviour, it remains an open question whether cooperative behaviour itself is regulated by the SDMN.

5 DEVELOPMENTAL REGULATION OF SOCIALITY/COOPERATIVE BEHAVIOUR

Modes of development can have a huge impact on the evolution of early phenotypic specialization vs.

extended phenotypic plasticity (English, Browning, & Raihani, 2015a). Invertebrates, and in particular eusocial insects, are more prone to early developmental specialization because they have to commit to the development of a certain phenotype before metamorphosis (Wilson, 1971). Most social insects show a strict behavioural and morphological caste differentiation determined by different developmental trajectories, which leads to a division of labour in insect colonies (Wilson, 1971). Arguably, the most famous example is the development of queens in honeybees induced by the ingestion of royal jelly (Kaftanoglu, Linksvayer, & Page, 2011). Early caste determination is a common phenomenon in most eusocial insects where nutrition and inhibitory pheromones play an important role (Schwander, Lo, Beekman, Oldroyd, & Keller, 2010). There are, however, a number of social insect species that are cooperative breeders without morphological specializations, which can switch between the role of subordinates and dominants within a lifetime (Field & Leadbeater, 2016; Gadagkar, 2016).

In contrast to many social insects, most social vertebrates remain morphologically and behaviourally flexible throughout their life. For example, dominant breeders and subordinate group members in cooperatively breeding vertebrates maintain their full reproductive capacity (Bell, Nichols, Gilchrist, Cant, & Hodge, 2012; Bruintjes, Bonfils, Heg, & Taborsky, 2011), but can adapt their social roles and behaviours contingent on the social context and environmental conditions (Bruintjes & Taborsky, 2011). Therefore, most social vertebrates do not develop morphological specializations based on their social rank or role (Carter, English, & Clutton-Brock, 2014; Huchard et al., 2014; Sanderson et al., 2015b; Taborsky et al., 2015; Zöttl et al., 2016; but see Jarvis, 1981; Fischer, Bessert-Nettelbeck, Kotrschal, & Taborsky, 2015). Nevertheless, early behavioural specialization might be beneficial, for instance, when deciding if and when to disperse (Fischer, 2014; Zöttl, Chapuis, Freiburghaus, & Taborsky, 2013), if and when to challenge the dominant individual in the home territory (Sharp & Clutton-Brock, 2011), and whether to rear offspring communally or solitarily (Manning, Dewsbury, Wakeland, & Potts, 1995). All of these decisions require specific behavioural repertoires. Bolder, more risk-prone phenotypes are more successful dispersers (Chapman et al., 2011) while larger individuals with superior fighting abilities are better able to challenge dominants for territory takeovers (Huchard, English, Bell, Thavarajah, & Clutton-Brock, 2016). A communal nest requires individuals to express prosocial behaviours towards breeding partners and foreign young (Dugdale, Ellwood, & Macdonald, 2010; Weidt, Hofmann, & König, 2008; Weidt, Lindholm, & König, 2014). Social behaviour can be costly (Cram, Blount, & Young, 2015; Grantner & Taborsky, 1998), and misdirected behaviours may have high fitness costs and can lead to evictions from the group (Bell et al., 2012), infanticide (Schmidt

et al., 2015) and even to fatal conflicts (Enquist & Leimar, 1990). Thus, environmentally induced developmental programming of behavioural strategies, for example, via parental effects or own early experience, might be also important in cooperatively breeding vertebrates.

The cues responsible for early phenotypic specialization are diverse and can induce phenotypic specializations between and within social groups. For example, intragroup caste specialization is dependent on group size (Ferguson-Gow, Sumner, Bourke, & Jones, 2014) or the level of competition between nests (Passera, Roncin, Kaufmann, & Keller, 1996) in ant species. In cooperatively breeding vertebrates, group size can influence maternal investment in eggs. Smaller eggs are produced when more helpers are available to compensate for the reduced maternal investment in individual eggs (Russell et al., 2007; Taborsky et al., 2007). In turn, offspring developing in larger groups may express different behavioural phenotypes than offspring from small groups as a result of developmental plasticity (Fischer et al., 2015). Within-group factors such as the provision of more or better food to particular group members can lead to divergent behavioural phenotypes such as the development of different caste phenotypes in social insect societies (Schwander et al., 2010) or different degrees of competitiveness in some vertebrates (Buston, 2003; Heg, Bender, & Hamilton, 2004; Huchard et al., 2016).

A second important role of developmental plasticity in social organization is the regulation of conflict within groups. The level of conflict in cooperative societies is particularly high when subordinates are fertile and therefore have a vested interest their own breeding opportunities. Subordinates queuing for a dominant position may compete with other subordinate group members about the position in the social hierarchy (Huchard et al., 2016). If access to reproductive opportunities is strongly skewed towards a few dominant individuals, conflicts over reproduction can also arise between dominant breeders and maturing subordinates (Heg et al. 2004). As social rank is often size-dependent, developmental plasticity of growth strategies may play a key role in either reducing or enhancing conflict. In response to social cues obtained from other group members, growth may be strategically enhanced to outcompete rivals or reduced to lower potential conflict with dominant group members. In her talk "Measuring cooperation and associated phenotypes in the field: developmental trajectories and genetic basis," Elise Huchard showed that in cooperatively breeding meerkats, growth rates remain flexible throughout the entire ontogeny (Huchard et al., 2014). In this species, rank position depends on size

and age, and subordinate females queue for the position of the dominant female, which is usually the oldest and heaviest female of the group. When Huchard and colleagues (Huchard et al., 2016) experimentally increased the growth rate of a subordinate by supplemental feeding, same-sex rivals responded by accelerating their own growth and food uptake. Conversely, subordinates of the cooperatively breeding cichlid fish, *N. pulcher*, inhibit their growth if their size difference to the same-sex dominant breeder becomes too small, as subordinates reaching body sizes too close to that of dominants risk expulsion from the group (Heg et al. 2004).

Finally, developmental processes may mediate conflict between dominant breeders and their offspring and future helpers or workers. In cooperative societies, not only are offspring dependent on care, but become carers themselves later in ontogeny. The optimal contribution to alloparental care required by dominant breeders vs. the optimal contribution subordinate helpers are willing to give may diverge and depend on the options for dispersal and independent breeding by subordinates (Russell & Lummaa, 2009). For instance, in his talk "Hormonal signals, epigenetic regulation, maternal effects, and their consequences for cooperation and conflict," Nikolaus von Engelhardt suggested that breeding females endow eggs with hormones or RNA transcripts, which might influence growth and behavioural propensities of offspring in a way that optimizes maternal fitness. These maternal effects may then influence the offspring's future willingness to contribute to alloparental care of younger broods. At the prenatal stage, offspring depend on parental cues to adjust their development, as they do not directly experience their environment. However, offspring may use cues obtained postnatally to "disagree" with the maternal programme and reverse their behavioural tendencies (Fischer et al., 2015).

Because of the important role of developmental plasticity in the regulation of cooperative behaviours, it is conceivable that in the course of the evolution of cooperation, environmentally induced phenotypic plasticity precedes, or even facilitates, genetic adaptation (known as the "plasticity-first hypothesis" West-Eberhard, 2003; see Levis & Pfennig, 2016 for a review). In a first step, plasticity enables a rapid adaptive response to changing environments through phenotypic accommodation. In a second step, genetic accommodation allows for the relatively slow refinement of genotypes by accumulating beneficial genetic mutations. This, together with the co-option of genes as discussed in previous sections, could provide an answer to the long-standing question of how novel cooperative traits emerge when cooperative species evolve from noncooperative ancestors. As the underlying genetic architecture

of cooperative behaviour is arguably complex and polygenic, genetic adaptation alone is unlikely to account for these relatively fast transitions. Thus, phenotypic plasticity that precedes genetic adaptation as described above might offer another explanation for the fast emergence of cooperative traits.

6 OUTLOOK

In previous sections, we discussed ways in which cooperative behaviour can be transmitted from one generation to the next, either genetically, through heritable epigenetic changes, or through social learning and culture. We also outlined reasons why the evolutionary dynamics of cooperative traits might be less straightforward than generally assumed. Following Anna Lindholm's talk and the ensuing discussion, we now focus on practical considerations and we provide promising avenues for future research in the genetics and development of cooperation.

6.1 Which systems are suitable?

The suitability of a system will ultimately depend on the exact question under investigation. In general, information on individuals is required for quantitative genetic approaches and desirable for molecular genetic approaches. Some taxa show a naturally occurring array of closely related species with a range of cooperative social behaviours. For example, Hymenoptera display a wide cooperative continuum from solitary to subsocial to eusocial species (Wilson, 1971), and species of the teleost family Cichlidae represent a wide range of social systems from nonsocial to highly social (Heg & Bachar, 2006; Taborsky, 1994). The parasitoid bethylid wasps presented by Ian Hardy at the workshop provides an excellent example of a tractable social study system. In one of these species, *Sclerodermus harmandi*, multiple unrelated foundresses cooperatively rear each other's offspring on a single host resource (Kapranas, Hardy, Tang, Gardner, & Li, 2016). There is a broad scope for experimental manipulation of resource size, relatedness, foundress number, and offspring survival in bethylid wasps (e.g., Sreenivas & Hardy, 2016). The quasisocial nature of this species makes it a particularly suitable candidate for the study of cooperative behaviours in insects at the threshold of the evolution of complex sociality.

Comparisons between the genomes and transcriptomes of species along the continuum of sociality can indicate likely genes and pathways for further investigation (Kapheim, 2016; Rehan & Toth, 2015; Robinson, Grozinger, & Whitfield, 2005; Toth & Rehan, 2017; Trapp, McAfee, & Foster, 2016).

Comparisons within species are also useful to examine possible molecular causes of phenotypic variance. Systems in which individuals differ in their tendency to cooperate or cheat in social situations (Santorelli et al. 2008), or in the amount of alloparental care to provide (Fischer, 2014; Kasper et al., 2017), are particularly well suited to studies of the underlying genetic architecture or gene expression patterns on the basis of cooperative phenotypes. Furthermore, the evolution of cooperative behaviours might not only depend on interactions within, but also between species (West, Griffin, & Gardner, 2007) or between different organizational levels of sociality (West & Gardner, 2013). We provide an example for multilevel cooperation, namely between microbiota and their host, in the Supporting Information.

6.2 Which specific traits and parameters should be measured?

It is of paramount importance to understand the biology of a system well enough in order to be able to accurately quantify fitness, and to decide which traits to measure. It is especially important to carefully consider if the phenotype measured is indeed a target of selection. In some instances, it might be better to measure the underlying mechanism, for instance an individual's physiology or cognitive ability, instead of the behavioural phenotype (behavioural gambit, Fawcett et al., 2013). Moreover, the interaction coefficient ψ could itself be considered a trait that varies genetically between individuals and is thus subject to selection (Bleakley & Brodie, 2009) and of particular importance for the evolution of cooperative traits. For instance, ψ can be estimated empirically as the partial regression coefficient of a phenotype on its partner's phenotype while keeping the direct genetic influence constant. However, this requires isogenic lines or large‐scale breeding designs with repeated measures of the same genotype with different social partners. Measuring individual‐level phenotypic proxies could provide a more feasible approach for vertebrates, assuming a close phenotype–genotype resemblance (Edenbrow et al., 2017). Those proxies could be estimates of the extent to which traits covary between interaction partners, for example, spatial proximity.

6.3 What type of approach should be followed?

Ideally, questions about the genetic basis of cooperative traits should combine both field observations and controlled laboratory studies. Moreover, insights gained from theoretical modelling of mechanisms underlying cooperation (see Supporting Information) and quantitative genetic modelling, for instance indirect genetic effects, should be considered. While the study of wild populations provides a more

realistic picture of selective pressures in nature, a laboratory setting allows for easier control of confounding nongenetic effects (e.g., parental or other transgenerational effects) that potentially distort estimates of heritability (Kasper et al., 2017). Ideally, field studies should use cross-fostering techniques to account for and estimate those effects (Hadfield, Heap, Bayer, Mittell, & Crouch, 2013). Likewise, laboratory experiments should use offspring of wild-caught individuals to preserve natural patterns and breadth of genetic variation within the population and avoid artefacts due to genetic drift or laboratory-specific selection. Furthermore, studying individuals in highly artificial test settings that do not properly reflect the actual biology of a species could lead to ecologically or evolutionarily meaningless results. This caveat is corroborated by recent studies that found an effect of laboratory rearing on gene expression, physiology, behaviour and social dynamics in paper wasps *Polistes fuscatus* (Jandt, Thomson, Geffre, & Toth, 2015) and an effect of the laboratory environment on prosocial behaviour of chimpanzees (Tennie, Jensen, & Call, 2016).

6.4 Which methods should be employed?

As with selection of study species, approach, trait and setting, the most appropriate experimental method depends on the questions being asked. Quantitative genetic methods provide insight on the relative proportions of heritable and several types of environmental variance of cooperative traits and their covariance with other traits, and thus on the inheritance and genetic architecture of a cooperative trait. Combined with selection experiments, they can be used to predict how traits respond to selection. This could be followed up by quantitative trait locus or genomewide association study approaches to search for candidate genetic polymorphisms that are responsible for phenotypic differences in cooperative tendency. Recent association studies in humans have shown that particular genotypes for the oxytocin receptor (OXTR) gene were highly associated with Asperger syndrome, a type of autism (Di Napoli et al. 2014). Particular genotypes may also be associated with OXTR and social empathy as measured through cooperative games (Thompson et al. 2013). Several new technologies are available for the manipulation of gene expression at the transcriptomic level (e.g., RNA interference, Castel & Martienssen, 2013), or by altering genes at the DNA level (e.g., gene editing via CRISPR-Cas, Hsu, Lander, & Zhang, 2014). These approaches could be employed to verify and validate candidate genes once identified by the above approaches. Future studies should incorporate new technologies for detecting genetic and epigenetic signatures of cooperation. For example, comparing genomes between closely related species exhibiting a continuum from solitary lifestyle to advanced sociality may provide

insights into the genomic structure underlying cooperation and the evolution of sociality along phylogenetic trees (Fischman, Woodard, & Robinson, 2011; Kapheim et al., 2015). Furthermore, exploring correlations of epigenetic marks with phenotypic variation in cooperativeness may provide insight into how gene expression is regulated in response to environmental factors (Jensen, 2015; Li-Byarlay, 2016). Investigating the stability of those epigenetic marks over time can shed light on the molecular pathways connecting previous social experience to future cooperative behaviour (Cardoso, Teles, & Oliveira, 2015; Shpigler et al., 2017). In conclusion, we advocate a holistic approach that integrates complementary methods to unravel the proximate and ultimate causation of cooperation and social evolution, including comparative phenotypic and genomic approaches to tackle questions of adaptation and convergent evolution, the study of norms of reaction and shifts in gene regulatory networks to appreciate the role of phenotypic plasticity, and the study of interactions between individuals and their social and physical environment to unravel the role of natural selection.

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AUTHOR CONTRIBUTION

C.K. and M.V. organized the workshop and led the writing of the manuscript. All authors contributed original ideas and helped draft the manuscript

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