Social and nutritional factors shape larval aggregation, foraging, and body mass in a polyphagous fly

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Data accessibility

Data will be made available in Dryad upon the acceptance of the manuscript.

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Abstract

The majority of insect species have a clearly defined larval stage during development. Larval nutrition is crucial for individuals' growth and development, and larval foraging success often depends on both resource availability and competition for those resources. To date, however, little is known about how these factors interact to shape larval development and behaviour. Here we manipulated the density of larvae of the polyphagous fruit fly pest *Bactrocera tryoni* ('Queensland fruit fly'), and the diet concentration of patches in a foraging arena to address this gap. Using advanced statistical methods of machine learning and linear regression models, we showed that high larval density results in increased larval aggregation across all diets except in extreme diet dilutions. Larval aggregation was positively associated with larval body mass across all diet concentrations except in extreme diet dilutions where this relationship was reversed. Larvae in low-density arenas also tended to aggregate while those in high-density arenas tended to disperse, an effect that was observed for all diet concentrations. Furthermore, larvae in high-density arenas displayed significant avoidance of low concentration diets – a behaviour that was not observed amongst larvae in low-density arenas. Thus, aggregation can help, rather than hinder, larval growth in high-density environments, and larvae may be better able to explore available nutrition when at high-density than when at low density.

Introduction

In holometabolous insects, larval foraging behaviour largely determines individual fitness (Chapman, 1998). Poor developmental conditions marked by low resource availability – such as when food is scarce and there is high larval competition – often affects both larval developmental time and body size in adulthood [e.g. [1-10]]. Adult body size tends to correlate positively with female fecundity as well as male mating performance and reproductive success [5,11]; accordingly, larval foraging behaviour is under productivity selection in females and sexual selection in males [11-15], with profound effects on behavioural and evolutionary processes such as cognitive task performance, survival, reproduction, and ultimately sexual selection and sexual conflict [6,16-18].

The quantity of resources in a food patch and the number of competing foragers are important determinants of larval responses to developmental conditions [19]. To maximize resource acquisition for investment in fitness traits of adulthood [20,21], larvae are expected to avoid competition with conspecifics, and to prefer patches of highest resource availability. The rationale for this is simple; if the resources are poor or the number of individuals sharing a finite resource is high, the benefits of foraging on that patch may be outweighed by the potential benefits of leaving that patch to seek resources elsewhere. Thus, the ideal situation may be that in which larvae forage in resource-rich food patches without competition. Research across insect taxa has shown that insect larvae have well-defined optimum diets that sustain development and growth, and produce high quality adults [22-27], that an excess of nutrients can be detrimental and even compensated for when larvae have a choice to select their food [e.g. [28-31]]. For social interactions, however, the rule is far less intuitive. Larval aggregations are common in many insects [32,33]. Although such social interactions may increase foraging competition, larval aggregations can confer physiological and behavioural

64 benefits that sustain larval growth and development [34-45]. As a result, larvae may maximize
65 development in a high-quality diet with some degree of social interactions and aggregation,
66 provided that competition is not so high that the benefits of aggregation are negated. For
67 instance, *Drosophila* larvae can benefit from occupying patches that are shared with
68 conspecifics, although the increase in competition can in some cases offset the benefits of
69 social behaviour [45] [see also [46-48]]. This hypothesis is derived from the premise that social and
70 nutritional factors interact to shape larval behaviour and growth during development. To date,
71 however, there have been very few direct empirical tests of this hypothesis.
72
73 An early attempt to demonstrate interactions between nutritional and social factors as
74 determinants of larval development showed that, in the gregarious caterpillar *Hemileuca*
75 *lucina,* social environment interacts with the quality of the food source to determine larval
76 growth at mild temperatures [37]. This investigation only contrasted caterpillars in solitary and
77 groups of a fixed size (10 individuals), and only investigated development on two related-
78 food sources, young vs. mature leaves of *Spiraea latifolia*. Although providing a useful
79 demonstration of concept, this dichotomous approach – i.e. solitary *vs* groups, young *vs*
80 mature leaves – has limited scope for understanding the interaction between social and
81 nutritional factors driving the ecology of larval development. Other studies have shown the
82 importance of larval aggregation in feeding and growth rates, insect-plant interactions, larval
83 defence against predators, and larval thermoregulation [e.g. [34-44]]. However, there has been no
84 detailed investigation of how the social and nutritional environments of larvae interact to
85 shape development and performance. Key questions remain unanswered though, as to 'how
86 does the number of foraging larvae with access to a common resource pool, which increase
87 the potential for social interactions, influence larval aggregation?'; 'When resource
88 availability decreases, do larvae aggregate to the same extent as to when resources are

89 abundant?'; and 'What are the implications of density- and diet-dependent larval aggregation
90 to larval growth and foraging behaviour?'
91
92 In the present study, we addressed these key questions of the interaction between nutritional
93 and social factors driving larval foraging decisions and performance in the tephritid fruit fly
94 *Bactrocera tryoni* (aka 'Queensland fruit fly' or 'Qfly'). Some tephrtids are highly
95 polyphagous and are amongst the most damaging insect pests of horticulture globally [49-51].
96 *Bactrocera tryoni* is able to infest more than 150 different fruits [49,52]; the wide diversity of
97 fruit that are exploited by *B. tryoni*, and variability of nutrients available in infested fruit,
98 make this species well suited for investigation of larval nutritional ecology. Here we first
99 designed circular foraging arenas containing patches of varying macronutrient concentration,
100 where different densities of larvae were allowed to forage. Larvae foraged freely in choice
101 and no-choice arenas, which allowed us to investigate the diet- and density-dependent effects
102 of larval developmental environment on foraging behaviour and larvae body mass. Using
103 statistical methods of machine learning and linear regression, we tested whether tendency to
104 aggregate and size of aggregations depended on the larval density and diet, by allowing
105 groups of several larval densities to forage in arenas of varying diet concentration within
106 which each arena contained multiple patches of the same diet. We then tested how larval
107 density and aggregation affected larval body mass across different diets. Finally, we
108 investigated how larval density influenced larval foraging decisions when facing choices
109 amongst patches with varying resource availability.
110
111 **Predictions**
112 1) Previous studies in other species have shown that larvae prefer to occupy patches that
113 are shared with conspecifics [e.g., [45]]. Thus, we predicted that an increase in larval

density should increase aggregation formation as well as aggregation size amongst diet patches. However, this effect could be diet-dependent, whereby macronutrient-poor diets could support smaller aggregations whereas macronutrient-rich diets would support larger aggregations. As a result, we predicted that aggregates should be smaller in macronutrient-poor diets than in macronutrient-rich diets;

2) In other insects, larval aggregation can facilitate feeding [e.g., [40]]. We therefore predicted that treatments with high larval aggregations should have larvae with higher body mass. However, macronutrient-poor diet is known to reduce larval body mass (see 'Introduction'). As a result, we predicted that larval body mass should be lower in macronutrient-poor diets compared with macronutrient-rich diets;

Materials and Methods

Fly stock and egg collection

We collected eggs from a laboratory-adapted stock of *B. tryoni* (>17 generations-old). The colony has been maintained in non-overlapping generations in a controlled environment room (humidity 65 ± 5%, temperature 25 ± 0.5ºC) with light cycle of 12h light: 0.5h dusk:11h dark: 0.5h dawn). Adults were provided a free-choice diet of hydrolysed yeast (MP Biomedicals, Cat. nº 02103304) and commercial refined sucrose (CSR® White Sugar), while larvae were maintained using the Chang-2006 gel-based diet formulation of Moadeli, et al. [53] for the last 7 generations (previously maintained on a carrot-based diet). We collected the eggs in a 300mL semi-transparent white plastic bottle that had numerous perforations of <1mm diameter through which females could insert their ovipositor and deposit eggs. The bottle contained 20mL of water, to maintain high humidity. Eggs were collected for 2h, and were then transferred to larval diet with a soft brush, where eggs were allowed to hatch and larvae to develop until they reached 2nd instars.

Experimental diets and foraging arena

We used 5 experimental diets that varied in macronutrient (i.e., yeast for protein and sugar for carbohydrate) concentration: our control and reference 100% Chang-2006 gel-based diet, which has proven effective for the larvae of this species [53], followed by diets with 80%, 60%, 40%, and 20% macronutrient concentration relative to the control diet (see Supplementary Tables for recipes). 20mL of diet was poured into 90mm diameter Petri dishes and allowed to set. We also prepared an agar solution that contained the same components as the gel diets except that no yeast or sugar was included. 20mL of the agar solution was used to cover 90mm diameter Petri dishes that then served as "foraging arenas". After setting, five equally spaced holes were made in the agar base of each foraging arena by perforating it with a 25mm diameter plastic tube. The same tube was used to cut discs from the experimental diets. The discs of experimental diets were then deposited – in order or randomly – in the holes that had been cut in the agar base of the foraging arenas (see Fig S1). Because the agar solution did not contain macronutrients, we considered the remaining areas of agar base as 'no choice' foraging option. Thus, larvae had a total of 6 options (i.e., 5 experimental diets + agar base). The pH of all experimental diets and the agar base was adjusted to 3.8-4 with citric acid. For the experiment, hydrolyzed yeast and sucrose were obtained from MP Biomedicals (Cat. nº 02103304 and 02902978, respectively), Brewer's yeast was obtained from Lallemand (Cat nº LBI2250), Nipagin was obtained from Southern Biological (Cat nº MC11.2), and all other chemicals composing the diet (e.g., citric acid [see [53]]) were obtained from Sigma Aldrich®.

Experimental procedures and statistical analyses

For all experiments, we placed 2nd instar larvae at the centre of the foraging arena (see Fig S1) that was then covered with the lid to minimize the loss of moisture. To minimize potential for effects of visual cues on larval diet choices, the foraging arenas were placed in a dark room. Foraging arenas were set up at 4 larval densities: 10, 25, 50, and 100 larvae. All larvae were released in the arena simultaneously. We did not observe cannibalism or escapes (larval counts were the same at the beginning and at the end of the experiments). All statistical analyses were performed using R version 3.4.0 and plots were performed using the package 'ggplot2' [54,55].

Experiment 1: Larval aggregation

To test effects of density and diet on larval aggregation and growth, for all diets and across all larval densities, we set up foraging arenas that contained 5 food patches of the same diet concentration (e.g., all patches with 100% diets) (see Fig S1). We then numbered the patches, and assessed the number of larvae in each of the diet patches as well as on the agar base at 1h, 2h, 4h, 6h, 8h, and 24h after larvae were placed in the arena. We observed that larvae could move across the diameter of the foraging arena in less than 1min, meaning that the time points used in the experiment were ample to allow larvae to explore the entire foraging arena. Four replicates were set up per larval density per diet (N = 80 foraging arenas). After 24h, 3 larvae per diet per larval density per replicate were selected from each foraging arena and weighed on a ME5 Sartorius® scale (0.001g precision) to obtain an estimate of average larval body mass. We tested the effects of larval density, diets, and their interaction, using two-way ANOVA model that included replicate as a covariate. To measure larval aggregation, we calculated an 'aggregation index' (*AI*) which was the sum of the absolute residuals of our observed data against the machine learning random predictions of a density-dependent random distribution; the procedure to obtain *AI* was as following:

1. We simulated the choices of larvae in foraging arenas with density 10, 25, 50, 100, and 200 larvae choosing amongst 6 patches, where the larvae were equally likely to display choice for any of the options (i.e., the choices for each patch were displayed with equal probability $p_n = {}^{1}/_{6}$, where p_n is the probability of a larvae choosing a given patch). We extrapolated our simulation for larval densities of 10, 25, 50, 100, and 200 larvae in order to build a robust function of density-dependent aggregation (see Fig S2).
2. We then obtained the residual distribution of our empirical data and the simulated density-dependent model against the exact random distribution, calculated simply by dividing the larval density by the number of patch options (i.e. ${}^{\delta}/_{6}$, where δ = larval density); We then fitted a random forest machine learning regression using the package 'randomForest' [56] to obtain a model that predicted the behaviour of the residuals. The random forest regression was cross-validated using the package 'rfUtilities' [57] (Fitted Mean Square Error of the model: 0.009; Median Cross-validation RMSE: 0.036); To build the model, 80% of the simulated data was used in the training phase while 20% was used in the test phase. The model performed accurately during the test phase (Mean Square Error in the Test dataset: 0.038);
3. Next, we used the machine learning model to predict the expected distribution of residuals in our dataset using the 'predict' function, and calculated the aggregation index *(AI)* as the difference between the observed sum of residuals and the predicted sum of the residuals obtained with the machine learning regression algorithm.

The machine learning model provides more accurate predictions of the expected distribution of the residuals than conventional linear model. For instance, the MSE (mean square error) of

the machine learning model in the test data set was 0.00404 whereas the MSE estimated using conventional linear model was 0.0107, suggesting that the machine learning model was ~2.7 times more accurate in its prediction. We therefore opted to use the machine learning approach to account for non-linear behaviour of the residuals as the density of larvae in the foraging arenas increases (see Fig S2). When we modelled *AI* using general linear model followed by a two-way ANOVA to determine the effect of time, larval density, diet, and their two-way interactions, we transformed *AI* (i. e. $AI^{2.25}$) in order to stabilize the variance across larval densities (Levene's test: $F_{3,476} = 0.560$, $p = 0.641$) and diets (Levene's test: $F_{4,475} = 0.548$, $p = 0.700$). To test for the effects of aggregation on larval body mass, we used an ANOVA with the average aggregation index over time, larval density, and diet, as well as the two-way interactions between these factors. For statistical inference, we transformed larval body mass (i.e., $Larval\ mass^{\ 0.3}$) for homogeneity of variances across larval densities (Levene's test: $F_{3,76} = 0.591$, $p = 0.622$). To calculate the average size of the largest aggregation, we sampled the aggregation with the highest larval count, and calculated the proportion of individuals of the group that were found in that aggregation (ρ) as $\rho = \alpha/\delta$, where α = the number of larvae in the largest patch and δ = the larval density of the group. To test for the effects of time, larval density, diet, and their two-way interactions we used a generalized linear model (GLM) with *Binomial* distribution – as we were dealing with proportion data – and *quasi* extension, to account for overdispersion of the data. Plots are of the raw data.

Experiment 2: Larval foraging

For larval foraging assays, the foraging arena contained one patch of each experimental diet, and we assessed the number of larvae selecting each diet across all larval densities (see above) at 1h, 2h, 4h, 6h, and 8h after larvae were placed in the arena. Foraging arenas

238 contained food patches (i.e. 100%, 80%, 60%, 40% and 20% macronutrient concentration) in
239 different orders within the arena (see Fig S1); we controlled for the order of the patches in all
240 models, which had no effect in the results (see ESM). We fitted a multinomial logistic
241 regression model using the 'multinom' function of the "nnet" package [58]. To test for foraging
242 propensity, we controlled for the order of the food patches while investigating the main
243 effects of time, larval density, and their interaction. Agar base (no choice) was the reference
244 level. To test for dietary choices, we used the same multinomial logistic regression, but this
245 time only considering those larvae that chose to forage. By using the standard diet (100%
246 macronutrient concentration) as our reference level, we could then infer the relative dietary
247 preferences of larvae that foraged. Statistical inferences for multinomial logistic regressions
248 were made based on 95% and 99% confidence intervals for each larval density separately.
249
250 **Results**
251 ***Experiment 1: High larval density increases larval body mass***
252 We first tested the influence of larval density on growth. Our results showed highly
253 significant positive effects of diet concentration and larval density on body mass (Table S1),
254 although there was no significant interaction between these factors. Body mass increased
255 steadily with larval density in the foraging arena and consistently across all diets (Fig 1).
256 However, diet concentration also affected larval body mass, as larvae from foraging arenas
257 with diluted diets (i.e. 40% and 20% macronutrient concentration) had lower body mass than
258 larvae from arenas containing more concentrated diets (Fig 1).
259
260 ***Experiment 1: Larval density affects larval aggregation in a diet-dependent manner***
261 We investigated whether larval density modulated larval aggregation, and whether this
262 relationship was affected by diet concentration. We found significant interaction between

effects of diet concentration and larval density on the aggregation index (Table S2), whereby larvae in high-density arenas aggregated more in high macronutrient concentration diets (>40%) and less in low macronutrient concentration diets (20%, Fig 2a-b).

There was a significant interaction between time and larval density, whereby larvae in low-density arenas (10 larvae) increased aggregation as time foraging passed, while the opposite pattern was observed for high-density arenas (100 larvae) (Table S2, Fig 2a-b). This was particularly evident for low-density arenas with low macronutrient concentration diets (see Fig 2a). This is important because if the larvae were simply coalescing in the same location (i.e., not seeking to aggregate but converging to the same location with high quality food substrate), we would expect larvae in low-density arenas to show the same pattern for high- and low diet concentration. Instead, the results show the opposite is true, whereby larvae in low-density arenas tended to aggregate more over time with low diet concentration than with high diet concentration (Fig 2a). This provides evidence that larvae seek to aggregate, especially when foraging in low-density arena and with low-resource food substrates. Arenas with density of 25 and 50 larvae showed the same trend as arenas with 10 and 100 larvae, respectively, although with lower magnitude (Fig 2a-b).

Experiment 1: The relationship between larval aggregation and larval body mass is diet-dependent

Next, we tested the relationship between larval aggregation and body mass. We found that aggregation had an overall highly significant positive effect on larval body mass when diet concentration was 40% or greater but that a negative trend was instead observed when diet concentration was 20% (Fig 2c, Table S3). There was a significant effect of diet concentration and larval density, but there were no significant interactions between larval

density and diet concentration, larval density and aggregation index, nor between diet concentration and aggregation index (Table S3). These results provide evidence for a positive relationship between larval aggregation and larval body mass, and revealed that in some cases nutrient concentration in the diet can be a strong modulator of this relationship.

Experiment 1: Larval density and diet influence the size of larval aggregations

Previous studies have shown that larval aggregation can help larvae to feed more efficiently, potentially leading to an increase in larval body mass (see for instance [40,59]). If this is true, an aggregation could become a 'hotspot' for other larvae, and we would expect that arenas with high larval densities would have few large aggregations. This could explain the relationships between larval aggregation and body mass and also the relationship between larval density and larval aggregation. Alternatively, high larval density could make larvae more inclined to disperse in order to minimize competition and, as a result, form smaller aggregations at more locations, hence exploiting a greater number of food patches. Our results showed a significant interaction between the effects of larval density and time, and larval density and diet concentration on the proportion of individuals in the largest aggregation (see Table S4, Fig 3). These results demonstrate that i) arenas containing diluted diets (i.e., 20% and 40%) had relatively more larvae in the largest aggregations than did arenas containing more concentrated diets, ii) low larval density arenas (i.e., 10 larvae) had aggregations that contained relatively more larvae compared with higher density arenas (i.e., 25, 50, 100), iii) high density arenas (i.e., 100 larvae) were more evenly distributed compared with low density arenas, whereas the opposite effect was found for low density arenas (i.e., 10 larvae), and iv) the proportion of larvae in the most numerous aggregation decreased in diluted diets in high density arenas, an effect that was not observed for low-density arenas (see Fig 3).

These findings support the hypothesis that high larval density promotes larval movement, whereby larvae formed smaller aggregations that exploit patches more evenly.

Experiment 2: Larval density shapes larval foraging behaviour

Next, we measured how larval density influenced larvae foraging propensity, as well as larvae foraging decisions when larvae have a choice amongst patches with varying diet concentrations. By using a multinomial logistic regression model that used 'no choice' (i.e. agar base) as our reference level, we could assess larval foraging propensity over time. Our results showed that larvae were more likely to forage in any given patch than to not forage at all, and the propensity of foraging was particularly high for patches of high nutrient concentration independent of larval density (Fig 4a, Table S5, Fig S3). Interestingly, the range of diets in which larvae foraged was greater for arenas containing 50 and 100 larvae and included the patch with 40% diet in addition to the 100%, 80% 60% patches that were more dominant for arenas of lower larval density (Fig 4a). These findings show that larvae are generally more prone to forage in high-quality patches, and that larval foraging propensity is density-independent.

We then tested whether larval density affected larval diet choices, using again a multinomial logistic regression although this time we used the standard diet (i.e. 100%) as our reference diet and excluded non-foraging larvae, while modelling the behaviour of larvae that were actively foraging in one of the food patches in the previous experiment. In arenas with low larval density (10 larvae), larvae displayed a significant preference for diets with 60% macronutrient concentration relative to the standard (100%) diet (Fig 4b, Table S6). However, as larval density increased (25 and 50 larvae), there was a shift in preference toward the patch containing 80% macronutrient concentration (Fig 4b), and finally, when

larval density was the highest (100 larvae), larvae displayed statistically significant preferences for both 60% and 80% macronutrient patches compared to the standard diet (Fig 4b-e). More importantly, though, is that only larvae in arenas with high density (50 and 100 larvae) displayed significant avoidance of low concentration patches of 20% macronutrient concentration (Fig 4d-e).

Discussion

In this study, we demonstrate how key ecological factors interact to determine larval foraging behaviour and growth in *B. tryoni*. Our findings showed that larval aggregation increased with larval density in a diet-dependent manner, and promoted larval body mass across all diets. Importantly, larval density modulated the size of larval aggregations, and influenced larval foraging behaviour when larvae experienced patches with varying concentrations, highlighting a role of social interactions and population density for larval behaviour. Our findings provide insight into larval foraging decisions of fruit flies, and more generally, provide insight into broad ecological patterns arising from nutrition and intraspecific competition within groups and populations. Fruit fly larvae are commonly found in aggregations within a fruit [9,10,60]. Furthermore, fruits can be heterogeneous foraging environments for larvae [e.g., [61]], and the nutritional composition of fruit can change as larvae develop [see [62-64]]. Therefore, the density of larvae and local diet quality might determine larval movement within a fruit in search of more nutritious and less competitive foraging sites. It is important to note that it is unlikely that our findings apply to movement of larvae between fruits. Crawling out of fruits is dangerous owing to risks of predation [[65], reviewed by [66]] and desiccation. In nature, *B. tryoni* females modulate their oviposition behaviour to minimize intra-specific competition amongst larvae [67], and it is reasonable to expect the larvae to very rarely move between fruits.

High population density can force animals to change their behaviour and expand their niche due to inter- and intra-specific competition, and this is a well-established ecological principle observed in both the laboratory and in nature [68,69]. Even though larvae are prone to aggregate, an increase in larval density could increase larval competition within large aggregations, which could in turn drive larvae to disperse and form smaller aggregations across different locations. The smaller aggregation size observed in high-density arenas support this idea, meaning that larval aggregations formed in high-density arenas were proportionally smaller than those formed at lower densities. Moreover, larval aggregations were proportionally smaller as the density increased and the larvae spent more time foraging, suggesting that social interactions within larger aggregations are likely to induce more frequent movement by the larvae. As the larvae move more often, they are more likely to find new (and unexplored) food patches, and are therefore more likely to explore patches more evenly. The influence of larval density on larval aggregation and growth could therefore be a plastic response to intraspecific competition because it could lead to better larval foraging decisions and a broader niche exploration [45,70]. The findings that high larval density also influence larval foraging behaviour in ways that decrease larval foraging propensity on resource-poor diet patches provide further support for the idea that high larval density promotes exploration of the foraging environment and effective exploitation of nutritional resources. Individuals of many species use social cues when making decisions [71], and recent models have predicted that social interactions could improve individual foraging success, especially when food is scarce and distributed heterogeneously [72]. It is also possible that larval aggregation alters the nutritional composition and the microbial communities of the diets. For instance, larvae of some insect species can be cannibalistic [73,74], and because larvae are a rich source of nutrients, cannibalism could affect the nutrient status of a food patch. Moreover, in *D.*

387 *melanogaster*, larval foraging behaviour is determined by the bacterial communities in the
388 diet [75], and in *B. tryoni*, gut-microbial fungi in the diet have been found to promote larval
389 development under nutrient-limiting conditions [76]. If larval density affected the relative
390 abundance of these fungi in the diet, this could in turn have influenced larval foraging
391 behaviour and larval body mass. Future studies that investigate the impact of larval density
392 on the occurrence of cannibalism, and that compare changes in larval and diet microbial
393 profiles in high- and low-density social environments will provide insights into the
394 mechanisms underpinning the effects of larval environments on foraging behaviour and
395 growth.
396
397 A negative relationship between population density and individual fitness is often assumed in
398 ecology [reviewed by [77]]. In invertebrates, including tephritid fruit flies, high-densities at the
399 larval stage can decrease nutrient availability, and reduce adult body mass, reproductive
400 success, and survival [e.g. [1,3-9,60]], which can lead to a density-dependent effects on fitness
401 that extends through generations [6]. However, high densities can also mitigate the negative
402 effects of environmental stresses, and act as a buffering factor for individual fitness and
403 survival [reviewed by [77]]. Therefore, high-density environments can sometimes confer fitness
404 benefits. Our findings support this view, as they reveal that the density of larvae can trigger
405 behavioural responses early in life that can benefit larval growth. This positive effect is likely
406 due to an increase in exploratory behaviour when at high-densities, which can increase niche
407 exploration and nutrient acquisition. It is important to mention that competition amongst
408 conspecifics should determine threshold in which sociality provides benefits to the larvae,
409 after which further increase in density should incur costs that offset the benefits to
410 individuals' fitness [45]. This threshold is currently unknown, but we predict that further
411 increase in the density of larvae in our experiments (e.g., 400 larvae) should result in

412 measurable costs such as decrease in body mass of the larvae. Determining the threshold is
413 out of the scope of this study, but remains an important topic for future investigations.
414 Nonetheless, our findings are applicable to biological scenarios where intraspecific
415 competition increases and resources are heterogeneous, and thus represent a logical
416 consequence of the interaction between the nutritional and social environments.
417
418 It is important to mention that as density increases, larvae may be displaced from the patch
419 due to the competition with conspecifics for space. This is a natural consequence of high
420 larval density (i.e., defined as more larvae per unit of space), and understanding how the
421 competition for space underlies larval behaviour is out of the scope of this study. Also, patch
422 quality could have decreased over time, especially in treatments with high larval densities,
423 and influenced some of the results found in our study. This is unlikely, however, because the
424 number of individuals in each patch sharply increased and stabilised in a plateau, with no
425 evidence of larvae evasion from the chosen patches throughout the 24h in which the
426 experiment was conducted (see e.g., Fig S3). Thus, our results demonstrate how the
427 interactions between larval density and larval nutritional environment shape larval foraging
428 behaviour.
429
430 **Conclusion**
431 The present study provides a new perspective on density-dependent effects on larval
432 development. Fruit fly larvae respond to a range of social and nutritional factors, with
433 important implications for larval foraging and growth. Together, our findings help us
434 understand the ecological factors underpinning larval development in insects, and serve as an
435 important stepping-stone for future research aimed at better understanding the behavioural
436 and nutritional aspects of development in group-living insects.

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Authors' Contribution

J.M, P.W.T, and F.P designed the experiment. J.M, F.P., B.N and S.T.T collected the data. J.M, F.P, B.N and P.W.T analysed the data. All authors wrote the manuscript, approved the submission, and agree to be accountable for all aspects of the work.

Conflict of interests

The authors have no competing interests to declare.

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Figure Legends

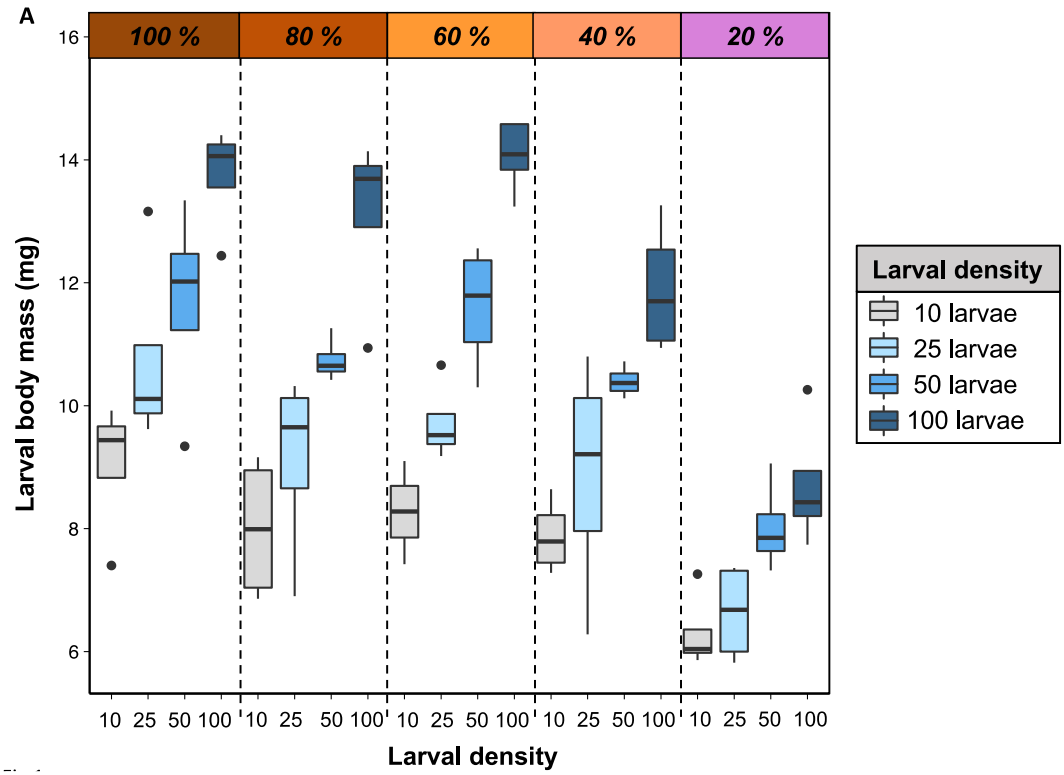


Figure 1 – Larval density increases larval body mass across diet dilutions. Body mass (mg) of larvae from different larval densities and from across diets, at the end of our experiment (24h, see Methods for details).

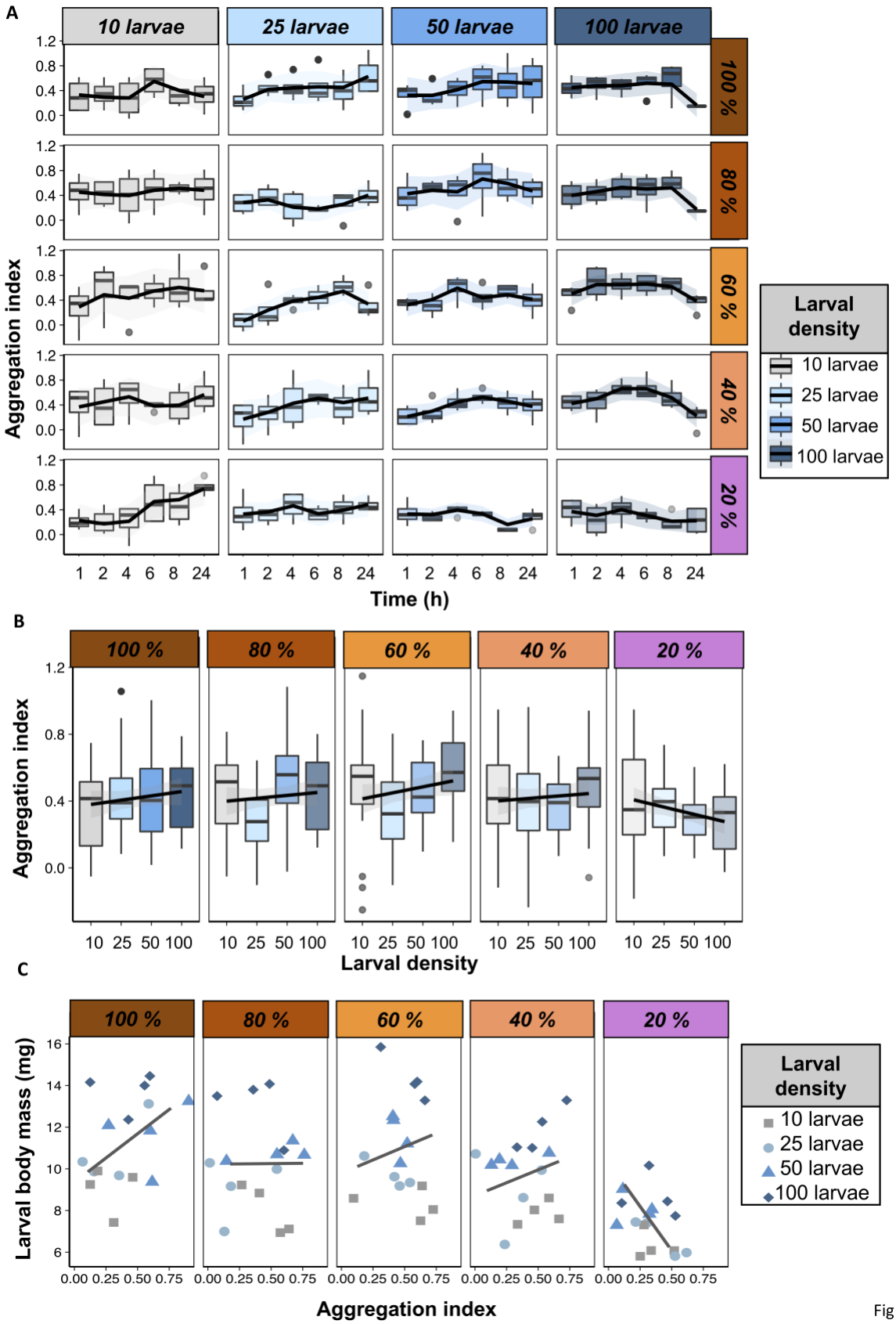
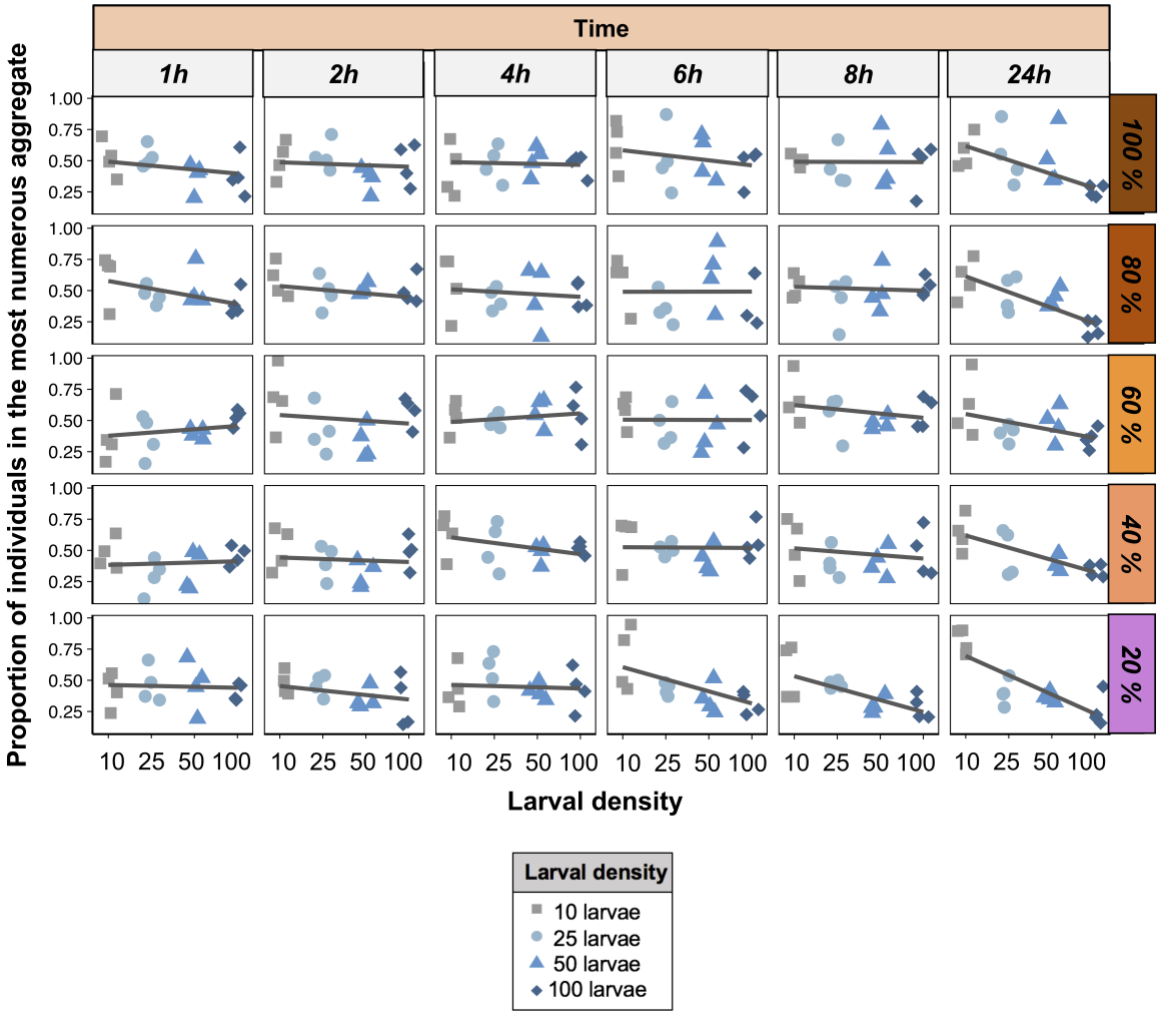


Fig 2

Figure 2 – Body mass and the relationship between body mass and aggregation.

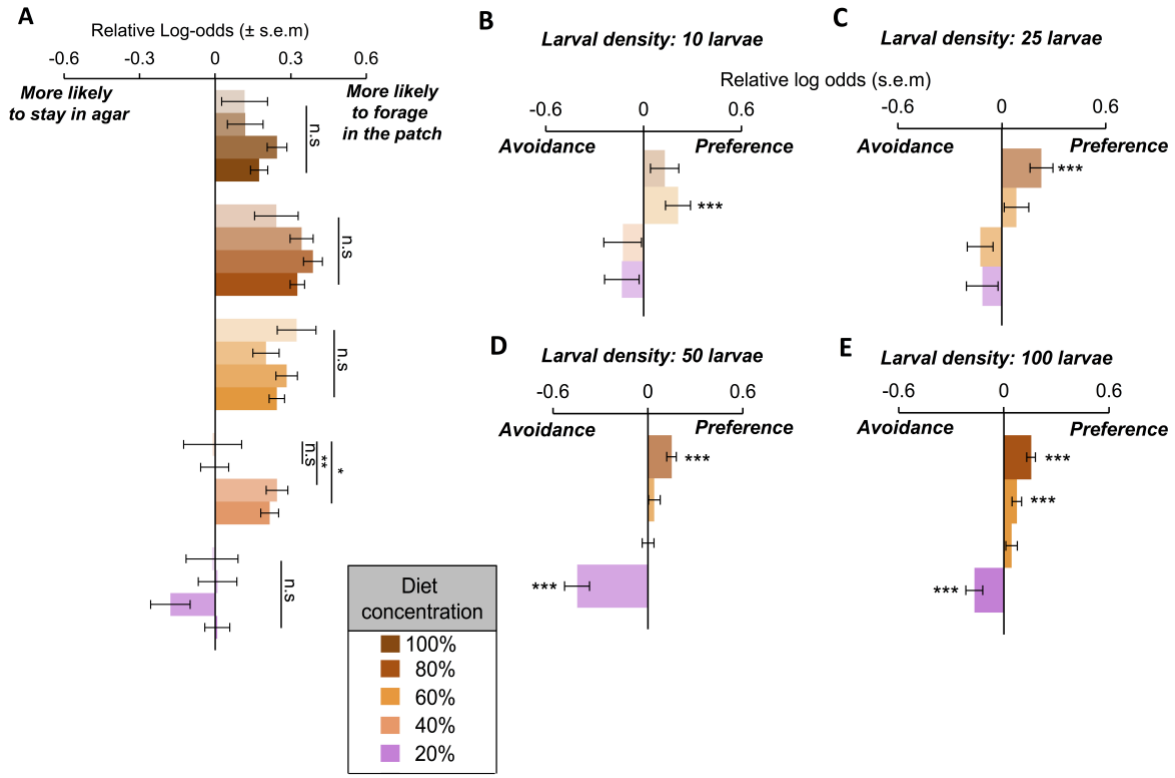
652 (a) Larval aggregation index (*y*-axis) over time (*x*-axis) across larval densities (horizontally)
653 and across diets (vertically). Lines were drawn using the 'loess' method in the package
654 'ggplot2' in R, and indicate the trend in the data. (b) Average larval aggregation index (*y*-
655 axis) on larval density (*x*-axis) over all time points in our experiment. Lines were drawn
656 using the 'lm' method in the package 'ggplot2' in R, and indicate the trend in the data. (c)
657 The relationship between larval body mass and the average aggregation index. Colours and
658 shapes indicate the larval density. Lines were drawn using the 'loess' method in the package
659 'ggplot2' in R, and indicate the trend in the data.



660

661 **Figure 3 – Proportion of larvae in aggregates.** The proportion of individuals in the most
662 numerous aggregate over time (horizontally) across diets (vertically). Shapes and colours

663 indicate larval density. Lines were drawn using the 'lm' method in the package 'ggplot2' in
664 R, and indicate the trend in the data.



665

666 **Figure 4 – Larval foraging propensity.** (a) Relative log-odds of larvae making a choice to
667 forage in a given food patch relative to staying in agar (no choice). Shades represent different
668 larval densities: 10, 25, 50, and 100 larvae. p-values obtained using Students' *t*-distribution.
669 Note that relative log-odds are calculated using the control 100% diet as reference. Log-odds
670 > 0: more likely to choose a given patch relative to staying in agar, Log-odds < 0, less likely
671 to choose a given patch relative to staying in agar. s.e.m = standard error of the mean. (b-e)
672 Relative log-odds of larvae patch preferences. Patch with standard diet (100% macronutrient
673 concentration) was used as the reference level. *** non-overlapping 99% confidence
674 intervals. s.e.m = standard error of the mean.

675

676 **Supplementary Information**

677 **Supplementary Information file –** Supplementary figures and tables with the complete
678 outputs of the statistical models.

679 **Figure S1 - Design of the foraging arenas.** (a) Schematic representations of the foraging
680 arena used in our larval dietary choice experiments. (b) Schematic representations of the
681 foraging arena used in our larval aggregation experiments. Note that the arenas were
682 designed exactly as in (a), although all patches contained the same diet concentration.

683 **Figure S2 – Box plots showing the behaviour of the residuals from the density-**
684 **dependent simulation.** Note that we extrapolated our simulations to include foraging groups
685 with density of 200 larvae (see Methods). Line was drawn using the 'loess' method in the
686 package 'ggplot2' in R to highlight the trend in the data.

687 **Figure S3 – Larval foraging behaviour.** The number of larvae in each foraging patch over
688 time across the larval density treatments.

689 **Table S1 – Complete analysis of larvae body mass. Bold:** $p < 0.05$

690 **Table S2 – Complete analysis of larvae aggregation index.** Data was transformed (square-
691 rooted) for statistical testing. **Bold:** $p < 0.05$

692 **Table S3 – Complete analysis of the relationship between larvae body mass and**
693 **aggregation index. Bold:** $p < 0.05$

694 **Table S4 – Complete analysis of the proportion of larvae in the most numerous**
695 **aggregate.** GLM with Binomial distribution and quasi extension to account for
696 overdispersion of the data.

697 **Table S5 - Complete analysis of larvae willingness to forage.** Agar (no choice) is the
698 reference level. **Bold:** $p < 0.05$

699 **Table S6 - Complete analysis of larvae dietary choices.** Standard diet (100% macronutrient
700 concentration) as reference level. *** non-overlapping 99% CI**.**

701 **Diet recipes –** The formulations for the diets used in the experiments.