

Loss of lipid synthesis as an evolutionary consequence of a parasitic lifestyle

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Evolutionary loss of traits can result from negative selection on a specific phenotype, or if the trait is selectively neutral, because the phenotype associated with the trait has become redundant. Even essential traits may be lost, however, if the resulting phenotypic deficiencies can be compensated for by the environment or a symbiotic partner. Here we demonstrate that loss of an essential metabolic trait in parasitic wasps has evolved through environmental compensation. We tested 24 species for the ability to synthesize lipids de novo and collected additional data from the literature. We found the majority of adult parasitoid species to be incapable of synthesizing lipids, and phylogenetic analyses showed that the evolution of lack of lipogenesis is concurrent with that of parasitism in insects. Exploitive host manipulation, in which the host is forced to synthesize lipids to the benefit of the parasitoid, presumably facilitates loss of lipogenesis through environmental compensation. Lipogenesis re-evolved in a small number of parasitoid species, particularly host generalists. The wide range of host species in which generalists are able to develop may impede effective host manipulation and could have resulted in regaining of lipogenic ability in generalist parasitoids. As trait loss through environmental compensation is unnoticed at the phenotypic level, it may be more common than currently anticipated, especially in species involved in intricate symbiotic relationships with other species.

coevolution | environmental compensation | fat reserves | host manipulation | parasitoids

Evolutionary changes are frequently associated with the acquisition of novel traits, but loss of traits can also curb the course of evolution (1, 2). Trait loss can be the result of negative selection for a specific phenotype, for example to reduce costs associated with the trait (3). Alternatively, a trait may become selectively neutral due to ecological or evolutionary shifts which render the phenotype associated with the trait redundant. Well-known examples are the loss of eyes in cave-dwelling organisms (4) and the loss of wing function in birds and insects (5). In these examples, respectively, vision and ability for flight have become redundant, resulting in mutation accumulation and loss of function.

The evolutionary loss of a trait, however, is not inevitably accompanied by a loss of the phenotype associated with that trait. If the loss of function is compensated by environmental or biotic factors, the phenotype will be maintained. For instance, several plant species have lost the ability for photosynthesis (6), although the photosynthetic requirements are met through exploitation of other plants. Similarly, despite the loss of vitamin C production in humans, vitamin C is still an essential vitamin for human health but it is provided through dietary intake (7, 8). Environmental compensation can therefore release traits from selection because the phenotype is not affected by loss of such traits. Moreover, if a trait is energetically costly, environmental compensation can even result in selection against expression of this trait, because reduced expression will save energetic expenditure.

Environmental compensation of trait loss is frequently observed in species that are involved in symbiotic, coevolutionary relation-

ships, such as occurs in instances of mutualism or parasitism. The supply of essential resources by a symbiotic partner makes the production of such resources superfluous in the receiving organism, and renders the genes involved prone to mutation accumulation. As a consequence, coevolution may lead to a loss of genes that is unnoticed at the phenotypic level. To illustrate this point, many obligate endosymbionts such as *Buchnera* and *Baumannia* have undergone massive genome reduction, although which genes are lost depends on their specific host (9, 10). Other relevant examples include the parasitic fungi, which have lost several genes involved in metabolism (11). Trait loss or a reduction in trait functioning is only expected to evolve in long-term, stable coevolutionary physiological relationships. However, the relative frequent occurrence of such tight interspecific interactions implies that environmental compensation for trait loss may be much more prevalent than currently appreciated (12–14).

Here we unravel a case of parallel evolution of compensated trait loss in insect parasitoids. The exceptional lifestyle of parasitoids provides unique opportunities to study environmental compensation for trait loss (15). Parasitoids develop in or on arthropod hosts during the larval stage; the larvae are therefore completely dependent on their host for nutrient acquisition (16). Various ways have evolved in which parasitoid larvae can manipulate their host's physiology to increase nutrient availability, including host exploitation for lipids (17, 18). For example, parasitism by the hymenopteran *Euplectrus separatae* results in a release of fat particles from the host's fat body and an increase in hemolymph free fatty acids of the host (19). Direct uptake of lipids from the host tissue is highly advantageous for parasitoid larvae because they can avoid substantial metabolic costs that are associated with lipogenesis (20). In addition, it has been shown that some parasitoid species are unable to accumulate lipids as adults due to the absence of de novo lipid synthesis (21, 22), even under conditions that would induce enhanced lipogenesis in other animals (23).

We propose that the loss of lipogenesis is an evolutionary consequence of the parasitoid lifestyle, because parasitism facilitates redundancy of traits that are involved in lipid production. So far, lack of adult lipid accumulation has been found in two parasitic dipterans and nine closely related parasitic hymenopterans (22). To demonstrate phylogenetic congruence between loss of lipogenesis and a parasitoid lifestyle, data are needed from multiple independent phylogenetic groups. We used a two-pronged approach to obtain these data. First, we performed an exhaustive survey of the literature and acquired data on lipogenic ability of 70

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species. Second, we tested an additional 24 species for their lipogenic ability by conducting feeding experiments and physiological measurements. The final dataset included almost 30 parasitoid species from three different orders, which enabled us to answer the question of whether the evolution of parasitism results in loss of lipogenic ability in insects. Furthermore, we tested for correlated evolution between lipogenic ability and key parasitoid traits associated with the parasitoid lifestyle.

Results

Feeding Experiments and Lipogenic Ability. The lipogenic ability of species was determined by comparing the lipid levels of individuals at emergence and after several days of feeding. Of the 24 species tested, 18 were demonstrated to lack lipid accumulation in a situation of excess food. The staphylinid beetle *Aleochara bilineata* (Coleoptera) and the rhinophorid fly *Paykullia maculata* (Diptera) showed a significant decrease in lipid levels between newly emerged individuals and fully fed individuals, which is indicative of lack of lipogenesis (Fig. 1 and Table S1). Furthermore, nearly all hymenopteran parasitoids tested lacked lipid accumulation. Individuals in the feeding treatments either had decreased lipid levels or failed to increase their lipid levels, despite the fact that they had access to a surplus of carbohydrates. Only five parasitoid species proved an exception to this pattern: *Gelis agilis*, *Lysibia nana*, *Acrolyta nens*, *Pteromalus puparum*, and *Leptopilina heterotoma* were shown to significantly increase their lipid levels during their lifetimes when fed. The only nonparasitoid species in our feeding experiment was the symphytan *Athalia rosae*, belonging to the most basal lineage within Hy-

menoptera. This species significantly increased its lipid levels when fed (Fig. 1 and Table S1).

Evolution of Lack of Lipid Accumulation in Relation to Parasitism. Our literature survey and feeding experiments resulted in data on the lipogenic ability of 94 species. Twenty-six species exhibited a lack of lipid accumulation, including 1 coleopteran parasitoid, 3 dipteran parasitoids, and 20 hymenopteran parasitoids. All other, nonparasitoid hymenopteran species showed an increase in lipid levels when fed, with the exception of the gall wasp *Diplolepis rosae* and its inquiline *Periclistus brandtii* (Fig. 1 and Table S1). Phylogenetic analysis of lipogenic ability is complicated by uncertainties in the hymenopteran phylogeny (24, 25). We therefore consider three possible consensus trees, but the conclusions of our analyses are qualitatively the same for all trees. The parasitoid lifestyle can be inferred to have evolved independently four times and lost twice when lack of lipogenesis is considered the ancestral state at the root of the tree (for the consensus trees in which Ichneumonoidea and Aculeata, as well as Proctotrupomorpha and Aculeata, are considered sister groups). The evolution of lack of lipid accumulation is concurrent with that of parasitism at least one time in Coleoptera, two times in Diptera, and one time in Hymenoptera, demonstrating that lack of lipid accumulation has evolved four times independently in parasitoid lineages throughout the insects. Lack of lipogenesis is significantly more likely to originate on parasitic than on nonparasitic branches in the phylogeny (concentrated changes test, $P < 0.001$). One of the consensus trees, in which Ichneumonoidea and Proctotrupomorpha are considered sister groups, shows the ancestral state at the root of the hymenopteran tree to be equivocal. The ancestral state could be either nonparasitic or parasitic and lipogenic ability could be absent or present (Fig. 2). We compared all possible combinations of character evolution and found lack of lipogenesis to have evolved concurrently with parasitism three, four, or five times. Each comparison showed concurrent evolution between lack of lipogenesis and parasitism to be significant (concentrated changes test, $P < 0.05$ for each comparison using Benjamini and Hochberg's (26) false discovery rate as a correction for multiple testing).

Evolution of Lipogenesis in Relation to Parasitoid Traits. Not all parasitoid species showed a lack of lipogenic ability. Within the parasitic Hymenoptera, five parasitoid species were found to be capable of lipogenesis (Fig. 1 and Table S1), showing an exception to the general pattern. Phylogenetic reconstruction of lipogenic ability in insects suggested the ability for lipogenesis in these five species to be a secondarily derived character rather than an ancestral trait, suggesting they have regained lipogenic ability (Fig. 2). To identify possible selection pressures that may have caused a reversal to lipogenic ability, we analyzed the conditional probability of reversed lipogenesis for several parasitoid traits. For each of the consensus trees, as shown in Table 1, no significant relationship was found with developmental characters (mode of parasitism, host stage attacked, host developmental arrest, and facultative hyperparasitism), nor with adult diet (host-feeding behavior) when lack of lipogenesis is the ancestral state at the root of the tree. Reversals from lack of lipogenesis to lipogenesis are not significantly associated with these parasitoid traits. When Ichneumonoidea and Proctotrupomorpha are considered sister groups, lipogenesis could have been the ancestral state at the root of the tree. In that case, similar results are obtained, except for pupal host stage attacked and facultative hyperparasitism, which are significantly correlated with the ability for lipogenesis (Table 1). In contrast, reversals to lipogenesis were concentrated to lineages with a generalist host species range, that is, which attacked 10 or more host species (Fig. 3) when either lack of lipogenesis or lipogenesis are considered the ancestral state. Parasitoids attacking many different host species seem to be capable of lipogenesis, as opposed to more specialist parasitoids, attacking relatively few

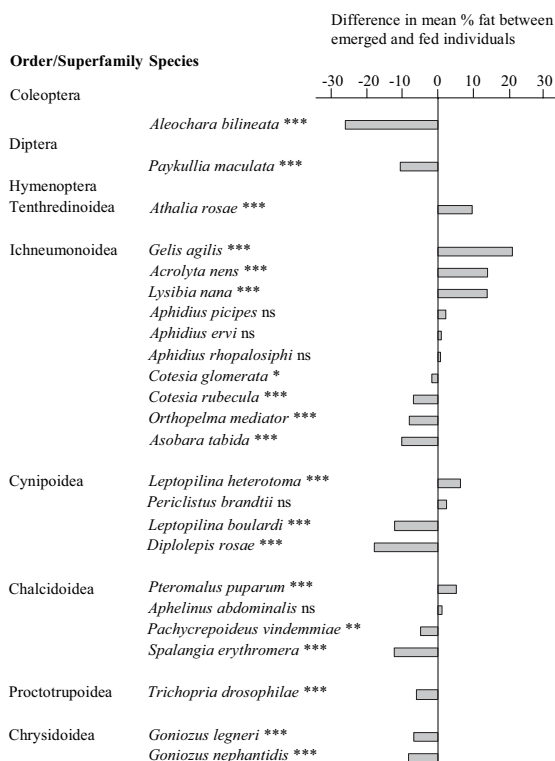


Fig. 1. Difference in mean lipid content (%) between emerged and fed individuals as measured in the feeding experiments. Asterisks indicate significant differences between emerged and fed individuals: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant. Sample sizes for each treatment and species are listed in Table S1.

Ichneumonidae and Proctotrupomorpha as sister groups

Results for the other two consensus trees are in agreement with the results obtained if lack of lipogenesis is ancestral.

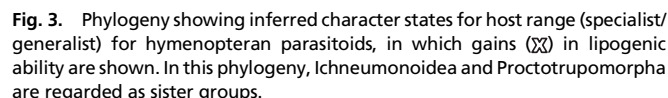
[†]Significant differences after correction for multiple testing using Benjamini and Hochberg's false discovery rate.

Larval Exploitation and Host Range. Adaptation by parasitoids to their host has resulted in highly specific mechanisms to increase lipid levels and optimize host exploitation (19, 38, 39). In agreement with the specialized nature of host–parasitoid interactions, our phylogenetic analysis indicates that breadth of host range is a key trait in the evolution of lipogenic abilities. Lack of lipogenesis is predominantly found in specialist parasitoids, whereas

Nonetheless, some generalist parasitoids were found incapable of synthesizing lipids as adults, such as *Nasonia vitripennis* and *Pachycrepoideus vindemmiae*. In *N. vitripennis*, it has been shown that although this species can develop on over 60 different host species, it has a preference for sarcophagous flies (40). From this perspective, it is a specialist because the injection of venom during oviposition can only increase lipid levels in *Sarcophaga* species (38). A certain level of specialism, in which parasitoids have adapted to specific hosts to manipulate their physiology, is thus required to obtain sufficient resources during development.

Toward Further Understanding of the Evolutionary Loss of Traits. The evolutionary loss of lipogenesis in parasitic insects is one of few examples of repeated trait loss through environmental compensation (6–8). This study demonstrates that compensated trait loss of physiological traits can be masked by parasitoid–host interactions. We found that species with superficially similar developmental strategies can vary considerably in physiological dependence on their host. Closer examination of other stable coevolutionary relationships can be expected to yield more such examples.

Parallel evolution of traits might be the result of quite different underlying genetic mechanisms. For example, similar coat patterns and pigmentation in mice species have been shown to have evolved through different genetic mechanisms (44). Similar to the evolution of novel traits, parallel trait loss can also be expected



to occur through various genetic mechanisms. Further genome analysis is needed to assess whether specific genes have been preferred targets of mutation accumulation. A prime candidate to explain lack of lipogenesis would be fatty acid synthase (FAS), which has a central role in the production of lipids. FAS has previously been documented to be absent in the genome of a parasitic fungus that is dependent on external lipids for growth (11). Recent completion of the genome of the parasitoid *N. vitripennis* (45) has revealed a homolog to the FAS gene of *Drosophila*, although its functionality remains to be tested.

A fundamental question regarding the evolutionary loss of traits is whether physiological abilities that have been lost can be regained. Indeed, a striking finding is that lipogenesis has re-evolved three times within Hymenoptera, a fact which challenges several widespread views regarding trait loss. Trait loss is usually thought to involve one-way directional evolution, in which a lost trait does not re-evolve (46). However, exceptions to this general rule have been found (47, 48), and lipogenic ability may be one of them. We propose that after the loss of lipogenic ability much of the lipogenic pathway may remain intact, perhaps due to pleiotropic effects on other physiological pathways. Conversion of carbohydrates to triglycerides employs key enzymes that are functional in other physiological processes, such as pyruvate metabolism, the citrate cycle, and biosynthesis of secondary metabolites (49). The lipogenesis pathway may be protected from severe degradation due to shared components with essential metabolic pathways, hence enabling a fully functional lipid synthesis to re-evolve. It is essential to identify the underlying genetic and physiological mechanisms involved to understand the evolutionary dynamics of the loss of this trait.

Materials and Methods

Insects. All insects were obtained from existing laboratory cultures or field collections, either as larvae and pupae or inside their host. The following species were obtained from existing cultures: *Cotesia glomerata*, *P. puparum*, *A. nens*, *L. nana*, and *G. agilis* (NIOO-KNAW, The Netherlands), *Cotesia rubecula* (University of Wageningen, The Netherlands), *Leptopilina boulardi* (University of Leiden, The Netherlands), *Aphidius ervi* and *Aphelinus abdominalis* (Koppert BV, The Netherlands), *A. rosae* (University of Bielefeld, Germany), *P. vindemmiae* (University of Lyon 1, France), *A. bilineata*, *Aphidius rhopalosiphii*, *Aphidius picipes*, *Leptopilina heterotoma*, *Asobara tabida*, *Spalangia erythromera*, and *Trichopria drosophilae* (University of Rennes 1, France), and *Goniozus nephantidis* and *Goniozus legneri* (University of Nottingham, United Kingdom). Galls containing *D. rosae*, *Orthopelma mediator*, and *P. brandtii* were collected at several sites near Wassenaar (The Netherlands) in September and October 2007. *Porcellio scaber*, hosts of *P. maculata*, were collected at several sites in the provinces of North and South Holland (The Netherlands) during January, February, and March 2008. All insects were kept at 20 °C, relative humidity (RH) 75%, and L:D 12:12, except *A. ervi* and *P. puparum*, which were kept at RH 50%, and *C. glomerata*, *G. agilis*, *L. nana*, *A. nens*, *L. boulardi*, *P. vindemmiae*, and *C. rubecula*, which were kept at 23 °C, RH 75%, and L:D 12:12. Galls containing *D. rosae*, *O. mediator*, and *P. brandtii* were kept subsequently at 20 °C, 10 °C, 5 °C, 10 °C, and 20 °C until emergence to mimic temperature conditions in their natural environment. Vials and galls were inspected daily for newly emerged females.

Feeding Experiments. To determine lipogenic ability, newly emerged females were randomly assigned to two treatments: emergence, in which females were frozen directly after emergence, or fed, in which individuals were allowed to feed on honey ad libitum for approximately half their average lifespan. During the experiments only adult females were used and were provided with water on cotton wool in addition to honey. An exception is *A. bilineata*, for which sex could not be determined and both sexes were used in experiments. *A. bilineata* was, furthermore, allowed to feed on *Drosophila* food medium, as this species is predaceous rather than nectivorous. Medium consisted of 20 g agar, 50 g saccharose, 35 g yeast, 9 g kalmus (10 parts acidum tartaricum, 4 parts ammonium sulfate, 3 parts potassium phosphate, and 1 part magnesium sulfate), and 10 mL nipagin (100 g 4-methyl hydroxyl benzoate per L of ethanol) per L of water. At the end of the experiment, females were frozen at −20 °C until further processing.

Fat content was determined based on the method by David et al. (50). Whole insects were freeze-dried for 2 days, after which dry weight was determined. Individuals were subsequently placed in a glass tube containing 4 mL of ether.

After 24 h, the ether was removed and insects were washed with fresh ether. Insects were freeze-dried for 2 days after ether extraction and dry weight was determined again. For *A. bilineata*, *P. maculata*, *D. rosae*, *O. mediator*, *P. brandtii*, and *A. rosae*, freeze-drying was extended to 4 days and the quantity of ether was 8 mL because of their increased cuticle toughness and larger size. Fat content was calculated by subtracting dry weight after ether extraction from dry weight before ether extraction, and then converted to the percentage of lipids to correct for differences in body size among individuals.

Reconstruction of Phylogenetic Relationships. Relationships between insect orders within the Hemimetabola are based on morphological data according to Kristensen (51), and the phylogeny of the endopterygotes was completed with molecular data on relationships between Holometabola, as reported in Castro and Downton (52). New insights suggest an alternative placement of Hymenoptera within the Holometabola, but vary considerably between studies (53–55). The phylogeny used in this study has been constructed with regard to the more conventional placement of Hymenoptera as a sister group of Mecoptera (Diptera + Lepidoptera). However, alternative placement of Hymenoptera does not affect our findings. Inference of phylogenetic relationships at the superfamily, family, and genus level has been made using available morphological and molecular data (SI Text). The enormous diversity of hymenopteran parasitoids poses difficulties in determining phylogenetic relationships, mainly at the superfamily level. Consensus between phylogenies based on morphology and molecular data has not yet been reached satisfyingly, and relationships among hymenopteran superfamilies are thus regarded as polytomous in this study. Within Hymenoptera, data on lipogenic ability have been obtained for parasitoids of the superfamilies Proctotrupoidea, Cynipoidea and Chalcidoidea (Proctotrupomorpha), and Ichneumonoidea and Chrysidoidea (Aculeata) (22, 56, 57). As the analysis does not allow polytomies, three alternative consensus trees were used for phylogenetic testing within Hymenoptera. The three trees assumed Ichneumonoidea and Aculeata, Proctotrupomorpha and Aculeata, or Ichneumonoidea and Proctotrupomorpha to be sister groups, respectively.

Statistical Analysis. Scatterplots and regression lines of dry weight before ether extraction compared with dry weight after ether extraction were used to find potential outliers in the data. Outliers were only removed if they deviated from a 99% confidence interval of the linear regression line. Normality of the data was determined by looking at the error structure of the residuals of the data. Nonnormal data were transformed to normality using a log or square-root transformation. Treatments showing normally distributed data were compared using independent sample t test if variances were equal and Welch's t test if variances were unequal. If data were not normally distributed, the nonparametric Kruskal–Wallis test was used. Statistical analyses were done using R project Version 2.9 (The R Foundation).

Phylogenetic tests were performed with each of the three consensus trees using all 94 insect species as a target clade. Lipogenic ability and parasitic lifestyle were scored as presence/absence characters. To test for correlations between parasitic lifestyle (independent character) and lipogenic ability (dependent character), ancestral states were reconstructed using parsimony for the three consensus trees. Concentrated changes tests (58) using minstate and maxstate simulations with a sample size of 10,000 were carried out using MacClade Version 4.08 (59).

Correlated evolution between parasitoid traits (independent characters) and lipogenic ability (dependent character) was tested using a phylogeny consisting only of parasitic Hymenoptera. Data on the presence or absence of parasitoid traits were obtained from the literature and each character was scored binomially, that is, ectoparasitoid or endoparasitoid (0/1), idiobiont or koinobiont (0/1), other host stage attacked or larval host stage attacked (0/1), pupal host stage attacked or other host stage attacked (0/1), other host stage attacked or adult host stage attacked (0/1), solitary or gregarious (0/1), primary or hyper and facultative hyperparasitoid (0/1), non-host-feeding or host-feeding (0/1), pro-ovigenic or synovigenic (0/1), and specialist or generalist (0/1). As clear definitions of generalism and specialism are lacking for hymenopteran parasitoids, we used two criteria based on Stireman (60): The first criterion assumes generalists to attack 5 or more host families and specialists 4 or fewer host families. The second criterion assumes generalists to attack 10 or more host species and specialists 9 or fewer host species. When reconstructing generalism and specialism, the ancestral state of the clade comprising *A. nens*, *L. nana*, *G. agilis*, and *P. turionellae* was equivocal. This has been resolved to generalism being the ancestral state of this clade, as the majority of species within the subfamily Cryptinae are known to be generalists (61, 62). Ancestral states were reconstructed using parsimony and analyses were carried out using concentrated changes tests with exact count.

For several trait states the sample sizes were low. A power analysis showed that even if maximal concurrence between trait state and lipogenic ability was assumed, a lack of significance was found; hence, for these traits, the power of the analyses was too low and they were excluded from the analyses. These trait states included endoparasitism, koinobiosis, larval host stage attacked, solitary, primary, synovigenic, and specialism. Concentrated changes tests were not performed if fewer than two gains occurred on branches of the trait state in question, which was the case for adult host stage attacked and gregarious and pro-ovigenic trait states.

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