Commensal bacteria differentially shape the nutritional requirements of *Drosophila* during juvenile growth

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Abstract

The interplay between nutrition and the microbial communities colonizing the gastro-intestinal tract (i.e. gut microbiota) determines juvenile growth trajectory. Nutritional deficiencies trigger developmental delays, and an immature gut microbiota is a hallmark of pathologies related to childhood undernutrition. However, how commensal bacteria modulate the impact of nutrition on juvenile growth remains elusive. Here, using gnotobiotic *Drosophila melanogaster* larvae independently associated with two model commensal bacterial strains, *Acetobacter pomorum*^{WJL} (Ap^{WJL}) and *Lactobacillus plantarum*^{NC8} (Lp^{NC8}), we performed a large-scale, systematic nutritional screen based on larval growth in 40 different and precisely controlled nutritional environments. We combined these results with genome-based metabolic network reconstruction to define the biosynthetic capacities of *Drosophila* germ-free (GF) larvae and its two commensal bacteria. We first established that Ap^{WJL} and Lp^{NC8} differentially fulfills the nutritional requirements of the ex-GF larvae and parsed such difference down to individual amino acids, vitamins, other micronutrients and trace metals. We found that *Drosophila* commensal bacteria not only fortify the host's diet with essential nutrients but, in specific instances, functionally compensate for host auxotrophies, by either providing a metabolic intermediate or nutrient derivative to the host or by uptaking, concentrating and sparing contaminant traces of micronutrients. Our systematic work reveals that, beyond the molecular dialogue engaged between the host and its commensal partners, *Drosophila* and its facultative bacterial partners establish an integrated nutritional network relying on nutrients sparing and utilization.

Introduction

Nutrition is the major environmental factor that determines to what extent an organism can realize its genetically-encoded growth potential [1]. The attributes of nutrition are defined by the quantity [2], quality [3] and bioavailability [4] of different nutrients in the diet. Nutrients are classified as non-essential or essential [3] based on the organism's biosynthetic capacities. Diets deficient in essential nutrients cause important growth and maturation delays or even growth arrest, or "stunting" characterized by low height-for-age score [5]. In addition, some nutrients are conditionally essential. These nutrients can be synthesized by the organism, but insufficiently so under certain metabolically demanding conditions such as juvenile growth. Therefore, these conditionally-essential nutrients need also to be retrieved from the diet, like the essential ones. Deficient consumption of conditionally essential nutrients can also be detrimental for growth [3].

The intricate relationship between nutrition and growth is modulated by gut microbes. In a classical twin study in humans, Smith *et al.* unequivocally demonstrated that the gut microbiota composition of the juvenile subject suffering from stunting is significantly different from that of the healthy twin. When the faecal microbiota from the discordant twins were transplanted into genetically identical germ-free (GF) mice fed a poor diet, the recipients of the microbiota from the stunted twin performed poorly in terms of growth gain and weight recovery compared to the recipients of the microbiota of the healthy twin [6]. Furthermore, genomic analyses of gut microbiota from children experiencing strong acute malnutrition showed significant under-representation in pathways of amino acid biosynthesis and uptake; carbohydrate utilization and B-vitamin metabolism [7]. Diets supplemented with nutrients favoring the growth of bacteria enriched in these under-represented

pathways increase plasma biomarkers and levels of mediators of growth, bone formation, neurodevelopment, and immune function in children with moderate acute malnutrition [7]. These studies clearly show that microbes strongly impact how organisms respond to changes in their nutritional environment.

Diverse animal models are employed to decipher the physiological, ecological, genetic and molecular mechanisms underpinning host/microbiota/diet interactions. Among them, *Drosophila melanogaster* is frequently chosen to study the impact of the nutritional environment on growth and development thanks to its short growth period as well as easy and cost-effective rearing conditions. During the juvenile phase of the *Drosophila* life cycle, larvae feed constantly and increase their body mass ~200 times until entry into metamorphosis [8]. However, the pace and duration of larval growth can be altered by the nutritional context and the host-associated microbes [9–11]. Like other animals, *Drosophila* live in constant association with commensal microbes, including bacteria and yeast [12]. The impact of the host-associated microbes can be systematically assessed by generating gnotobiotic flies associated with a defined set of commensals [13–15]. Lab-reared flies typically carry bacterial strains from only four to eight species. The microbiota from wild flies are more complex. Nevertheless, they are usually dominated by members of the genera *Acetobacter* and *Lactobacillus* [16–22]. Most bacterial strains from these dominant genera are easy to culture in the lab, and some have even been genetically engineered for functional studies of host-microbes interactions [23–25]. These model commensal bacteria are facultative symbionts that are constantly horizontally acquired [26–28]. Even though recent experimental evidence shows that wild bacterial isolates can persistently colonize the adult crop [22,29], the larval gut

commensals are in fact transient; they constantly shuttle between the larval gut and the food substrate to establish a mutualistic cycle with the host [30,31].

We and others have previously shown that GF larvae raised in poor nutritional conditions show important developmental delays, and single model bacterial strains behaving as commensals can accelerate *Drosophila* development under these nutritional challenges [20,25]. Specifically, *Acetobacter pomorum*^{WJL} (Ap^{WJL}) modulates developmental rate and final body size through the insulin/insulin-like growth factor signaling (IIS) pathway, and its intact acetate production machinery is critical [25]. *Lactobacillus plantarum*^{WJL} or *L. plantarum*^{NC8} (Lp^{NC8}) promote host juvenile growth and maturation partly through enhanced expression of intestinal peptidases upon sensing bacterial cell wall components by *Drosophila* enterocytes [20,23,32]. Interestingly, the growth-promoting effect of these bacteria is striking under nutritional scarcity, suggesting that, besides the molecular dialogue engaged between the commensal bacteria and their host to enhance protein digestion and compensate for reduced dietary macronutrients intake, bacteria-mediated growth promotion on globally scarce diets may also include specific compensation of essential nutrients, as recently reported for thiamin [33]. However, how the presence of such bacteria systematically alters the host's nutritional environment and satisfies the host's nutritional requirements remains unexplored. To do so, we assessed the bacterial contribution to *Drosophila* larval growth in 40 different and strictly controlled nutritional contexts based on chemically-defined Holidic Diets (HDs).

Holidic diets comprise a mixture of pure chemical ingredients that satisfy the different physiological requirements of the *Drosophila* host [34,35]. By altering the concentration of each or a combination of ingredients, one can exactly tailor the experiments by generating specific nutrient deficiencies or excess [36]. The first

development of HD supporting the growth of *Drosophila* can be traced back to the 1940s [37], and have been used to assess the direct impact of the nutritional environment on axenic larvae in the 1950s [38,39]. HDs were then used to investigate the links between nutrition and lifespan [40–43], fecundity [40–42,44], food choice behavior [45,46], nutrient sensing [47], growth and maturation [33,40–42,48–50]. In this study, we adopted the recently developed FLYAA HD where the amino acid concentrations are calculated so that they match the amino acid ratios found in the translated exome of the fly [40]. The FLYAA HD is optimal for both fecundity and lifespan of adults, and it can efficiently support larval growth albeit not to the optimal growth and maturation rate obtained with rich oligidic diets [34]. Using this chemically defined HD, we aimed to deconstruct in a systematic manner the microbial contribution to the host's nutritional requirements down to individual nutrients.

To do so, we first needed to establish the biosynthetic capacities of GF larvae and two model commensal bacteria of *Drosophila*: Ap^{WJL} and Lp^{NC8} on HD. We further complemented the *in vivo* study with automated metabolic network reconstruction based on the genome sequences of *D. melanogaster*, Ap^{WJL} and Lp^{NC8}. In recent years, metabolic approaches based on genome-driven network reconstructions have been applied to predict the potential metabolic dependencies and metabolic exchanges between hosts and associated microbes [51–56]. The mutualistic association between the pea aphid *Acyrthosiphon pisum* and its obligate intracellular symbiont *Buchnera aphidicola* was the first symbiotic association for which genomic information were available on both partners, and is a case study for a comprehensive survey of integrated host-symbiont metabolic interactions. In this model, decades of nutritional experiments using HDs and aposymbiotic aphids were

reinterpreted in the light of newly available genomic data, thus changing the traditional paradigm that proposed a clear separation between the pathways of the host and its symbionts and revealing a particularly integrated metabolic network, which is the result of the long coevolution of the insect with its obligate endosymbionts [57,58]. This example shows how important it is to integrate theoretical and experimental approaches to model metabolic pathways of symbiotic partners and properly dissect the functioning of their associations.

Here we report that association of GF larvae with Ap^{WJL} or Lp^{NC8} modifies the nutritional requirements of ex-GF larvae in a specific manner each. We show that Ap^{WJL} and Lp^{NC8} not only modify the nutritional environment of their host by fortifying diets with essential nutrients. Moreover, they functionally compensate host auxotrophies despite not synthetizing the missing nutrient, probably by either providing a nutrient derivative to the host or by uptaking, concentrating and sparing contaminant traces of the missing micronutrient.

Results and Discussion

Metabolic network reconstruction of the host (*Drosophila melanogaster*) and its two commensals, Ap^{WJL} and Lp^{NC8}, was automatically generated using the CycADS pipeline [59]. The resulting BioCyc metabolism databases are available at http://artsymbiocyc.cycadsys.org for annotation and analysis purposes. We generated the enriched functional annotations of all the predicted proteins from the complete genomes of *D. melanogaster* (*Drosophila*, RefSeq GCF_000001215.4 release 6), *A. pomorum* strain DM001 (Ap^{WJL}, accession NCBI Bioproject PRJNA60787) and *L. plantarum* subsp. *plantarum* NC8 (Lp^{NC8}, NCBI Bioproject PRJNA67175). From the genomic analyses, we inferred all pathways allowing production of the organic compounds that are present in the exome-based FLYAA holidic diet (HD) developed by Piper *et al* [40]: fly's essential and non-essential amino acids (EAA^{Fly} (n=10) and $NEAA^{Fly}$ (n=10)), B-vitamins (n=7), cholesterol (n=1), and nucleic acids and lipid precursors (NAL, n=4).

Drosophila melanogaster biosynthetic capabilities inferred from genome-based metabolic network reconstruction

Although a BioCyc metabolic reconstruction of *D. melanogaster* is already publicly available https://biocyc.org/FLY, we constructed an improved BioCyc database using a recent genome version and annotation [59]. This metabolic reconstruction identified 22,189 protein-encoding genes including 5,061 enzymes and 156 transporters associated with 1,610 compounds assembled in a network of 331 pathways (*versus* the 227 pathways found in BioCyc). Like other metazoans, *Drosophila* possesses the gene repertoire to produce all the $NEAA^{Fly}$, but is unable to produce the EAA^{Fly} (Fig 1A and Table S1). *Drosophila* can also produce myo-inositol,

inosine and uridine, but is unable to synthesize vitamins from simple precursors (Fig 1B and Table S2).

Ap^{WJL} biosynthetic capabilities inferred from genome-based metabolic network reconstruction

According to our metabolic reconstruction, Ap^{WJL} genome comprises 4,268 protein-encoding genes including 1,326 enzymes and 46 transporters associated with 1,306 compounds assembled in a network of 313 pathways. Ap^{WJL} is a complete autotroph for all amino acids, and possesses the genetic potential to produce the DNA bases inosine and uridine and five of the seven vitamins present in the HD: biotin, folate, pantothenate, riboflavin and thiamine (Fig 1A-B and Table S1-2). The first two steps of nicotinate pathway (EC 1.4.3.16 and 2.5.1.72) seem lacking in Ap^{WJL}. However, three candidate proteins (peg.1228, 1229 and 1231) encode the succinate dehydrogenase enzymatic activity (EC 1.3.5.1). This enzyme can alternatively use oxygen or fumarate as O-donor, depending on aerobic or anaerobic living conditions. Hence, this enzyme can switch between its aerobic condition activity (EC 1.3.5.1) towards its anaerobic condition activity (EC 1.4.3.16) using fumarate as a substrate and producing imminoaspartate. Hence, assuming that one of these genes can produce the activity at a sufficient rate in aerobic conditions in Ap^{WJL}, then the bacteria would be able to produce NAD^+ and $NADP^+$ from Asp (Fig 1B and Table S2). The biosynthesis of pyridoxine is almost complete in Ap^{WJL}. Although we were not able to detect specific activities for the first two steps of the pathway, we propose (see below) that the bacteria have the capability to produce vitamin intermediates using enzymes with very close activities (Table S2). It is to note that pyridoxine is reported as non-essential for acetic acid bacteria [60]. In summary, Ap^{WJL} genome

analysis predicts that it is able to synthesize all amino acids, DNA bases and the seven B-vitamins (biotin, folate, pantothenate, riboflavin, thiamine and intermediates of nicotinate and pyridoxine) present in HD. However, we found no genomic support for the synthesis of choline and myo-inositol in the Ap^{WJL} genome.

Lp^{NC8} biosynthetic capabilities inferred from genome-based metabolic network reconstruction

Metabolic reconstruction from Lp^{NC8} genome generated a database that includes 2,868 protein-encoding genes, consisting of 973 enzymes and 74 transporters associated with 1,154 compounds, all assembled in a network of 246 metabolic pathways. From a genomic perspective (Fig 1A and Table S1-2), Lp^{NC8} is able to produce most amino acids from glucose or inner precursors with the exception of Phe, sulfur-containing (Cys, Met) and branched-chain amino acids (BCAA, Ile, Leu, Val). Arg is known to be limiting [61] or essential to certain *L. plantarum* strains [62,63], yet Lp^{NC8} genome encodes a complete Arg biosynthesis pathway. A manual curation of the pathway showed that the $Lp^{NC8's}$ *argCJBDF* operon should be functional, as it does not contain stop codons, frame-shifts or deletions. Lp^{NC8} may produce Ala and Asp only using secondary metabolic routes (Table S1). Therefore, Lp^{NC8} is expected to acquire these amino acids from the diet, or to have an altered growth when they are absent from the diet. Similarly, biosynthesis of Thr is directly linked to Asp and Cys and is probably very limited in Lp^{NC8}.

Regarding vitamins and bases biosynthesis, Lp^{NC8} is able to produce folate, riboflavin, and thiamine (through the pyrimidine salvage pathway (2.1.7.49)), as well as all DNA bases including uridine and inosine (Fig 1B and Table S2). Lp^{NC8} is not able to synthesize biotin, pyridoxine, pantothenate, choline and myo-inositol. Based

on our genomic analysis, Lp^{NC8} is not able to achieve the entire nicotinate biosynthetic pathway from Asp nor from Trp as described in eukaryotes and in some bacteria [64] even if the first step of the pathway could possibly be accomplished by the succinate dehydrogenase, as described above for Ap^{WJL}, the other two enzymes of the initial part of the pathway are missing (Fig 1B and Table S2).

Collectively, our metabolic networks reconstruction shows that *Drosophila* and its associated bacteria have differential biosynthetic capacities. Indeed, some of the complete biosynthetic pathways are only present in one organism while others are present in two or all the three partners (Fig 2). In addition, we did not detect incomplete biosynthetic pathways potentially complemented between a commensal and its host (Fig 1-2), as previously observed for obligate mutualistic partners [57,58].

Experimental validation of commensal bacteria auxotrophies using holidic diets

In order to test experimentally the metabolic potential of *Drosophila* and its commensals, predicted by our automated genome annotations and subsequent metabolic pathway reconstructions (see above), we adopted the exome based FLYAA holidic diet (HD) [40]. We systematically removed a single component at a time to generate 39 different fly nutritional substrates (henceforth named HDΔX, X being the nutrient omitted), plus one complete HD medium. This medium can also be prepared in a liquid version by omitting agar and cholesterol from the recipe. Liquid HD can then be used to assess bacterial growth in 96-well plates, increasing the experimental throughput.

We first assessed Ap^{WJL} and Lp^{NC8} growth in each of the 40 different liquid HD for 72 hours, using maximal optical densities (OD_{Max}) as a readout (Fig 3A and Table S3). In the complete HD, both Ap^{WJL} and Lp^{NC8} grow well (Fig 3A, first line). On the deficient media, Ap^{WJL} can grow in HD∆Sucrose, presumably using acetate from the acetate buffer as a carbon source. Also, its growth is not altered in the absence of any of EAA^{Fly}, Vitamins or NAL. However, while Ap^{WJL} growth is not impacted by the lack of most $NEAA^{Fly}$, it grows poorly in HD∆Ala, HD∆Cys and HD∆Glu. In addition, Ap^{WJL} fails to grow in HD∆Cu, HD∆Fe and HD∆Mg (Fig 3A, first column and Table S3). The broad growth capacity of Ap^{WJL} in HDs correlates well with the wide range of environmental niches the genus *Acetobacter* can colonize. *Acetobacter* species are found in sugar-rich niches such as flowers and fruits, but also in poorer niches such as soil and water, where they need to synthesize all nutrients required for their own growth [65]. These findings corroborate our genome-based predictions (Fig 1). Furthermore, the genome-based metabolic pathway reconstruction predicted that Ap^{WJL} would not be able to synthesize choline and myo-inositol, however we observed that Ap^{WJL} grows in their absence. Choline is an important precursor of phosphatidylcholine (PC), which is a major component of *Acetobacter* membranes and plays an important role to confer acetic acid tolerance. Despite its importance, PC is not essential for *Acetobacter* growth. Indeed, mutants precluding PC synthesis show a shift towards increased membrane content of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) and do not show any growth defect in standard medium [66]. Similarly, Ap^{WJL} likely does not need myo-inositol for its growth since inositol compounds are absent from the membrane of most bacteria [67]. Regarding nicotinate and pyridoxine, the biosynthesis pathways of these two vitamins are only partial and do not support the production of the final molecules (Fig 1B and Table

S2), however intermediates such as pyridoxine-phosphate, pyridoxal-5-phosphate and pyridoxamine or nicotinate-D-ribonucleotide, NAD^+ and $NADP^+$ may be synthesized and would support bacterial growth in nicotinate or pyridoxine depleted diets. Interestingly, Ap^{WJL} growth was only precluded in the absence of some metal ions: Cu, Fe, and Mg. Metal ions are important co-factors required for enzymatic activities [68]. Specifically, in acetic acid bacteria, Cu is an important co-factor of the energy-producing cytochromes of the respiratory chain [69] making it essential for Ap^{WJL} growth.

We detected far more nutritional auxotrophies for Lp^{NC8} on HDs (Fig 3A, second column and Table S3). Lp^{NC8} fails to grow in HDΔSucrose, since sucrose is the only suitable carbon source for this strain in the liquid HD. Also, Lp^{NC8} growth is precluded in the absence of nine amino acids, including six EAA^{Fly} (Arg, Ile, Leu, Phe, Thr, Val) and three $NEAA^{Fly}$ (Ala, Asp, Cys). It also grows poorly in media lacking the EAA^{Fly} Lys, Met and Trp and the $NEAA^{Fly}$ Asn and Glu. Moreover, Lp^{NC8} does not grow in HDΔBiotin and HDΔPantothenate. However, it slightly grows in absence of nicotinate, despite the prediction from our genome-based metabolic pathway reconstruction that nicotinate cannot be produced (Fig 1B and Table S2). Finally, Lp^{NC8} growth is not affected by the lack of any NAL and even increased in the absence of certain metal ions such as Ca, Cu, Mg, Zn. In contrast, Lp^{NC8} growth is significantly reduced in HDΔMn. These relatively elevated nutritional requirements of Lp^{NC8} were expected as *L. plantarum* is a species adapted to nutrient-rich environments [70]. Hence, many *L. plantarum* strains have lost the capacity to synthesize various nutrients that can easily be found in their natural habitats [70,71]. The inability of *L. plantarum* to synthesize important nutrients such as BCAA (Ile, Leu and Val) or the B-vitamin pantothenate was previously identified by both genome

analyses [62] and growth studies in chemically defined minimal media [61,72,73]. Moreover, it is known that *L. plantarum* needs Mn to resist to oxidative stress [74], which explains its poor growth in HD∆Mn.

Our experimental data only partially correlate with the results of the genome-based predictions. Predicted auxotrophies for Ile, Leu, Val, Phe, Cys, pantothenate and biotin were confirmed *in vivo*. The identified Arg auxotrophy was not surprising because, as mentioned above, Arg is often described as essential to *L. plantarum* in high metabolic demanding conditions even though all genes necessary for Arg biosynthesis are present. However, auxotrophies of Lp^{NC8} to Thr, Ala, Asp, were not expected (Fig 3A, denoted by "*"), even though these amino acids were predicted to be limiting (see above). As mentioned previously, bacterial growth in liquid HD was assessed in 96-well plates using a microplate reader (see Material and Methods). Every cycle includes an agitation step to homogenize the solution to improve OD reading accuracy. This agitation step may oxygenate the media and thus negatively affects Lp^{NC8} growth in suboptimal nutritional conditions since *L. plantarum* strains are aerotolerant but optimal growth is achieved under microaerophilic or anaerobic conditions [75]. To challenge these unexpected auxotrophies, we assessed Lp^{NC8} growth in liquid HD∆Thr, HD∆Ala, HD∆Asp in 15-mL closed falcon tubes without agitation. After 72 hours of incubation, we determined CFU counts in each media (Fig 3B). As predicted by our genomic analyses, Lp^{NC8} was now able to grow in each of the three deficient media in static conditions to the same extent as in the complete HD (Fig 3B). Therefore, Lp^{NC8} auxotrophies observed for Thr, Ala and Asp in 96-well plates are likely due to excessive oxygenation. This could also explain the poor growth of Lp^{NC8} in the absence of the EAA^{Fly} Lys, Met and Trp and the $NEAA^{Fly}$ Asn and Glu.

Surprisingly, the ability of Lp^{NC8} to grow in HDΔCholine, HDΔMyo-inositol, HDΔNicotinate and HDΔPyridoxine do not correlate with our metabolic predictions. As for Ap^{WJL} (see above), Lp^{NC8} growth probably does not require choline or myo-inositol. A previous study quantified choline and inositol compounds in *L. plantarum* cell extracts and found them to be extremely low and therefore most likely due to contaminations from the medium rather than components of *L. plantarum* biomass [76]. Pyridoxine is a precursor of pyridoxal-5-phosphate, a cofactor necessary for amino acid converting reactions. Teusink *et al.* [62] showed that *L. plantarum*^{WCSF1} requires exogenous sources of pyridoxine only in a minimal medium lacking amino acids. Since the HDΔPyridoxine contains all amino acids, it is likely that pyridoxine is not essential for Lp^{NC8} growth in these conditions. Finally, the capacity of Lp^{NC8} to grow in HDΔNicotinate could be related with the presence of alternative pathways to nicotinate intermediates biosynthesis (Fig 1B and Table S2). Indeed, this possibility has been previously reported in the genus *Lactobacillus* [71] which would explain the capacity to grow in absence of exogenous nicotinate.

Altogether, the complete HD is a suitable nutritional environment that allows the two model *Drosophila* commensals, Ap^{WJL} and Lp^{NC8} to grow. Growth capacities in deficient media vary from one bacterium to another and are dictated by their individual genetic repertoires.

Germ free larvae exhibit 22 auxotrophies while developing on FLYAA holidic diets

We next sought to establish the nutritional requirements of GF larvae by assessing larval developmental timing (DT) in complete HD and in each of the 39 deficient HD (larvae were reared from eggs until pupae on the HDs, see Material and

Methods). DT is expressed as D_{50}, which represents the day when 50% of the larvae population has entered metamorphosis in a specific nutritional condition. In agreement with previous studies [38,39], GF larvae fail to develop in all HD∆EAA^{Fly}, all HD∆Vitamins, HD∆Choline, HD∆Cholesterol, HD∆Zn and HD∆Mg (Fig 4A, first column). Over 60 years ago, Sang *et al.* reported that Zn was dispensable for GF larval development [38]. We suspect that the casein in the medium used in Sang *et al.* inadvertently provided trace amount of Zn, which could account for the discrepancy between our observation and that of Sang *et al*. Also in accordance with previous studies [38,39,50], GF larvae were able to reach pupariation in HD∆NEAA^{Fly} (∆Ala, ∆Cys, ∆Gln, ∆Glu, ∆Gly, ∆Pro), HD∆Uridine, HD∆Myo-inositol, and HD∆Mn at the same rate as on a complete HD (Fig 4A first column, Table S4). The absence of sucrose, Tyr, inosine, Ca, Cu and Fe did not prevent pupae emergence, but increased the duration of larval development very significantly (Fig 4A first column, Table S4). Surprisingly, GF larvae were able to reach pupariation, albeit late, in HD∆Sucrose. Indeed, all the HDs developed to date include carbohydrates (either sucrose or fructose) as a carbon source [34]. Larval development in the absence of carbohydrates suggests that GF larvae may use other components of the HD, such as amino acids, as carbon source. In summary, GF *yw* larvae show 22 auxotrophies while developing on sterile FLYAA holidic diets.

Our observations correlate well with our genome-based predictions of the metabolic capabilities of the three partners (Fig 1) with one exception: GF larvae did not reach pupariation in HD∆Asn. This result was surprising because Asn is described as a NEAA in *Drosophila* and other animals [77]. To test whether Asn auxotrophy was specific to the *yw* fly line used in our lab, we assessed larval DT in two other *D. melanogaster* reference lines, the DGRP line DGRP-*RAL-25210* [78]

and w^{1118}. Unlike *yw*, both w^{1118} and *RAL-25210* larvae were able to develop in GF condition in HDΔAsn albeit with a severe developmental delay (Fig 5A). Therefore, the complete Asn auxotrophy seen with our *yw* strain is rather an exception than a rule, an observation that correlates with our metabolic pathway reconstruction that was based on the genome sequence of the *D. melanogaster* Reference Genome strain (Bloomington stock #2057). We next sequenced the coding region of the enzyme AsnS, which converts Asp to Asn in *yw* flies, and did not detect any non-synonymous mutation (Fig S1). Further studies may thus be required to determine the origin of the Asn auxotrophy in our *yw* line on HD. However, these results indicate that Asn is not an EAA per se but remains a limiting NEAA, an observation that also applies to Tyr.

Bacterial cell wall sensing contributes to Lp^{NC8} mediated larval growth promotion in complete chemically defined diets

We then investigated if and how the association with commensals affects the nutritional requirements of GF larvae during juvenile growth and maturation. To this end, we mono-associated GF embryos with Ap^{WJL} or Lp^{NC8} and measured D_{50} and egg-to-pupa survival in complete and deficient HDs (Fig 4A second and third columns, respectively and Table S4, S5). On a complete HD, mono-association with either Ap^{WJL} or Lp^{NC8} accelerated larval DT with a mean D_{50} of 8.4 and 7.7 days, respectively, whereas GF mean D_{50} is 10.1 days (Fig 4A, first line). These growth-promoting effects upon mono-association with either Ap^{WJL} or Lp^{NC8} have been previously reported on complex diets and insights on the underlying molecular mechanisms were provided [20,25]. Shin *et al*. showed that when the associated larvae grow on a low casamino-acid semi-oligidic diet, the pyrroloquinoline quinone–

398 dependent alcohol dehydrogenase (PQQ-ADH) activity of Ap^{WJL} modulates the
399 developmental rate and body size through insulin/insulin-like growth factor signaling
400 (IIS). PQQ-ADH disruption in the Ap^{WJL}::Tn*pqq* mutant severely reduces acetic acid
401 production, which has been proposed to alter the regulation of developmental and
402 metabolic homeostasis upon mono-association [25]. Lp^{NC8} promotes host juvenile
403 growth and maturation on a low yeast-based oligidic diet, partly through enhanced
404 expression of intestinal peptidases upon sensing of bacterial cell walls components
405 by *Drosophila* enterocytes [20,23]. Deletion of the *dlt* operon, which encodes the
406 molecular machinery involved in the D-alanylation of teichoic acids, leads to bacterial
407 cell wall alteration with a complete loss of D-alanylation of teichoic acids and
408 consequently cell walls purified from the $Lp^{NC8}\Delta dlt_{op}$ mutant trigger a reduced
409 expression of peptidases in enterocytes [23]. Therefore, we first probed the
410 importance of these molecular mechanisms on commensals mediated larval growth
411 promotion on complete HD. To this end, we tested in our HD setting the associations
412 with the loss of function mutants Ap^{WJL}::Tn*pqq* [25] and $Lp^{NC8}\Delta dlt_{op}$ [23]. In complete
413 HD, only the $Lp^{NC8}\Delta dlt_{op}$ mutant failed to support larval growth, reminiscent of the
414 previous observation on the low-yeast oligidic diets (Fig S2). Surprisingly, in
415 complete HD, the Ap^{WJL}::Tn*pqq* mutant actually triggered an enhanced growth
416 promotion as compared to its WT reference strain. Shin *et al.* reported that
417 Ap^{WJL}::Tn*pqq* associated larvae experienced growth delay, which can be rescued by
418 acetic acid provision [25]. Therefore, the acetic acid-based buffer in HD may explain
419 why Ap^{WJL}::Tn*pqq* no longer behaves as a loss of function mutant in this setting;
420 however, how it actually surpasses the WT strain on complete HD remains elusive.
421 Collectively, these results establish that sensing bacterial cell walls containing D-
422 alanylated teichoic acids is also an important feature of the intrinsic growth promoting

ability of Lp^{NC8} in a complete chemically defined HD. Thus, the previously reported molecular sensing mechanism that mediates the growth-promoting effect of Lp^{NC8} during chronic undernutrition is also at play in synthetic diets.

Association with Ap^{WJL} fulfills 19 of the 22 nutrient requirements of GF larvae

Association with Ap^{WJL} sustained larval development (albeit to different degrees) in the absence of 19 out of 22 GF larvae essential nutrients (Fig 4A second column). Ap^{WJL} associated larvae reached pupariation in the absence of each EAA^{Fly} (though their development was slower than on complete HD), Asn, vitamins, choline and Zn. Association with Ap^{WJL} also rescued the developmental delay observed in GF larvae in HDΔTyr, HDΔinosine, HDΔCu and HDΔFe. The only nutritional requirements of GF larvae that were not fulfilled by Ap^{WJL} were cholesterol, pantothenate and Mg.

Association with Lp^{NC8} fulfills 12 of the 22 nutrient requirements of GF larvae

Compared to Ap^{WJL}, mono-association with Lp^{NC8} compensated for a reduced number of the GF larvae nutritional deficiencies (12 out of 22; Fig 4A third column). Lp^{NC8} associated larvae reached pupariation in the absence of some EAA^{Fly} (HDΔHis, HDΔLys, HDΔMet, HDΔPhe, HDΔThr), Asn, certain vitamins (HDΔBiotin, HDΔFolate, HDΔNicotinate, HDΔRiboflavin, HDΔThiamine), and Zn. Moreover, Lp^{NC8} rescued the developmental delay observed in GF on HDΔTyr, HDΔinosine, HDΔCu and HDΔFe.

Bacteria need to be metabolically active in order to fulfill larval nutritional requirements

Bacteria were grown in rich medium before association with larvae (see Material and Methods). Therefore, they may have accumulated nutrients that can be used later by the larvae to fulfill their nutritional requirements. To test for the nutritional input brought by the initial bacterial inoculum, we associated GF larvae with 10X heat-killed (HK) bacteria (mimicking the maximal bacterial biomass found in the diet during the experiment, Fig S3B) and measured D_{50} in complete and deficient HDs (Fig 4A fourth and fifth columns). In most cases, the D_{50} of larvae in HK and GF conditions were similar. Therefore, bacteria need to be metabolically active to fulfill the larval nutritional requirements on HDs. However, we found some exceptions. In HDΔAsn, HDΔBiotin, HDΔFolate, HDΔCu and HDΔFe, addition of HK bacteria allowed the larvae to develop, though not as fast as in association with living bacteria. These results suggest that larvae only require a very small amount of these nutrients, which can be sufficiently derived from the inert bacterial inoculum.

On a low-protein oligidic diet, larval growth promotion by commensal bacteria correlates positively with the quantity of microbes [79]. We wondered whether the differences that we observed in growth-promotion efficiency were due to differences in bacterial loads. Thus, we tested the correlation between bacterial loads and benefit to host growth in three contexts; (1) conditions where both bacteria are beneficial to their host: complete HD; (2) conditions where each bacterium impacts differently the host: HDΔMet, HDΔPhe, HDΔNicotinate; (3) conditions where only one bacterium compensates for the lack of a nutrient: HDΔCys, HDΔPyr and HDΔCholine. We found no correlation between bacterial load in the larval gut (Fig 4B; Fig S3A) or in the diet (Fig 4B-C; Fig S3B) and the ability of the bacteria to impact host developmental timing on the tested diets. These results reinforce the notion that, in our experimental settings, *Drosophila*-associated bacteria are biologically active partners and their

load, either in the diet or in the gut, does not dictate their functional impact on their host's nutrition or development.

The ability of bacteria to compensate nutritional deficiencies does not always correlate with the ability of bacteria to synthesize the nutrient

Next, based on the genome-based predictions and the experimentally revealed auxotrophies of GF larvae on FLYAA HD, we correlated the ability of each commensal bacterium to synthesize a nutrient to its ability to fulfill the larval requirements in this nutrient. We identified four distinct situations related to the 19 compensations of the 22 auxotrophies shown by GF larvae.

<u>Situation 1: the bacteria synthesize the missing nutrient in the diet and compensate for the related larval auxotrophy (15/19 auxotrophies compensations).</u>

In most of the tested conditions, when the bacteria can synthesize a nutrient, they can also fulfill the related nutritional requirements of the GF larvae. For Ap^{WJL} this includes all EAA^{Fly}, Asn and most vitamins (except pantothenate). For Lp^{NC8}, the correlation between the nutritional complementation of ex-GF larva and the ability of Lp^{NC8} to synthesize the missing nutrient is more limited and only applies to the requirements of His, Lys, Met, Thr, Asn and most vitamins. Nonetheless, these results suggest that bacteria can actively supply the nutrients lacking in the HD to the larvae. This phenomenon is reminiscent of previous observations using conventional and gnotobiotic hosts, in which microbial sparing of riboflavin or thiamine by differential commensals have been proposed [33,80]. Exceptions to this case seem to be Ap^{WJL} on HDΔPantothenate and Lp^{NC8} on HDΔTrp. Specifically, Ap^{WJL} can

produce pantothenate and grows in HD∆Pantothenate and similarly Lp^{NC8} can produce Trp and grows in HD∆Trp. However, neither supported larval development on the respective depleted HD. It is therefore probable that Ap^{WJL} and Lp^{NC8} produce enough pantothenate and Trp, respectively, to sustain their own growth in the depleted HD but not sufficiently or in a manner inaccessible to the larvae and thus fail to fulfill larval requirements for these nutrients.

Situation 2: the bacteria do not synthesize a nutrient and they cannot fulfill larval nutrient requirements.

Expectedly, we observed that when bacteria do not synthesize a nutrient, they do not fulfill ex-GF larvae requirements for this nutrient. For instance, Lp^{NC8} cannot produce the BCAA (Ile, Leu and Val), nor grow in their absence, and thus it cannot fulfill larval requirements for these amino acids. In some depleted diets bacteria were able to grow (Fig 3A) even though they cannot synthesize the missing nutrient (Fig 1 and Table S1-2), and they failed to fulfill the larvae requirements of these specific nutrients. This is observed for Ap^{WJL} and Lp^{NC8} on HD∆Cholesterol. The likely explanation is that cholesterol is an animal sterol but is dispensable for bacterial growth [67,81]. Similarly, on HD∆Choline and HD∆Pyridoxine, Lp^{NC8} grows (Fig 3A) but it is unable to fulfill larval requirements (Fig 4A), as according to genome-based predictions it cannot synthesize these compounds (Fig 1 and Table S2).

Situation 3: the bacteria do not synthesize a nutrient but they can fulfill larval nutrient requirements (3/19 auxotrophies compensations)

In most cases, we observe growth rescue by bacteria provision of the missing nutrients, but there are interesting exceptions. According to genome-based

521 predictions, Ap^{WJL} is unable to synthesize *de novo* choline, pyridoxine and nicotinate
522 (Fig 1B and Table S2). Surprisingly, it compensates larval auxotrophies on
523 HD∆Choline, HD∆Pyridoxine and HD∆Nicotinate. Similarly, genome analysis predict
524 that Lp^{NC8} cannot synthesize nicotinate (Fig 1B and Table S2) but it compensates
525 larval auxotrophy on HD∆Nicotinate.

526 To confirm that the bacteria are uncapable to synthesize these compounds,
527 we assessed the presence of these compounds in bacterial supernatants using
528 Nuclear Magnetic Resonance spectroscopy (NMR) and High Potential Liquid
529 Chromatography coupled with Mass Spectrometry (HPLC-MS). We were able to
530 quantify choline in a complete HD using NMR spectroscopy (at 0.531 ± 0.003 mM for
531 a theoric concentration of 0.477 mM). However, we failed to detect it in the
532 supernatant of Ap^{WJL} culture in HD∆Choline (see Material and Methods section).
533 Similarly, we did not detect any production of nicotinate by either Ap^{WJL} and Lp^{NC8},
534 nor production of pyridoxine by Ap^{WJL} in HD∆Nicotinate or HD∆Pyridoxine although
535 the analytical method used (HPLC-MS, see Material and Methods) was very
536 sensitive with a limit of detection (LOD) of 15,625 nM and 0,977 nM for nicotinate and
537 pyridoxine, respectively.

538 In the case of choline, Ap^{WJL} may synthesize other compounds that *Drosophila*
539 can use to functionally replace choline. As stated before, *Acetobacter* mutants
540 precluding phosphatidylcholine (PC) synthesis shift their membrane composition
541 towards increased content of phosphatidylethanolamine (PE) and
542 phosphatidylglycerol (PG) [66]. PE and PG have been reported to be part of the
543 phospholipidic repertoire of *Drosophila* membranes [82], in which PE represents
544 approximately 50% of their lipid composition [83]. We posit that ex-GF larvae growing

545 on HD∆Choline capitalize on ethanolamine or glycerol phospho-derivatives produced
546 by Ap^{WJL} to compensate for the lack of choline in their diet.

547 In the case of pyridoxine, despite its inability to synthesize pyridoxine, Ap^{WJL}
548 may fulfill larval requirements through the production of intermediates such as
549 pyridoxine-phosphate, pyridoxal-5-phosphate or pyridoxamine, which are predicted to
550 be synthesized based on genome analysis (Fig 1B and Table S2).

551 Regarding nicotinate, both Ap^{WJL} and Lp^{NC8} grow on HD∆Nicotinate and also
552 can fulfill the larval requirements in this vitamin, even though they cannot synthesize
553 it. However, genome-based metabolic predictions suggest that Ap^{WJL} may
554 compensate for the lack of nicotinate by producing intermediates such as nicotinate-
555 D-ribonucleotide, NAD^+ and $NADP^+$. In the case of Lp^{NC8}, we postulate the existence
556 of alternative metabolic pathways leading to nicotinate intermediates biosynthesis.

557 Lp^{NC8} cannot grow in the absence of Phe (Fig 3A). The genomic analyses
558 point to the possible loss of the gene coding for the enzyme prephenate dehydratase
559 (4.2.1.51), the penultimate step on Phe biosynthesis. Yet Lp^{NC8} can fulfill larval
560 requirements for Phe (Fig 4A). We wondered if the Phe auxotrophy we observed in
561 96-well plates (Fig 3A) was due to the oxygenation generated by the agitation
562 through OD readings, as for Thr, Ala and Asp (Fig 3B). To test this, we set cultures of
563 Lp^{NC8} in HD∆Phe in static 15-mL closed falcon tubes and assessed bacterial growth
564 after three days of culture. In contrast to agitation, Lp^{NC8} grows in HD∆Phe up to max.
565 10^6 CFUs in static conditions (Fig 5B), whereas in the complete media (Fig 3B),
566 Lp^{NC8} grows up to 5.10^8 CFUs. These results indicate that the rescue of larvae
567 developmental timing by Lp^{NC8} in HD∆Phe is still mediated by bacterial nutrient
568 supply. However, the poor growth of Lp^{NC8} in HD∆Phe suggests the existence of an
569 alternative pathway for Phe biosynthesis in absence of the prephenate dehydratase

570 (Fig 1A). As suggested by Hadadi et al. [84], Phe might be produced from L-
571 arogenate using a derivative catalysis through the 2.5.1.47 activity which is encoded
572 in Lp^{NC8} by the *cysD* gene (nc8_2167) (Table S2).

573 A second such interesting case is larval development rescue by Lp^{NC8} on
574 HDΔCys. Lp^{NC8} is auxotroph for Cys (Fig 3A), even in static conditions (Fig 5B).
575 Lp^{NC8}-associated larvae develop faster than GF larvae in HDΔCys, though GF larvae
576 are not auxotroph for Cys (Fig 4A). This beneficial effect of Lp^{NC8} on ex-GF larvae
577 development on HDΔCys is similar to what is observed on a complete HD (Fig 4A,
578 first row). Therefore this result probably reflects the basal nutrient-independent
579 growth-promoting effect of Lp^{NC8}, which relies on the sensing and signaling of Lp^{NC8}
580 cell wall by its host (Fig S2) [23] and requires Lp^{NC8} to be metabolically active (Fig
581 4A, fifth column). Taken together, our results suggest that Lp^{NC8} is able to grow in
582 HDΔCys only in the presence of *Drosophila* larvae. To test this hypothesis, we
583 assessed Lp^{NC8} growth in solid HDΔCys in the absence and the presence of larvae
584 (Fig 5C). Without larvae, Lp^{NC8} grew one log above the inoculum level (~5.10^5
585 CFU/tube) on solid HDΔCys (Fig 5C, "on diet only"). This minimal growth on solid
586 HDΔCys could be due to the Cys reserves from Lp^{NC8} growth in rich media (MRS)
587 prior to inoculation, or from contaminants in the agar and cholesterol added to
588 prepare the solid HD. Interestingly, in the presence of larvae in the HDΔCys, Lp^{NC8}
589 CFU counts increased over time, reaching ~10^8 CFU/tube at day 6 (Fig 5C, "on diet
590 with larvae"). These results indicate that in HDΔCys, larvae support Lp^{NC8} growth,
591 probably by supplying Cys or a precursor/derivative. In turn, Lp^{NC8} sensing and
592 signaling in the host promotes larval development and maturation. This observation
593 extends the recent demonstration that *Drosophila* and *L. plantarum* engage in a
594 mutualistic symbiosis, whereby the insect benefits the growth of the bacterium in their

shared nutritional environment [30]. Here, we discover that Cys is an additional *Drosophila* symbiotic factors also previously referred to as “bacteria maintenance factors” [30].

Situation 4: Bacterial compensation of minerals and metal deficiencies by concentrating traces or by functional compensation (1/19 auxotrophy compensation).

We observed that both Ap^{WJL} and Lp^{NC8} would compensate for Cu, Fe and Zn deficiencies but not Mg (Fig 4A, second and third columns). Requirements in Cu and Fe were also fulfilled by HK bacteria (Fig 4A, fourth and fifth columns) although larvae associated with HK bacteria in these conditions developed much slower than larvae associated with living bacteria. This suggests that the inert bacterial inoculum contains traces of Cu and Fe accumulated during the overnight growth in rich medium prior to the inactivation and inoculation. These accumulated quantities allowed the larvae to develop when Cu and Fe were not supplied in the HD. Surprisingly, Zn requirements were fulfilled by living bacteria only (Fig 4A). We hypothesize that bacteria may concentrate contaminating traces of these elements in the HD and make them more available to larvae. Alternatively, this could be an interesting case of functional complementation that requires further investigation. Indeed, Zn is an important enzymatic cofactor in the biosynthesis of several metabolites by the larva [85]. In the absence of Zn, GF larvae would not produce these compounds; instead they could be produced by the bacteria and supplied to the ex-GF larvae similarly to the nutritional complementation we observed above for choline (situation 3). Interestingly, a link between Zn response and the microbiota of *Drosophila* have been described in previous studies. Expression of the Zn transporter *zip-3* is higher in GF *Drosophila* adults midguts than in their conventionally raised

620 (CR) counterparts [27]. Moreover, the genes encoding Metallothioneins B and C
621 (*MtnB* and *MtnC*) are more expressed in flies harboring a microbiota than in GF flies
622 [86]. Metallothioneins are intracellular proteins that can store Zn. Their expression, as
623 well as expression of Zn transporters such as *zip-3*, is regulated by intracellular
624 levels of Zn [87]. Altogether, these results suggest that commensal bacteria may play
625 an important role in the uptake of metals (especially Zn) by *Drosophila* larvae. This
626 idea is reminiscent of recent reports in *Caenorhabditis elegans,* whereby a
627 commensal bacterium promotes worm development upon Fe scarcity by secreting a
628 scavenging siderophore [88].
629
630 **Commensal bacteria spare amino acids essential to larval development**
631 Despite the interesting exceptions detailed above, our data establish that in
632 many cases, bacterial commensals complement the nutritional requirements of their
633 host by synthesizing and supplying essential nutrients. Bacteria can actively excrete
634 amino acids in their environment when they are produced in excess as intracellular
635 by-products of metabolic reactions [89]. Moreover, the bacterial cell wall is rich in D-
636 amino acids, and it undergoes an important turnover [90,91]. In certain bacterial
637 species, D-amino acids accumulate in the supernatant during growth and act as a
638 signal to undergo stationary phase [92]. Thus, D-amino acids may also contribute to
639 larval nutrition. Indeed, it has been previously shown that D-amino acids (D-Arg, D-
640 His, D-Lys, D-Met, D-Phe, and D-Val) can support growth of GF larvae probably
641 through the action of amino acid racemases [48]. We thus hypothesized that Ap^{WJL}
642 and Lp^{NC8} could provide amino acids to their host by releasing them in the HD. To
643 directly test this hypothesis, we cultured Ap^{WJL} and Lp^{NC8} in liquid HD lacking each
644 EAA^{Fly} and quantified the production of the corresponding missing EAA^{Fly}. We

645 focused on EAA^{Fly}, whose deficiency could be compensated by the commensals in
646 our DT experiments (Fig. 4A). In these assays, Ap^{WJL} was cultured under agitation
647 and Lp^{NC8} cultures were grown in both agitated and static conditions (see Material
648 and Methods). After three days, we quantified the amino acids concentration from
649 bacterial supernatants using high performance liquid chromatography (HPLC). We
650 quantified amino acid production by Ap^{WJL} under agitation while growing in HDΔArg,
651 HDΔHis, HDΔIle, HDΔLeu, HDΔLys, HDΔMet, HDΔPhe, HDΔThr, and HDΔVal and
652 observed accumulation of all missing AAs expect for Lys and Met (Fig 6A). For Lp^{NC8},
653 we analyzed the supernatants of HDs that support Lp^{NC8} growth under agitation (Fig
654 3A): HDΔHis, HDΔLys and HDΔMet. We also analyzed supernatants from static
655 conditions, HDΔHis, HDΔLys, HDΔMet, HDΔPhe and HDΔThr. Surprisingly, from all
656 tested conditions we only detected His accumulation in the supernatant of Lp^{NC8}
657 grown on HDΔHis under agitation (Fig 6B). We did not detect Lys and Met in Ap^{WJL}
658 culture supernatant or Lp^{NC8} culture under agitation supernatant nor His, Lys, Met,
659 Phe or Thr in Lp^{NC8} static culture supernatants. However, Ap^{WJL} or Lp^{NC8} can both
660 fulfill larval requirements in a HD lacking these AA (Fig 4A). We only analyzed
661 supernatants after 72h of growth, it is therefore possible that we missed the peak of
662 accumulation of the targeted AA, which may have taken place at another time point
663 during the growth phase. Also, Ap^{WJL} and Lp^{NC8} may only secrete precursors or
664 catabolites of these AA that we did not target in our analysis. Such AA derivatives
665 may also be used by the larvae to compensate for the lack of the cognate amino
666 acids in the diets (like nicotinate or pyridoxine intermediates, see above).
667 Alternatively, the culture conditions of bacteria on liquid holidic diet are likely to differ
668 from the conditions encounter in the larval guts, and both Ap^{WJL} and Lp^{NC8} could be
669 receiving cues from the larva itself to produce and/or secrete these nutrients while in

the gut. However, we detected Arg, His, Ile, Leu, Phe, Thr and Val production by Ap^{WJL} and His by Lp^{NC8}, a production which correlates with the respective ability of Ap^{WJL} and Lp^{NC8} to compensate for the lack of these AAs in the respective depleted HD. Of note, the concentration of newly synthesized amino acids accumulating in the supernatant is low compared to their concentration in a complete HD (20-150 µM in the former vs 1-5 mM in the latter). However, the bacterial supply of amino acids to the larvae is probably a continuous process, which may also be stimulated upon uptake and transit through the larval intestine. Thus, amino acids would directly be supplied to the larvae and will fulfill its nutritional requirements without the need to accumulate in the surrounding media.

Altogether, our results show that Ap^{WJL} and Lp^{NC8} are able to synthesize and excrete some EAA^{Fly} in their supernatants. These results confirm our hypothesis that *Drosophila* commensal bacteria Ap^{WJL} and Lp^{NC8} produce these EAA^{Fly} while growing on $HD\Delta EAA^{Fly}$. When associated to *Drosophila* larvae, Ap^{WJL} and Lp^{NC8} will therefore supply these amino acids to the larvae, allowing larval development on these deficient media as observed upon mono-associations (Fig 4A).

Conclusion

In this study, we have unraveled the interactions between the nutritional environment of *D. melanogaster* and two of its associated bacteria, as well as the functional importance of these interactions for *Drosophila* juvenile growth. We systematically characterized, both *in silico* and *in vivo*, the biosynthetic capacities of growing GF larvae and two model bacterial strains behaving as natural commensals of *Drosophila* (Ap^{WJL} and Lp^{NC8}). We show that both bacteria, each in its unique manner, alleviate the nutritional constraints in the environment to accelerate host growth and maturation in diets depleted in essential nutrients (Fig 7). The capacity of the bacteria to fulfill 19 of the requirements in 22 essential nutrients for larvae correlated with their metabolic activity and in most cases (15 out of 19) their ability to produce the missing nutrient. In contrast to obligate symbioses, our results highlight the clear separation between the metabolic pathways of the host and its commensals and reveal a particularly integrated nutritional network between the insect and its facultative commensals around the sparing and utilization of nutrients.

Importantly, we further substantiate that the host requirement for essential nutrients can be fulfilled by bacterial provision of a metabolic intermediate of such nutrient (2 out of 19), for example nicotinate intermediates by both Ap^{WJL} and Lp^{NC8} or pyridoxine intermediates by Ap^{WJL}. Interestingly, we also detected two situations where nutrient compensation is not explained by a direct supply of the given nutrient or a metabolic intermediate: (1) the compensation of choline deficiency by Ap^{WJL} and (2) the compensation of Zn deficiency by both Ap^{WJL} and Lp^{NC8}. We propose the existence of functional compensation mechanisms whereby Ap^{WJL} would complement choline deficiency by synthesizing and providing functional analogues of choline derivatives, such as ethanolamine or glycerol derivatives. In addition, both

commensals would compensate Zn deficiency by uptaking, concentrating and sparing contaminant traces of Zn to the host.

Previous works have shown different mechanisms of growth-promotion by microbes in a global low-nutrient context: *Drosophila* larvae can feed on inert microbes to extract nutrients [30,79,93]; alive commensal microbes can improve amino acid absorption by increasing the host's intestinal peptidases activity [23,32] and increase nutrient sensing-related hormonal signals [20,25]. Here we show that in addition, the metabolic activities of alive *Drosophila*'s commensal bacteria correct host auxotrophies. These results reveal a novel facet of the facultative nutritional mutualism engaged between *Drosophila* and its commensal bacteria, which supports the host's nutritional versatility and may allow its juvenile forms to better cope with changes in nutrients availability during the critical phase of post-natal growth; hence ensuring optimal host fitness. Our work lays the basis for further mechanistic studies to investigate if and how commensals regulate the synthesis and release of essential nutrients for the host and if the host influences this process. Dissecting how commensals functionally compensate for nutrients that they cannot produce, catabolize excess of nutrients or detoxify toxic molecules also constitute attractive perspectives for future investigations.

In some cases, the genome-based predictions of bacterial biosynthetic capabilities were incongruent with our *in vivo* assessment of bacterial auxotrophies (Table S6). Such seeming discrepancy served as an entry point for us to discover novel phenomena and interactions that would have been missed had we only adopted a single approach. One such interesting example is Asn auxotrophy unique to the *Drosophila yw* line in GF conditions. Another one is the larval provision of Cys (or its derivatives) to Lp^{NC8} to maintain a mutualistic nutritional exchange between

host and commensal. Previously, combination of *in silico* and *in vivo* approaches has been successfully used for bacteria [62] but not applied to complex symbiotic systems such as facultative host-commensals nutritional interactions. Indeed, reports characterize these interactions *in silico* [94] but they were not confirmed *in vivo*. Our work fulfills this gap and emphasizes the importance of using parallel systematic genome-based annotation, pathway reconstruction and *in vivo* approaches for the understanding of the intricate relationships between the microbial and the nutritional environments and their impact on animal juvenile growth.

Materials and Methods

Expert automated genome annotation and reconstruction of the biosynthetic potential of *D. melanogaster, A. pomorum*^{WJL} *and L. plantarum*^{NC8}

We used the Cyc Annotation Database System (CycADS) [59], an automated annotation management system, to integrate protein annotations from the complete genomes of *Drosophila melanogaster* (RefSeq GCF_000001215.4 release 6), *A. pomorum* strain DM001 (accession NCBI- Bioproject PRJNA60787) and *L. plantarum* subsp. *plantarum* NC8 (NCBI Bioproject PRJNA67175). CycADS collects protein annotation results from different annotation methods including KAAS [95], PRIAM [96], Blast2GO [97,98], and InterProScan [99] in order to obtain Enzyme Commission numbers and Gene Ontology annotations. All annotation information was then processed in the CycADS SQL database and automatically extracted to generate appropriate input files to build the three BioCyc databases using the Pathway Tools software v22.5 [100]. The BioCyc databases and their associated metabolic networks are available in the EcoCyc database [101]. From the genomic analyses, we inferred the biosynthetic capabilities of the three organisms and manually inspected all pathways allowing production of the organic compounds that are present in the exome-based FLYAA holidic diet (HD) [40]. For each gap found in biosynthetic pathways or non-conventional enzymatic catalysis, TBLASTN [102] searches were performed in the three genomes to look for unpredicted protein activities. Alternative pathways were searched in the literature or using the BioCyc “Metabolic Route Search” tool [103].

Drosophila diets, stocks and breeding

D. melanogaster stocks were reared as described previously [32]. Briefly, flies were kept at 25°C with 12/12-hour dark/light cycles on a yeast/cornmeal medium containing 50 g/L of inactivated yeast, 80 g/L of cornmeal, 7.4 g/L of agar, 4 mL/L of propionic acid and 5.2 g/L of nipagin. Germ-free stocks were established as described previously [86] and maintained in yeast/cornmeal medium supplemented with an antibiotic cocktail composed of kanamycin (50 µg/mL), ampicillin (50 µg/mL), tetracycline (10 µg/mL) and erythromycin (5 µg/mL). Axenicity was tested by plating fly media on nutrient agar plates. *D. melanogaster yw* flies were used as the reference strain in this work. Other *D. melanogaster* lines used include a wild-type strain from the DGRP collection, DGRP-*RAL*-25210 [78], and the w^{1118} line [104].

Experiments were performed on Holidic Diet (HD) without preservatives. Complete HD, with a total of 8 g/L of amino acids, was prepared as described by Piper at al. using the fly's exome matched amino acid ratios (FLYAA) [40]. Briefly, sucrose, agar, amino acids with low solubility (Ile, Leu and Tyr) as well as stock solutions of metal ions and cholesterol were combined in an autoclavable bottle with milli-Q water up to the desired volume, minus the volume of solutions to be added after autoclaving. After autoclaving at 120°C for 15 min, the solution was allowed to cool down at room temperature to ~60 °C. Acetic acid buffer and stock solutions for the essential and non-essential amino acids, vitamins, nucleic acids and lipids were added. Single nutrient deficient HD was prepared following the same recipe excluding the nutrient of interest (named HD∆X, X being the nutrient omitted). Tubes used to pour the HD were sterilized under UV for 20 min. HD was stored at 4°C until use, for no longer than one week.

Bacterial strains and growth conditions

795 *A. pomorum*^{WJL} (Ap^{WJL}) [25], *L. plantarum*^{NC8} (Lp^{NC8}) [105], Ap^{WJL}::Tn*pqq* [25]
796 and Lp^{NC8}Δ*dlt*$_{op}$ [23] were used in this study. Ap^{WJL} is a commensal of *Drosophila* lab
797 stocks and has been isolated from the midgut of an adult *Drosophila* [19]. Lp^{NC8} is not
798 a natural commensal of the *Drosophila* gut as it has been isolated from grass silage
799 [105] but we previously showed that it behaves as a commensal [23] and we use this
800 strain as a model *Drosophila* commensal thanks to its genetic tractability (no plasmid
801 and high transformation efficiency). *A. pomorum* strains were cultured in 10 mL of
802 Mannitol Broth (Bacto peptone 3 g/L, yeast extract 5 g/L, D-mannitol 25 g/L) in 50 mL
803 flask at 30°C under 180 rpm during 24h. *L. plantarum* strains were cultured in 10 mL
804 of MRS broth (Carl Roth, Germany) in 15 mL culture tubes at 37°C, without agitation,
805 overnight. Liquid or solid cultures of Ap^{WJL}::Tn*pqq* were supplemented with
806 kanamycin (Sigma-Aldrich, Germany) at a final concentration of 50 µg/mL. CFU
807 counts were performed for all strains on MRS agar (Carl Roth, Germany) plated
808 using the Easyspiral automatic plater (Intersciences, Saint Nom, France). The MRS
809 agar plates were then incubated for 24-48h at 30°C for Ap^{WJL} or 37°C for Lp^{NC8}. CFU
810 counts were done using the automatic colony counter Scan1200 (Intersciences, Saint
811 Nom, France) and its counting software.
812
813 **Bacterial growth in liquid HD**
814 To assess bacterial growth in the fly nutritional environment we developed a
815 liquid HD comprising all HD components except agar and cholesterol. Liquid HD was
816 prepared as described for HD. Single nutrient deficient liquid HD was prepared
817 following the same recipe excluding the nutrient of interest. After growth in culture
818 media, PBS washed Ap^{WJL} or Lp^{NC8} were inoculated at a final concentration of ~ 10^6
819 CFU/mL in 200 µL of either complete liquid HD or nutrient deficient liquid HD.

820 Cultures were incubated in 96-well microtiter plates (Nunc™ Edge 2.0. Thermo
821 Fisher Scientific) at 30°C for 72h. Growth was monitored using an SPECTROstar^{Nano}
822 (BMG Labtech GmbH, Ortenberg, Germany) by measuring the optical density at 600
823 nm (OD_{600}) every 30 minutes. Heatmap in Fig 3A represents the maximal OD
824 detected during the 72h of growth (average of three replicates). The whole
825 experiment was repeated at least twice. Fig 3A was created using the imagesc
826 function on MATLAB (version 2016b. MathWorks, Natick, Massachusetts). Lp^{NC8}
827 growth in static conditions was performed in 10 mL of liquid HD in 15-mL falcon tubes
828 inoculated at a final concentration of ~10^4 CFU/mL. Cultures were incubated at 30°C
829 for 72h. After incubation, cultures were diluted in PBS and plated on MRS agar as
830 described above.
831
832 **Bacterial growth in solid HD**
833 Bacterial CFUs in HDΔCys were assessed in presence or absence of
834 *Drosophila* larvae. Microtubes containing 400 µL of HD and 0.75–1 mm glass
835 microbeads were inoculated with ~10^4 CFUs of Lp^{NC8}. 5 first-instar larvae, collected
836 from eggs laid on HDΔCys, were added. The tubes were incubated at 30°C for 0, 3 or
837 6 days. After incubation, 600µL of PBS were added directly into the microtubes.
838 Samples were homogenized with the Precellys 24 tissue homogenizer (Bertin
839 Technologies, Montigny-le-Bretonneux, France). Lysates dilutions (in PBS) were
840 plated on MRS and CFU counts were assessed as described above.
841
842 **Developmental timing determination**
843 Axenic adults were placed in sterile breeding cages overnight to lay eggs on
844 sterile HD. The HD used to collect embryos always matched the experimental

845 condition. Fresh axenic embryos were collected the next morning and seeded by
846 pools of 40 in tubes containing the HD to test. For the mono-associated conditions a
847 total of ~10^7 CFUs of Ap^{WJL} or ~10^8 CFUs of Lp^{NC8}, washed on PBS, were inoculated
848 on the substrate and the eggs. Inoculation of Ap^{WJL} was limited to ~10^7 CFUs
849 because higher inoculums decreased egg-to-pupa survival. For heat killed (HK)
850 conditions, washed cells of Ap^{WJL} or Lp^{NC8} were incubated 3h at 65°C. Once at room
851 temperature, embryos were inoculated with ~10^8 HK CFUs and ~10^9 HK CFUs,
852 respectively. In the germ-free conditions, bacterial suspensions were replaced with
853 sterile PBS. Tubes were incubated at 25°C with 12/12-hour dark/light cycles. The
854 emergence of pupae was scored every day until all pupae had emerged. The
855 experiment was stopped when no pupae emerged after 30 days. Each gnotobiotic or
856 nutritional condition was inoculated in five replicates. Means, standard error of the
857 mean and statistical tests (Dunn's test of multiple comparisons) are detailed in Table
858 S4. Since larvae are cannibalistic and can find missing nutrients by eating their
859 siblings [106,107], we therefore excluded replicates with low egg-to-pupa survival
860 (<25%,i.e. n<10). Moreover, we considered that larvae failed to develop in one
861 condition if the mean egg-to-pupa survival of the five replicates was inferior to 25%
862 (for details in egg-to-pupae survival, see Table S5). D_{50} was determined using
863 D50App (http://paulinejoncour.shinyapps.io/D50App) as described previously [23].
864 The whole experiment was repeated at least twice. D_{50} heatmap represents the
865 average of the five replicates of each gnotobiotic and nutritional condition. Fig 4A
866 was done using the imagesc function on MATLAB (version 2016b. MathWorks,
867 Natick, Massachusetts).
868
869 **Nicotinate and pyridoxine quantification by HPLC/MS**

After growth in culture media, PBS washed Ap^{WJL} or Lp^{NC8} were inoculated in triplicates at a final concentration of ~10^6 CFU/mL into 10 mL of liquid HDΔNicotinateΔPyridoxineΔCholine and HDΔNicotinate, respectively. Ap^{WJL} was grown under agitated conditions (50 mL flasks incubated at 30°C under 180 rpm). Lp^{NC8} was grown under static conditions (15-mL falcon tubes at 30°C). Samples were taken at times 0h and 72h. Samples were centrifuged (5000 rpm, 5 min). Supernatants were collected and stored at -20°C until use. Supernatants were separated on a PFP column (150 × 2.1 mm i.d., particle size 5 µm; Supelco Bellefonte, PEN, USA). Solvent A was 0.1 % formic acid in H20 and solvent B was 0.1 % formic acid in acetonitrile at a flow rate of 250 µL/min. Solvent B was varied as follows: 0 min: 2 %, 2 min: 2 %, 10 min: 5 %, 16 min: 35 %, 20 min: 100 % and 24 min: 100%. The column was then equilibrated for 6 min at the initial conditions before the next sample was analyzed. The volume of injection was 5 µL. High-resolution experiments were performed with a Vanquish HPLC system coupled to an Orbitrap Qexactive+ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a heated electrospray ionization probe. MS analyses were performed in positive FTMS mode at a resolution of 70 000 (at 400 m/z) in full-scan mode, with the following source parameters: the capillary temperature was 320 °C, the source heater temperature, 300 °C, the sheath gas flow rate, 40 a.u. (arbitrary unit), the auxiliary gas flow rate, 10 a.u., the S-Lens RF level, 40 %, and the source voltage, 5 kV. Metabolites were determined by extracting the exact mass with a tolerance of 5 ppm. The limit of detection was determined following the ERACHEM guideline [108]. Nicotinate, and pyridoxine standards were mixed at 5 µM and diluted 13 times up to 0.48 x 10^{-3} µM. Each solution was injected 3 times. The limit of detection was determined as: LOD=3 x s'0 where s'0 is the standard deviation of the intercept.

895

896 **Choline quantification by RMN**

897 After growth in culture media, PBS washed Ap^{WJL} were inoculated in triplicates
898 at a final concentration of ~10^6 CFU/mL into 10 mL of liquid
899 HD∆Nicotinate∆Pyridoxine∆Choline. Ap^{WJL} was then grown under agitated conditions
900 (50 mL flasks incubated at 30°C under 180 rpm). Samples were taken at times 0h
901 and 72h. Samples were centrifuged (5000 rpm, 5 min). Supernatants were collected
902 and stored at -20°C until use. Supernatants were analyzed by 1H 1D NMR on a
903 Bruker Ascend 800 MHz spectrometer equipped with a CPCI 5 mm cryoprobe. A
904 volume of 540 µL of supernatant was mixed to 60 µL of Trimethylsillyl Propionic Acid
905 (TSP) 10mM solution in D2O for spectra calibration. A 1D 1H NMR sequence with
906 water presaturation and a pulse angle of 30° and a complete relaxation delay of 7
907 sec was used. An acquisition of 64 k points was acquired (2 sec acquisition time) and
908 processed with 256 k points.

909

910 **DNA extraction and AsnS locus analyses**

911 Genomic DNA from 2 adults *yw* flies was extracted as previously described [109].
912 Briefly, flies were grinded in microtubes containing 0.75–1 mm glass microbeads and
913 500 µL of lysis buffer (Tris-HCl 10 mM, EDTA 1 mM, NaCl 1 mM at pH 8.2) using the
914 Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux,
915 France). Then, we added Proteinase K (PureLink Genomic DNA extraction kit,
916 Invotrogen) at a final concentration of 200 µg/mL and incubated the samples at 56°C
917 under 700 rpm agitation for 1h. Samples were centrifuged at 10000 g for 2 min and
918 we collected the supernatant. *AsnS* coding sequence was amplified by PCR (Q5 Pol
919 High Fidelity M0491S, New England BioLabs) using the primers AsnS_F

(CGGGCCGCTTCGTTAAAAA) and AsnS_R (TGGAATTCCTCAGACTTGCCA) with a Veriti Thermal Cycler (Applied BioSystems, USA). PCR products were purified using the NucleoSpin Gel and PCR Cleanup kit (Macherey-Nagel, Germany) following manufacturer's instructions. Sequencing was done by Sanger sequencing (Genewiz, Germany) using the following primers: AsnS_F, AsnS_R, AsnS1 (AGGATTATGGAAAGGATCTTCTGCA), AsnS2 (CTCCGGTCGGATTTGCATCA), AsnS3 (TAATGCCAAAGGGGTCTCGG) and AsnS4 (GTGCGCCAGCTGCATTTATC). The whole coding sequence was then assembled and analyzed using Geneious (version 10.1.3. Biomatters Ltd. New Zealand) by mapping on the reference *D. melanogaster* genome (RefSeq GCF_000001215.4 release 6).

Amino acid quantification by HPLC

After growth in culture media, PBS washed Ap^{WJL} or Lp^{NC8} were inoculated in triplicates at a final concentration of ~10^6 CFU/mL into 10 mL of each liquid HDΔEAA^{Fly} shown to support their growth and in which they fulfill larval requirements (Fig 3A, Fig 4A). For Ap^{WJL} this includes liquid HDΔArg, HDΔHis, HDΔIle, HDΔLeu, HDΔLys, HDΔMet, HDΔPhe, HDΔThr, HDΔVal in agitated conditions. For Lp^{NC8} this includes liquid HDΔHis, HDΔLys and HDΔMet in agitated conditions and liquid HDΔHis, HDΔLys, HDΔMet, HDΔPhe and HDΔThr in static conditions. For agitated conditions, cultures were done in 50 mL flasks and incubated at 30°C under 180 rpm. Static conditions were performed in 15-mL falcon tubes at 30°C. Samples were taken at times 0h and 72h. Samples were centrifuged (5000 rpm, 5 min). Supernatants were collected and stored at -20°C until use.

Amino acid quantification was performed by HPLC from the supernatants obtained at 0h and 72h. Samples were crushed in 320 µl of ultra-pure water with a known quantity of norvaline used as the internal standard. Each sample was submitted to a classical protein hydrolysis in sealed glass tubes with Teflon-lined screw caps (6N HCl, 115°C, during 22h). After air vacuum removal, tubes were purged with nitrogen. All samples were stored at -20°C, and then mixed with 50 µL of ultra-pure water for amino acids analyses. Amino acid analysis was performed by HPLC (Agilent 1100; Agilent Technologies, Massy, France) with a guard cartridge and a reverse phase C18 column (Zorbax Eclipse-AAA 3.5 µm, 150 × 4.6 mm, Agilent Technologies). Prior to injection, samples were buffered with borate at pH 10.2, and primary or secondary amino acids were derivatized with ortho-phthalaldehyde (OPA) or 9-fluorenylmethyl chloroformate (FMOC), respectively. The derivatization process, at room temperature, was automated using the Agilent 1313A autosampler. Separation was carried out at 40°C, with a flow rate of 2 mL/min, using 40 mM NaH2PO4 (eluent A, pH 7.8, adjusted with NaOH) as the polar phase and an acetonitrile/methanol/water mixture (45/45/10, v/v/v) as the non-polar phase (eluent B). A gradient was applied during chromatography, starting with 20% of B and increasing to 80% at the end. Detection was performed by a fluorescence detector set at 340 and 450 nm of excitation and emission wavelengths, respectively (266/305 nm for proline). These conditions do not allow for the detection and quantification of cysteine and tryptophan, so only 18 amino acids were quantified. For this quantification, norvaline was used as the internal standard and the response factor of each amino acid was determined using a 250 pmol/µl standard mix of amino acids. The software used was the ChemStation for LC 3D Systems (Agilent Technologies).

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Figure legends

Fig 1. Expert automated genome annotation and metabolic network reconstruction of Drosophila, Ap^{WJL} and Lp^{NC8}

(A) Amino acids biosynthetic pathways. (B) Vitamins and cofactors biosynthetic pathways. Left panels, *Drosophila melanogaster*. Central panels, *Acetobacter pomorum*^{WJL}. Right panels, *Lactobacillus plantarum*^{NC8}. Color code: blue, biosynthesized amino acids or vitamins; brown, limited amino acid or vitamin biosynthesis (biosynthesis of the metabolite may be possible, but it is limited and/or requires secondary metabolic pathways); black, non-biosynthesized amino acids or vitamins; grey, pathway intermediary metabolites. Red cross: non-functional pathway (lack of key enzyme(s)). Orange nods, major metabolic pathways. Abbreviations: α-

cglu: α-keto-glutarate. AceCoA: Acetyl-CoA. Ant: Antranilate. Aro: Arogenate. Cho: chorismate. Cit: Citrate. Cysta: Cystathionine. Dihyn-P3: 7,8-Dihydroneopterin-3'-P3. Dm-ribi: 6,7-Dimethyl-8-ribityllumazine. Ery-4P: Erythrose-4P. Fum: Fumarate. Glc: Glucose. Gly-3P: Glycerate-3P. Homocys: Homocysteine. Homoser: Homoserine. Ind: Indole. Orn: Ornithine. Oxa: Oxaloacetate. P-ra-imi: 1-(5'-Phospho-ribosyl)-5-aminoimidazole. Phoser: Phosphoserine. Pre: Prephenate. Pyn-P: Pyridoxine phosphate. Pyr: Pyruvate. Rib-5P: Ribose-5P.

Fig 2. *Drosophila*, Ap^{WJL} and Lp^{NC8} have differential biosynthetic capacities of nutrients contained in the holidic diet

Venn diagram represents the number of nutrients present in the FLYAA Holidic Diet that can be synthesized by each organism. The list of corresponding metabolites is provided. Dotted circles: biosynthesis of this metabolite by Lp^{NC8} (green) may be possible, but might be limiting.

Fig 3. Ap^{WJL} and Lp^{NC8} auxotrophies detected in liquid fly Holidic Diet (HD)

(A) Heat map representing the mean maximal optical density (OD_{Max}) reached by Ap^{WJL} or Lp^{NC8} after 72h of culture. Each line shows growth in a different version of liquid HD: complete HD (first line) or HD lacking nutrient X (ΔX, lines below). EAA^{Fly}: Fly's Essential Amino Acids, $NEAA^{Fly}$: Fly's Non-Essential Amino Acids, NAL: Nucleic Acids and Lipids. Cultures were made in 96-wells plate under agitation. Asterisks (*) pinpoint contradictions with our metabolic pathway automated annotations that are explained in panel B. (B) Growth of Lp^{NC8} in four versions of liquid HD: complete HD, HDΔThr, HDΔAla and HDΔAsp, in static conditions. Plot shows means with standard error based on three replicates by assay. Each dot represents an independent

replicate. The dashed line represents the level of inoculation at t0 (10^4 CFUs per mL).

Fig 4. Ap^{WJL} and Lp^{NC8} can differentially fulfill their host's nutritional requirements in holidic diets

(A) Heat map representing the mean D_{50} (*i.e.* day when 50% of the larvae population has entered metamorphosis) of GF larvae (first column) and larvae associated with Ap^{WJL}, Lp^{NC8}, $Ap^{WJL}{}_{HK}$ and $Lp^{NC8}{}_{HK}$ (columns 2, 3, 4, 5 respectively). Each line shows D_{50} in a different version of HD: complete HD (first line) or HD lacking nutrient X (ΔX, lines below). EAA^{Fly}: Fly's Essential Amino Acids, $NEAA^{Fly}$: Fly's Non-Essential Amino Acids, NAL: Nucleic Acids and Lipids. White means larvae did not reach pupariation in these conditions. Means, standard errors of the mean and statistical tests (Dunn's test of multiple comparisons) are detailed in Table S4. (B-D) Absence of correlation between time of development and quantity of bacteria. Y axis shows D50 (*i.e.* day when 50% of the larvae population has entered metamorphosis) and X axis shows quantity of bacteria (Log_{10} CFUs) in the larval gut (B), in the diet in presence of larvae 3 days after inoculation (C) and in the diet in presence of larvae 6 days after inoculation (D). Each dot shows a different condition. Complete HD: on complete HD. ΔX: on HD lacking nutrient X. Black dots: in mono-association with Ap^{WJL}, green dots: in mono-association with Lp^{NC8}. For each bacterium, we tested Pearson's product-moment correlation between D50 and quantity of bacteria. cor: Pearson correlation coefficient for each bacterium.

Fig 5. Evaluation of HDΔAsn, HDΔPhe and HDΔCys contexts

(A) D_{50} of *yw*, DGRP-25210 and w^{1118} larvae on HDΔAsn. Boxplots show minimum, maximum and median. Each dot shows an independent replicate. GF *yw* larvae did not reach pupariation. For the other two lines, we performed Kruskal-Wallis test followed by post-hoc Dunn's tests to compare each gnotobiotic condition to GF. ns: non-significant, **: p-value<0,005, ***: p-value<0,0005, ****: p-value<0,0001. (B) Growth of Lp^{NC8} in liquid HDΔPhe and liquid HDΔCys, in static conditions, 3 days after inoculation. Plot shows mean with standard error. Each dot shows an independent replicate. The dashed line represents the level of inoculation at t0 (10^4 CFUs per mL). (C) Growth of Lp^{NC8} on solid HDΔCys, in absence and in presence of larvae, 3 days and 6 days after inoculation. Plot shows mean with standard error. Each dot represents an independent replicate. The dashed line represents the level of inoculation at t0 (10^4 CFUs per tube). We performed two-ways ANOVA followed by post-hoc Sidak's test. ns: non-significant, **: p-value<0,005.

Fig 6. Ap^{WJL} and Lp^{NC8} can produce and release EAA^{Fly} during growth

(A) HPLC measured concentration of Arg, His, Ile, Leu, Phe, Thr and Val in the supernatant of an Ap^{WJL} culture in HDΔArg, HDΔHis, HDΔIle, HDΔLeu, HDΔPhe, HDΔThr, HDΔVal, respectively, 72h after inoculation. Plot shows mean with standard error. Each dot shows an independent replicate. Each AA was not detected prior to microbial growth (Table S7). (B) HPLC measured concentration of His in the supernatant of a Lp^{NC8} culture in HDΔHis, 72h after inoculation. Plot shows mean with standard error. Each dot shows an independent replicate (53.08 µM, 52.82 µM and 52.99 µM).

Fig 7. Ap^{WJL} and Lp^{NC8} differentially shape the nutritional requirements of their juvenile host

For each gnotobiotic condition, essential nutrients are represented in black and non-essential nutrients in color. Color code: blue, this nutrient can be synthesized by the bacteria; red: this nutrient cannot be synthesized by the bacteria, suggesting a mechanism of functional compensation. In purple: lack of this nutrient may be compensated by an intermediate metabolite or a derivative produced by the bacteria.

Supporting Information

Figure S1: The Asn auxotrophy of the *yw* line is not due to mutations in *AsnS* gene.

Pairwise alignment of AsnS coding region sequenced from *D. melanogaster yw* and AsnS coding region from *D. melanogaster* reference genome, Bloomington #2057.

Figure S2: $Lp^{NC8}\Delta dlt_{op}$, but not Ap^{WJL}::Tn*pqq*, show a loss-of-function of its intrinsic growth-promoting ability in HD.

D_{50} (*i.e.* day when 50% of the larvae population has entered metamorphosis) of GF larvae and larvae associated with Ap^{WJL}, Ap^{WJL}::Tn*pqq*, Lp^{NC8} and $Lp^{NC8}\Delta dlt_{op}$, reared on complete HD. We performed Kruskal-Wallis test followed by *post hoc* Dunn's tests to compare each gnotobiotic condition to GF. ns: non-significant, *: p-value<0,05, ****: p-value<0,0001.

Figure S3: Quantity of Ap^{WJL} and Lp^{NC8} on different HDs, in presence of larvae.

(A) Bacterial load per larva, at day 6 post-inoculation. Boxplots show minimum, maximum and median. Each dot shows an independent replicate. (B) Load of Ap^{WJL} and Lp^{NC8} in solid HD in presence of larvae, 3 days and 6 days after inoculation. Plot shows mean with standard error based on three replicates by assay. Each dot represents an independent replicate. The dashed line represents the level of inoculation at t0 (10^4 CFUs per tube).

Table S1: Inference from genomic analysis of the biosynthetic capabilities for amino acids production in *D. melanogaster, A. pomorum*^{WJL} and *L. plantarum*^{NC8}. f / i: targeted amino acid biosynthesis is feasible / impossible in a depleted medium.

Table S2: Inference from genomic analysis of the biosynthetic capabilities for vitamins production in *D. melanogaster, A. pomorum*^{WJL} and *L. plantarum*^{NC8}. f / i: targeted vitamin biosynthesis is feasible / impossible in a depleted medium.

Table S3: OD_{Max} of Ap^{WJL} and Lp^{NC8} grown in 39 HDs. Mean and Standard Error to the Mean (SEM) of OD_{Max} reached by Ap^{WJL} or Lp^{NC8} grown in complete liquid HD (first line) or liquid HD lacking nutrient X (ΔX, lines below) during 72h of growth.

Table S4: D_{50} of larvae in 40 HDs and 5 gnotobiotic conditions. Mean and Standard Error of the Mean (SEM) of D_{50} (*i.e.* day when 50% of the larvae population has entered metamorphosis) of GF larvae or larvae associated with Ap^{WJL}, Lp^{NC8}, Ap^{WJL}_{HK} and Lp^{NC8}_{HK}. n: number of independent replicates for each condition. For each gnotobiotic condition, we performed Kruskal-Wallis test followed by post-hoc Dunn's test to compare each nutritional environment to complete HD.

Table S5: Egg-to-pupa survival in 40 HDs and 5 gnotobiotic conditions. Mean and Standard Error of Mean (SEM) of egg-to-pupa survival of GF larvae or larvae associated with Ap^{WJL}, Lp^{NC8}, $Ap^{WJL}{}_{HK}$ and $Lp^{NC8}{}_{HK}$. n: number of independent replicates for each condition. For each gnotobiotic condition, we performed Kruskal-Wallis test followed by post-hoc Dunn's test to compare each nutritional environment to complete HD.

Table S6: Comparison of genome-based metabolic predictions with *in vivo* auxotrophies and bacterial complementation of larval nutritional deficiencies. Can partner A synthesize nutrient X?: prediction from automated annotation and metabolic reconstruction (from Fig 1, Table S1 and Table S2). Can partner A grow in the absence of nutrient X?: auxotrophy observed *in vivo* (from Fig 3A-B). Can bacterial partner A promote larval growth on HD ΔX?: *in vivo* complementation of ex-GF larvae requirements (from Fig 4A), y: yes (green), n: no (red). Hypothesis to explain contradiction: why the different approaches do not always lead to the same conclusion. NA: Non-applicable.

Table S7: All experimental data used to generate graphs of this manuscript.

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1476

Figure 1

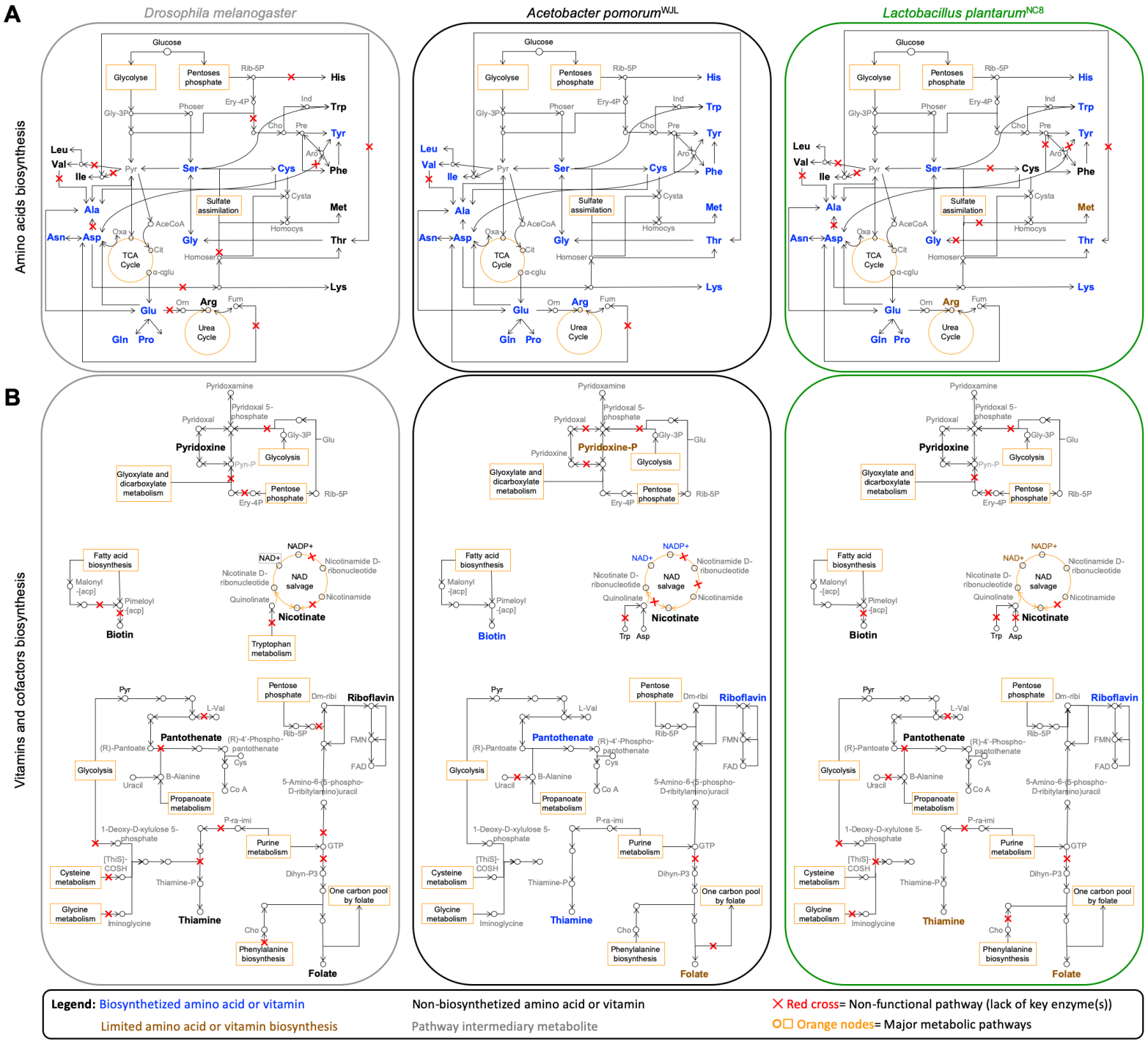

A
Drosophila melanogaster
Acetobacter pomorum WJL
Lactobacillus plantarum NC8
Amino acids biosynthesis
Glucose
Glycolyse
Pentoses phosphate
Rib-5P
His
Trp
Tyr
Phe
Leu
Val
Ile
Ser
Cys
Met
Thr
Lys
Ala
Asn
Asp
Gly
Glu
Gln
Pro
Arg
Sulfate assimilation
TCA Cycle
Urea Cycle
B
Vitamins and cofactors biosynthesis
Pyridoxine
Pyridoxine-P
Glycolysis
Glyoxylate and dicarboxylate metabolism
Pentose phosphate
Fatty acid biosynthesis
Biotin
Nicotinate
NAD salvage
Tryptophan metabolism
Pantothenate
Riboflavin
Propanoate metabolism
Cysteine metabolism
Glycine metabolism
Thiamine
Purine metabolism
One carbon pool by folate
Phenylalanine biosynthesis
Folate
Legend: Biosynthetized amino acid or vitamin
Limited amino acid or vitamin biosynthesis
Non-biosynthetized amino acid or vitamin
Pathway intermediary metabolite
Red cross= Non-functional pathway (lack of key enzyme(s))
Orange nodes= Major metabolic pathways

Figure 2

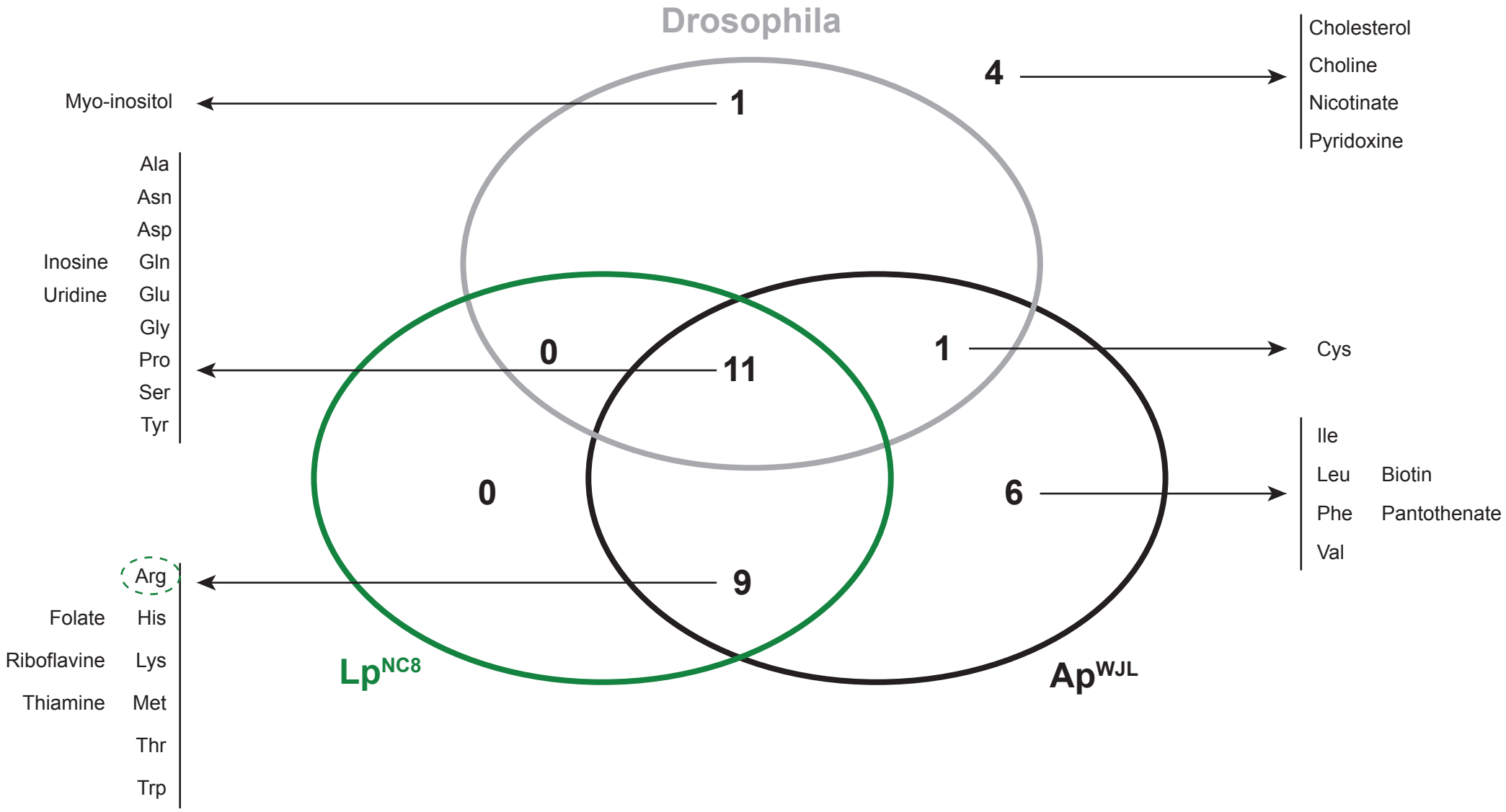
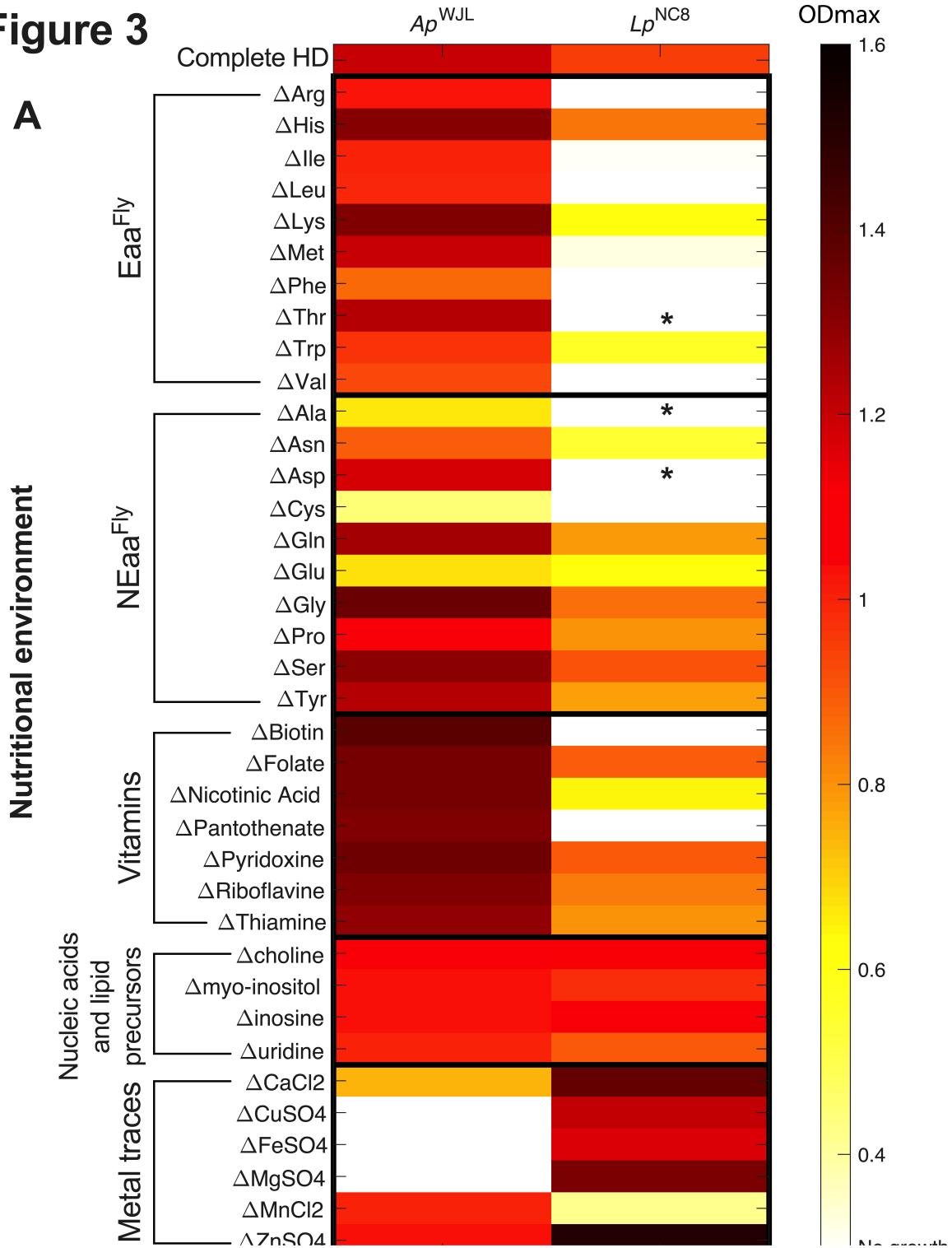

Drosophila
Lp^NC8
Ap^WJL
Myo-inositol
1
4
Cholesterol
Choline
Nicotinate
Pyridoxine
Inosine
Uridine
Ala
Asn
Asp
Gln
Glu
Gly
Pro
Ser
Tyr
0
11
1
Cys
0
6
Ile
Leu
Phe
Val
Biotin
Pantothenate
Arg
His
Lys
Met
Thr
Trp
Folate
Riboflavine
Thiamine
9

Figure 3


A
Ap^WJL
Lp^NC8
ODmax
Complete HD
ΔArg
ΔHis
ΔIle
ΔLeu
ΔLys
ΔMet
ΔPhe
ΔThr
ΔTrp
ΔVal
ΔAla
ΔAsn
ΔAsp
ΔCys
ΔGln
ΔGlu
ΔGly
ΔPro
ΔSer
ΔTyr
ΔBiotin
ΔFolate
ΔNicotinic Acid
ΔPantothenate
ΔPyridoxine
ΔRiboflavine
ΔThiamine
Δcholine
Δmyo-inositol
Δinosine
Δuridine
ΔCaCl2
ΔCuSO4
ΔFeSO4
ΔMgSO4
ΔMnCl2
ΔZnSO4
EaaFly
NEaaFly
Vitamins
Nucleic acids and lipid precursors
Metal traces
Nutritional environment
1.6
1.4
1.2
1
0.8
0.6
0.4

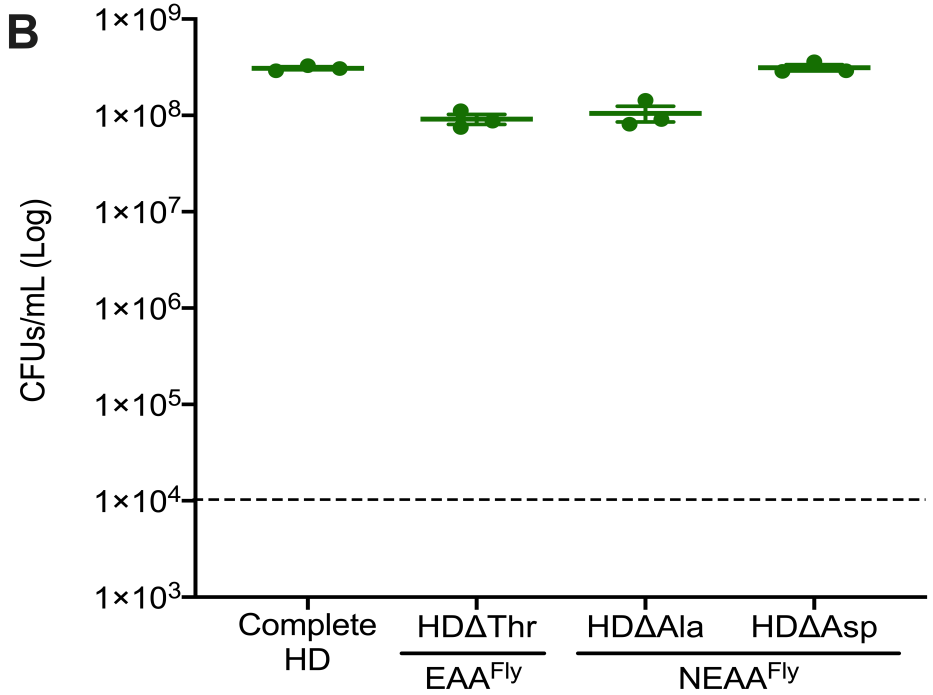

B
CFUs/mL (Log)
1×10^9
1×10^8
1×10^7
1×10^6
1×10^5
1×10^4
1×10^3
Complete HD
HDΔThr
HDΔAla
HDΔAsp
EAAFly
NEAAFly

Figure 4

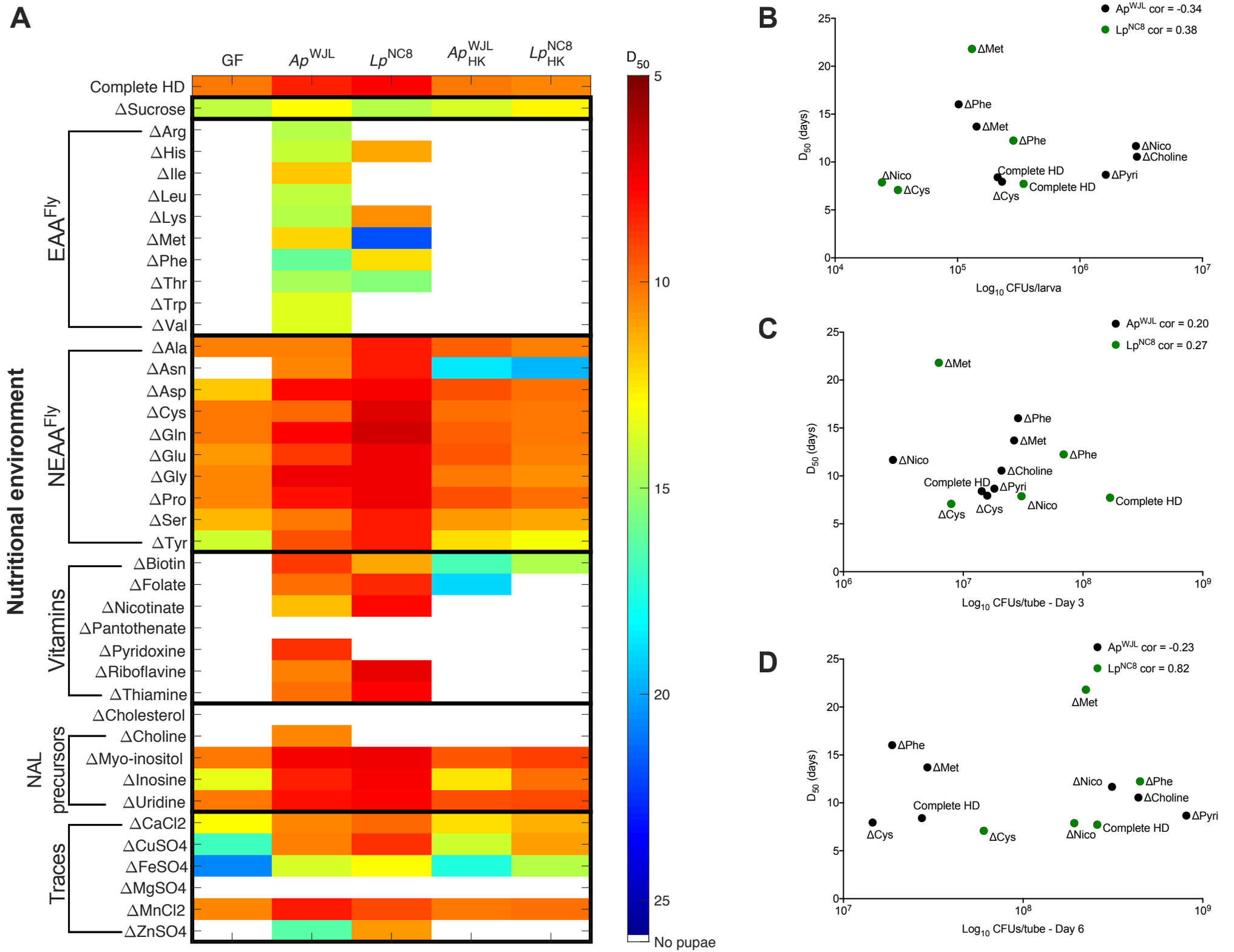

A
GF
Ap^WJL
Lp^NC8
Ap^WJL HK
Lp^NC8 HK
D50
Complete HD
ΔSucrose
ΔArg
ΔHis
ΔIle
ΔLeu
ΔLys
ΔMet
ΔPhe
ΔThr
ΔTrp
ΔVal
ΔAla
ΔAsn
ΔAsp
ΔCys
ΔGln
ΔGlu
ΔGly
ΔPro
ΔSer
ΔTyr
ΔBiotin
ΔFolate
ΔNicotinate
ΔPantothenate
ΔPyridoxine
ΔRiboflavine
ΔThiamine
ΔCholesterol
ΔCholine
ΔMyo-inositol
ΔInosine
ΔUridine
ΔCaCl2
ΔCuSO4
ΔFeSO4
ΔMgSO4
ΔMnCl2
ΔZnSO4
EAA^Fly
NEAA^Fly
Vitamins
NAL precursors
Traces
Nutritional environment
No pupae
B
Ap^WJL cor = -0.34
Lp^NC8 cor = 0.38
D50 (days)
Log10 CFUs/larva
C
Ap^WJL cor = 0.20
Lp^NC8 cor = 0.27
Log10 CFUs/tube - Day 3
D
Ap^WJL cor = -0.23
Lp^NC8 cor = 0.82
Log10 CFUs/tube - Day 6

Figure 5

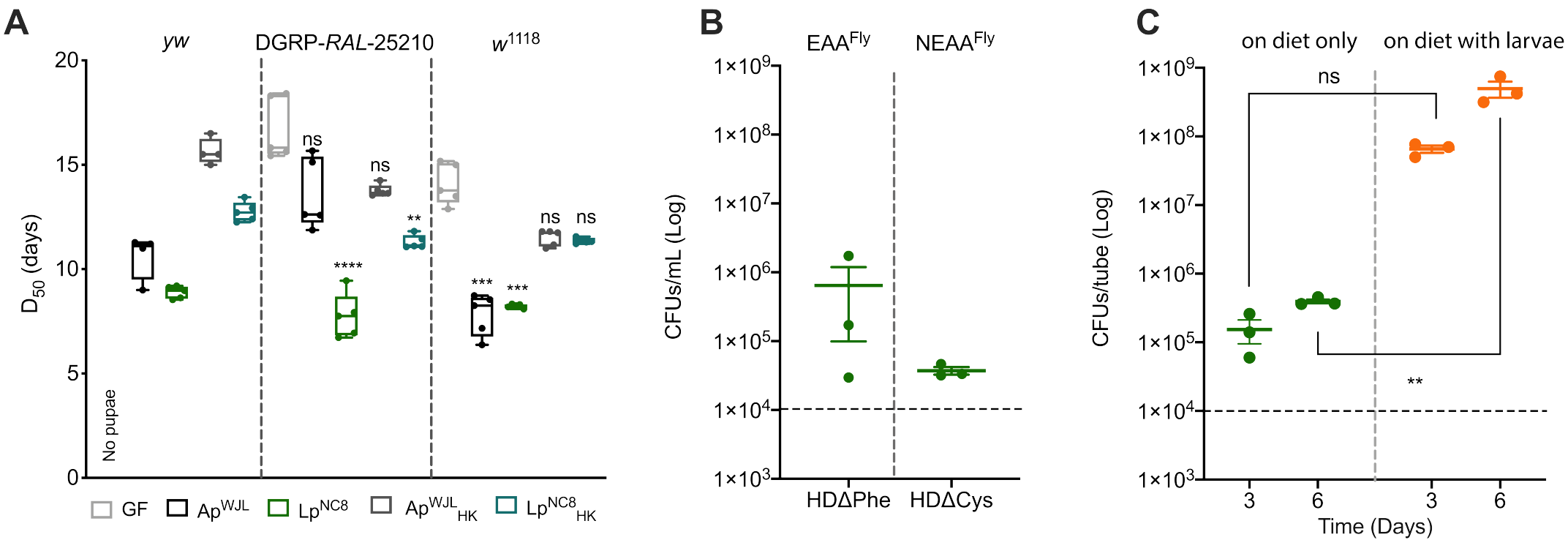

A
yw
DGRP-RAL-25210
w1118
D50 (days)
20
15
10
5
0
No pupae
ns
ns
****
**
ns
ns
***
***
GF
ApWJL
LpNC8
ApWJLHK
LpNC8HK
B
EAAFly
NEAAFly
CFUs/mL (Log)
1×10^9
1×10^8
1×10^7
1×10^6
1×10^5
1×10^4
1×10^3
HDΔPhe
HDΔCys
C
on diet only
on diet with larvae
CFUs/tube (Log)
ns
**
3
6
3
6
Time (Days)

Figure 6

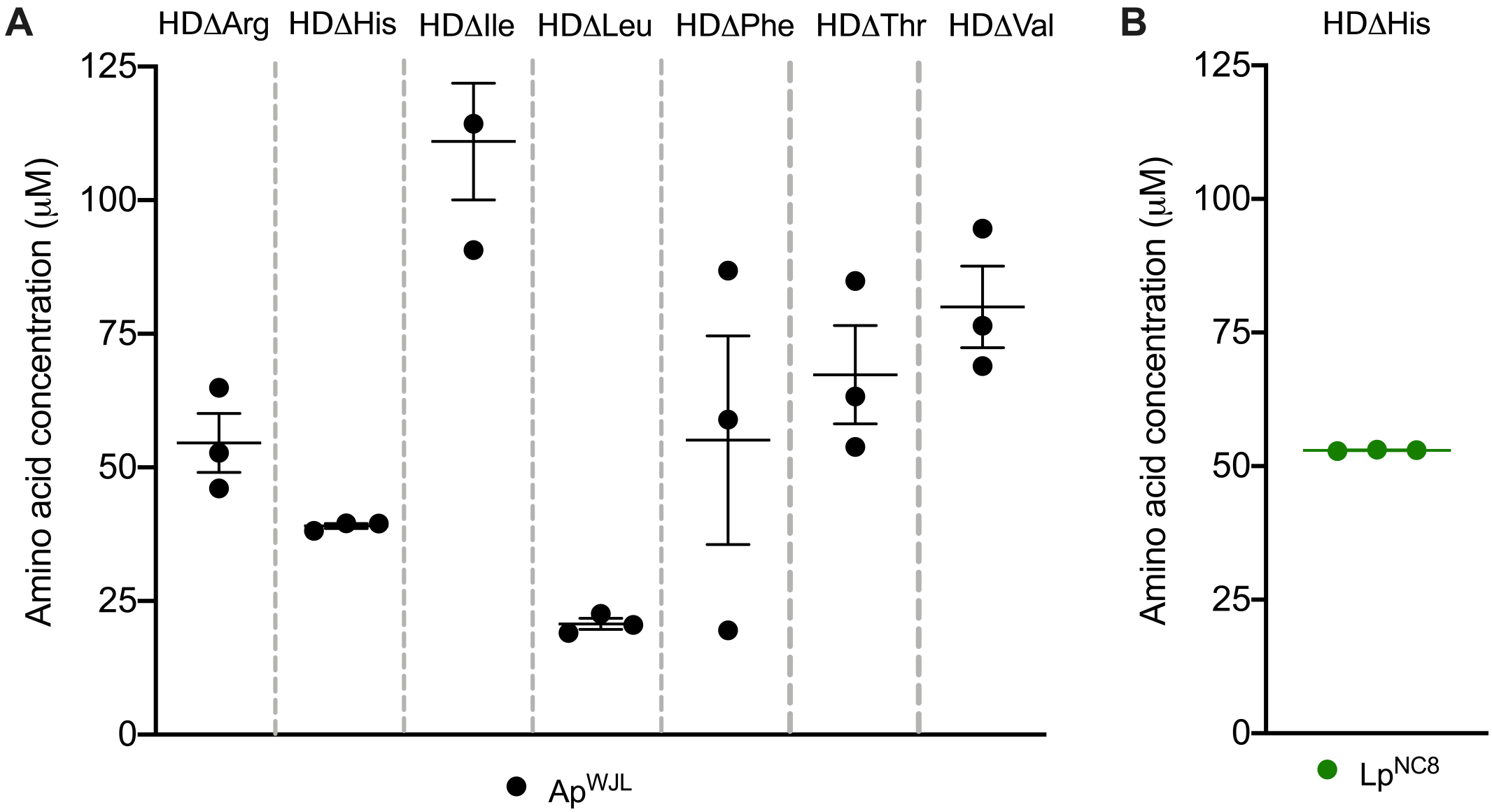
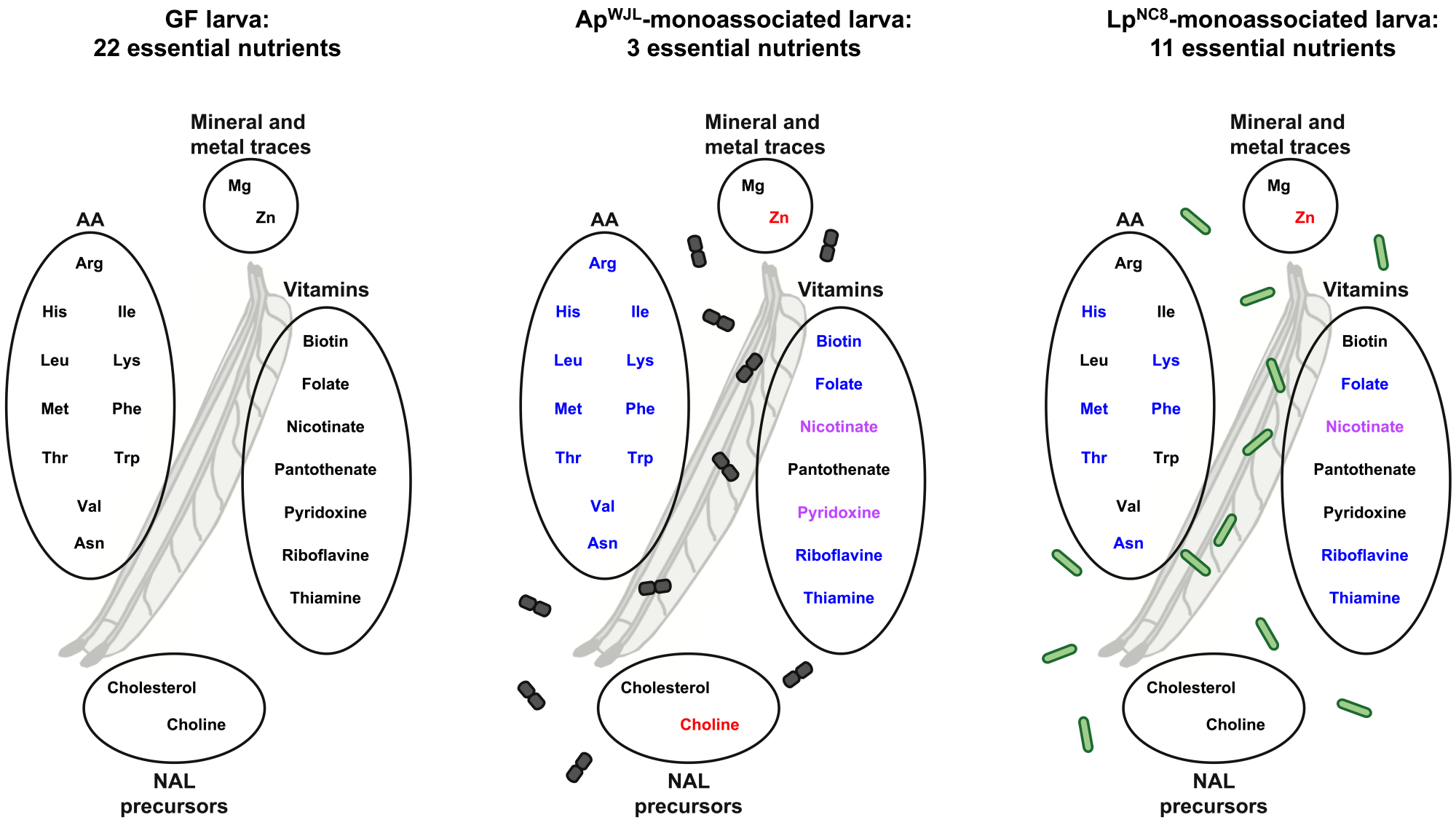

A
HDΔArg
HDΔHis
HDΔIle
HDΔLeu
HDΔPhe
HDΔThr
HDΔVal
Amino acid concentration (μM)
125
100
75
50
25
0
ApWJL
B
HDΔHis
Amino acid concentration (μM)
125
100
75
50
25
0
LpNC8

Figure 7



Legend:

- Drosophila essential nutrient.
- Drosophila essential nutrient, produced by the bacteria.
- Drosophila essential nutrient. Functionally compensated by bacteria (i.e. not produced).
- Drosophila essential nutrient. Compensated by bacteria through synthesis of derivatives.

Figure S1

```
                   1                                 10                                20                                30
AsnS Dm BL#2057    M  C  G  I  F  A  I  F  S  R  D  G  E  P  I  P  T  Q  I  L  H  G  S  K  H  S  L  R  E  L
AsnS Dm yw         M  C  G  I  F  A  I  F  S  R  D  G  E  P  I  P  T  Q  I  L  H  G  S  K  H  S  L  R  E  L
                                                 40                                50                                60
AsnS Dm BL#2057    A  Y  R  Q  S  G  K  H  R  H  R  G  P  D  S  T  G  V  Y  V  N  S  L  E  G  V  A  M  I  H
AsnS Dm yw         A  Y  R  Q  S  G  K  H  R  H  R  G  P  D  S  T  G  V  Y  V  N  S  L  E  G  V  A  M  I  H
                                                 70                                80                                90
AsnS Dm BL#2057    E  R  L  R  I  I  G  V  E  M  G  D  Q  P  F  V  S  E  D  G  N  L  I  L  V  A  N  G  E  I
AsnS Dm yw         E  R  L  R  I  I  G  V  E  M  G  D  Q  P  F  V  S  E  D  G  N  L  I  L  V  A  N  G  E  I
                                                100                               110                               120
AsnS Dm BL#2057    Y  N  Y  L  E  L  S  A  E  I  A  K  K  R  G  S  Y  N  P  M  S  D  C  H  V  I  L  E  L  Y
AsnS Dm yw         Y  N  Y  L  E  L  S  A  E  I  A  K  K  R  G  S  Y  N  P  M  S  D  C  H  V  I  L  E  L  Y
                                                130                               140                               150
AsnS Dm BL#2057    Q  D  Y  G  K  D  L  L  Q  Y  I  T  G  M  F  A  F  A  L  Y  D  R  K  T  K  E  V  L  L  A
AsnS Dm yw         Q  D  Y  G  K  D  L  L  Q  Y  I  T  G  M  F  A  F  A  L  Y  D  R  K  T  K  E  V  L  L  A
                                                160                               170                               180
AsnS Dm BL#2057    R  D  P  F  G  I  I  P  M  Y  V  G  E  D  A  S  G  N  L  W  V  A  S  E  M  K  C  L  V  D
AsnS Dm yw         R  D  P  F  G  I  I  P  M  Y  V  G  E  D  A  S  G  N  L  W  V  A  S  E  M  K  C  L  V  D
                                                190                               200                               210
AsnS Dm BL#2057    T  C  S  K  V  E  T  F  T  P  G  E  A  R  F  G  K  V  G  D  F  K  T  C  W  Q  F  Q  Q  S
AsnS Dm yw         T  C  S  K  V  E  T  F  T  P  G  E  A  R  F  G  K  V  G  D  F  K  T  C  W  Q  F  Q  Q  S
                                                220                               230                               240
AsnS Dm BL#2057    W  I  K  E  V  P  T  Q  T  C  E  L  S  L  L  R  A  N  L  E  F  A  V  R  S  H  L  Q  C  D
AsnS Dm yw         W  I  K  E  V  P  T  Q  T  C  E  L  S  L  L  R  A  N  L  E  F  A  V  R  S  H  L  Q  C  D
                                                250                               260                               270
AsnS Dm BL#2057    V  Q  M  G  A  L  L  S  G  G  V  D  S  S  L  I  A  S  I  A  T  K  I  M  R  E  R  D  P  N
AsnS Dm yw         V  Q  M  G  A  L  L  S  G  G  V  D  S  S  L  I  A  S  I  A  T  K  I  M  R  E  R  D  P  N
                                                280                               290                               300
AsnS Dm BL#2057    F  R  L  K  T  F  S  V  G  L  R  D  A  P  D  F  Q  A  A  R  S  V  A  K  Y  I  D  S  D  H
AsnS Dm yw         F  R  L  K  T  F  S  V  G  L  R  D  A  P  D  F  Q  A  A  R  S  V  A  K  Y  I  D  S  D  H
                                                310                               320                               330
AsnS Dm BL#2057    K  E  I  I  F  E  I  D  E  A  L  D  G  I  R  D  I  I  Y  H  L  E  T  Y  D  V  T  T  V  R
AsnS Dm yw         K  E  I  I  F  E  I  D  E  A  L  D  G  I  R  D  I  I  Y  H  L  E  T  Y  D  V  T  T  V  R
                                                340                               350                               360
AsnS Dm BL#2057    C  S  L  P  M  L  L  L  A  R  Y  I  K  S  T  G  I  K  M  I  L  S  G  E  G  A  D  E  I  F
AsnS Dm yw         C  S  L  P  M  L  L  L  A  R  Y  I  K  S  T  G  I  K  M  I  L  S  G  E  G  A  D  E  I  F
                                                370                               380                               390
AsnS Dm BL#2057    G  G  Y  L  Y  F  H  K  A  P  S  Y  N  D  F  H  E  E  L  V  K  R  V  R  Q  L  H  L  S  D
AsnS Dm yw         G  G  Y  L  Y  F  H  K  A  P  S  Y  N  D  F  H  E  E  L  V  K  R  V  R  Q  L  H  L  S  D
                                                400                               410                               420
AsnS Dm BL#2057    C  L  R  A  N  K  V  A  M  A  K  G  V  E  L  R  V  P  F  L  D  T  G  F  V  N  H  V  M  Q
AsnS Dm yw         C  L  R  A  N  K  V  A  M  A  K  G  V  E  L  R  V  P  F  L  D  T  G  F  V  N  H  V  M  Q
                                                430                               440                               450
AsnS Dm BL#2057    I  R  P  E  D  K  I  P  G  P  L  N  K  F  G  E  V  Q  Q  K  R  L  E  K  Y  V  L  R  A  A
AsnS Dm yw         I  R  P  E  D  K  I  P  G  P  L  N  K  F  G  E  V  Q  Q  K  R  L  E  K  Y  V  L  R  A  A
                                                460                               470                               480
AsnS Dm BL#2057    F  A  D  N  Y  L  P  D  E  V  L  W  R  Q  K  E  Q  F  S  D  G  V  G  Y  D  W  I  D  S  I
AsnS Dm yw         F  A  D  N  Y  L  P  D  E  V  L  W  R  Q  K  E  Q  F  S  D  G  V  G  Y  D  W  I  D  S  I
                                                490                               500                               510
AsnS Dm BL#2057    R  R  V  A  T  S  H  V  S  D  Q  E  F  S  A  A  A  L  R  F  P  F  N  T  P  T  T  K  E  A
AsnS Dm yw         R  R  V  A  T  S  H  V  S  D  Q  E  F  S  A  A  A  L  R  F  P  F  N  T  P  T  T  K  E  A
                                                520                               530                               540
AsnS Dm BL#2057    F  Y  Y  R  C  I  F  A  E  Q  F  P  G  E  S  A  A  R  T  V  V  R  W  V  P  R  L  D  W  G
AsnS Dm yw         F  Y  Y  R  C  I  F  A  E  Q  F  P  G  E  S  A  A  R  T  V  V  R  W  V  P  R  L  D  W  G
                                                550                               558
AsnS Dm BL#2057    C  P  E  D  P  S  G  R  A  Q  A  V  H  Q  V  N  K  N
AsnS Dm yw         C  P  E  D  P  S  G  R  A  Q  A  V  H  Q  V  N  K  N
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Figure S2

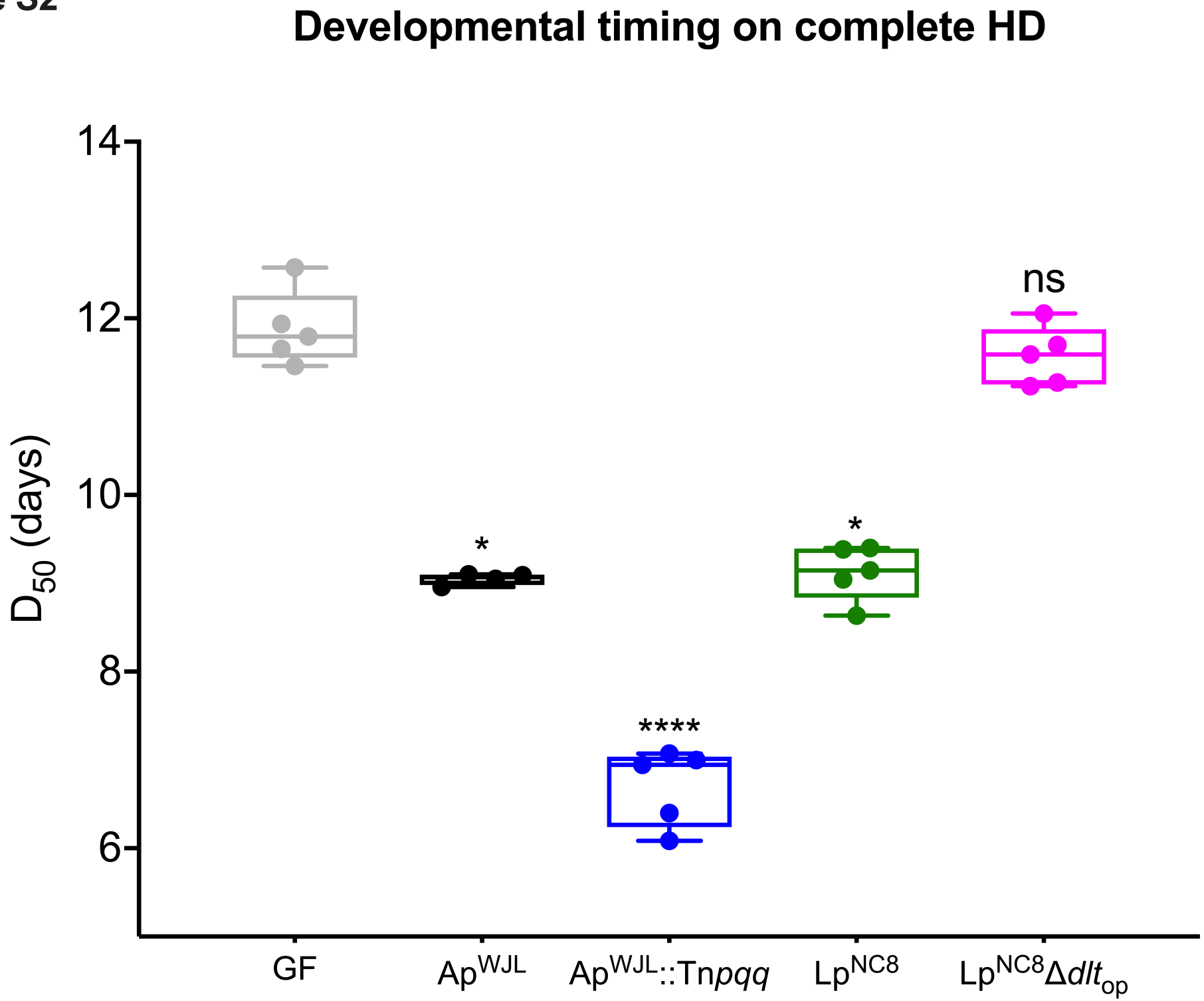
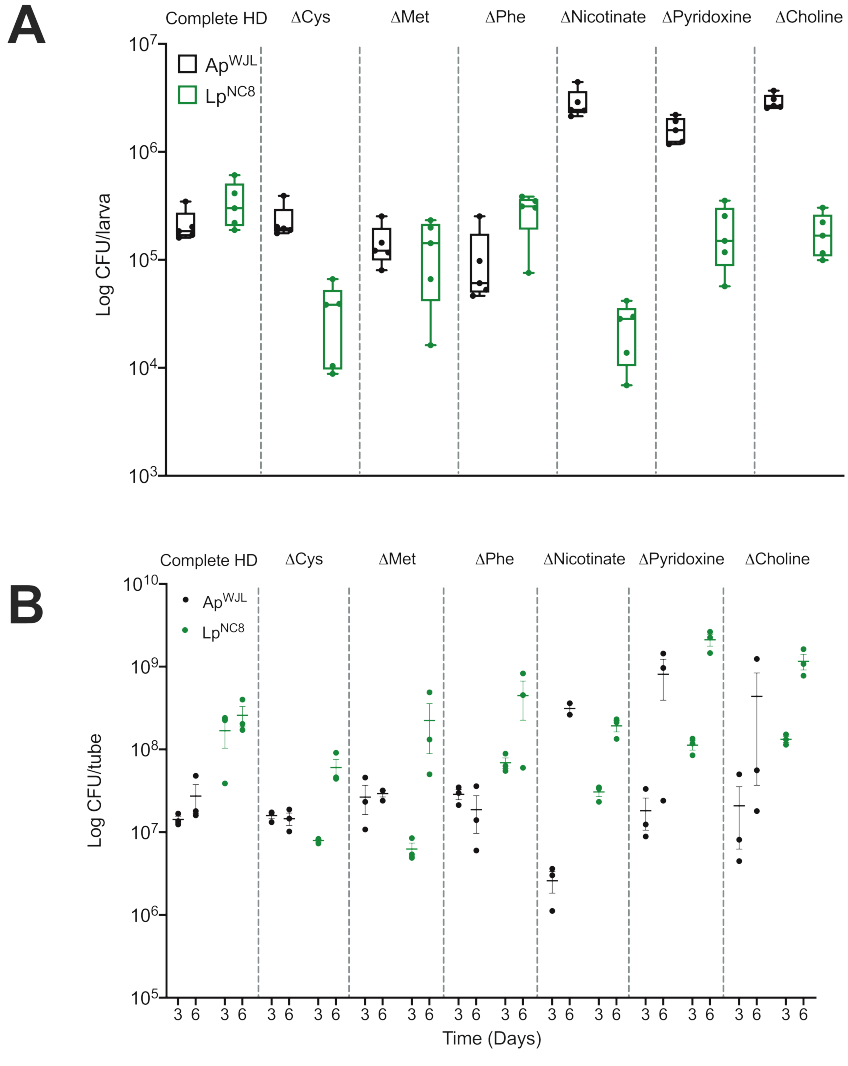

Developmental timing on complete HD
14
12
10
8
6
D50 (days)
ns
*
*
****
GF
ApWJL
ApWJL::Tnpqq
LpNC8
LpNC8Δdlt op

Figure S3


A
Complete HD
ΔCys
ΔMet
ΔPhe
ΔNicotinate
ΔPyridoxine
ΔCholine
Ap^WJL
Lp^NC8
Log CFU/larva
10^7
10^6
10^5
10^4
10^3
B
Complete HD
ΔCys
ΔMet
ΔPhe
ΔNicotinate
ΔPyridoxine
ΔCholine
Ap^WJL
Lp^NC8
Log CFU/tube
10^10
10^9
10^8
10^7
10^6
10^5
3 6
Time (Days)