

RESEARCH ARTICLE

Relatedness modulates density-dependent cannibalism rates in *Drosophila*

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Abstract

1. Cannibalism is taxonomically widespread and can have large impacts on individual fitness and population-level processes. As such, identifying how cannibalism rates vary in response to ecological cues is important for predicting species evolution and population dynamics.
2. In this study, we aimed to identify several eco-evolutionary factors that affect cannibalism rate and measure how they interacted with one another.
3. To do this, we conducted two experiments using complimentary methods to measure how cannibalism rates varied among larval *Drosophila melanogaster* and *Drosophila simulans* in response to changes in conspecific relatedness, social familiarity and density.
4. We found that larvae were more likely to cannibalise non-related larval victims in both species, and that this effect increased at high densities in *D. simulans*. We found no evidence that *Drosophila* larvae use social familiarity to assess relatedness. Finally, in *D. melanogaster*, cannibalistic larvae prefer to cannibalise larvae that are being attacked by a greater number of conspecifics, implying that cues linked to conspecific abundance encourage cooperative cannibalism.
5. By showing that cannibalism frequency in *Drosophila* spp. is sensitive to relatedness and several other factors, we reveal the complex relationship between cannibalism frequency and species ecology. Also, by showing that the effect of relatedness on cannibalism frequency is density dependent, we advance the current understanding of how ecological variables interact to affect kin selection.

KEYWORDS

cannibalism, density dependence, *Drosophila*, kin selection, relatedness cues

Adam M. Fisher and Sally Le Page joint first authorship

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1 | INTRODUCTION

How an organism behaves towards related individuals can have major impacts on its inclusive fitness (Hamilton, 1964). As such, in species where individuals can detect kin, variation in relatedness can drive behavioural variation (Mikát et al., 2019; Schneider & Bilde, 2008). This link, present in many species, means that an accurate understanding of how relatedness affects behaviour could be important for predicting species ecology. Individuals assess relatedness using either phenotypic cues that directly signal the presence of specific shared genes or genotypes (Queller et al., 2003), or indirect indicators that correlate with relatedness. For example, the proximity in which individuals emerge may be indicative of which brood an individual came from and is known to influence the behaviour of individuals towards conspecifics (Carazo et al., 2015; Hopper et al., 1996; Ward & Hart, 2003). Thus, it is possible that individuals assess the specific phenotypic traits of those they have emerged near and associate these traits with relatedness. Kin recognition often facilitates the evolution of behaviours in which individuals actively help relatives survive and breed (Hatchwell et al., 2001; Khwaja et al., 2019), but it can also prevent individuals harming or killing relatives (Dobler & Kölliker, 2009; Pfennig et al., 1994).

Cannibalism can be a significant source of mortality and has evolved in species across many taxonomic groups, including fish (Pereira et al., 2017), amphibians (Pfennig et al., 1994), insects (Fisher, Holwell, et al., 2020; Hopper et al., 1996) and spiders (Elgar, 1991). Cannibalism can also drive the evolution of phenotypic traits such as size variation (Elgar, 1991; Pfennig et al., 1994; Wilder & Rypstra, 2008) and can have a large impact at the population level by stabilising population fluctuations and affecting extinction risk (Fisher et al., 2018; Fisher, Cornell, et al., 2020; Ricker, 1954; Via, 1999). Hypotheses for the adaptive function of cannibalism are diverse and range from cannibalism as a foraging strategy (Barry et al., 2008; Petersen et al., 2010) to cannibalism as a mechanism of mate choice (Prenter et al., 2006). Evidence also suggests that cannibalism rates increase with population density in some species (Claessen et al., 2004; Ricker, 1954). Apart from a few exceptions (Andrade, 2003; Schwartz et al., 2016), cannibalism is thought to have an extremely negative effect on the fitness of the victim. As such, one would expect strong selection for individuals that can avoid cannibalising kin, particularly in species where kin encounter rates are likely to be high. Invertebrate larvae often emerge en masse and thus are likely to encounter individuals from the same brood. Relatedness has been shown to play a significant role in determining cannibalism rates in the larvae of several invertebrate species (Dobler & Kölliker, 2009; Hopper et al., 1996). High encounter rates among siblings and the extreme fitness consequences of cannibalism make predatory invertebrate larvae ideal organisms for studying kin selection.

Drosophilid fruit flies have an almost ubiquitous global distribution and collectively play a major role in the decomposition of decaying organic matter. The larvae of most Drosophilid fruit fly species feed on microorganisms growing on decaying vegetable

matter. However, there is evidence from laboratory studies that *Drosophila melanogaster* larvae will readily devour the carcasses of dead invertebrates when alternative food sources are absent (Ahmad et al., 2015). It has also been shown that *D. melanogaster* can increase their survival rate when starved by actively hunting and cannibalising living conspecific larvae. Moreover, the mouthparts of *D. melanogaster* develop a greater number of teeth when reared on a cannibalistic diet (Vijendravarma et al., 2013) which could be an adaptation for facilitating cannibalism, as is seen in several amphibian larvae (Pfennig et al., 1994). There is evidence that *Drosophila* larvae can be food limited in the wild (Atkinson, 1979; Grimaldi & Jaenike, 1984), which would promote the evolution of cannibalism. In addition, the pre-existing plasticity of *Drosophila* mouthparts implies that cannibalistic behaviour may have evolved in response to natural fluctuations in food availability.

As cannibalism can have important evolutionary and ecological impacts, understanding how the frequency of cannibalism is affected by environmental cues could be important for predicting population change. The preferential cannibalism of non-kin over kin would also provide evidence that cannibalism has been under persistent selection to promote the survival of relatives. Literature on kin recognition in adult and larval *Drosophila* has, to date, been conflicting. Some studies suggest that *Drosophila* have an ability to detect genetic relatedness (Khodaei & Long, 2019, 2020; Lizé et al., 2014). Laboratory studies observing fruit flies and other invertebrates suggest that social familiarity (i.e. emerging in close proximity to one another and being reared together) is important for kin recognition (Carazo et al., 2015; Hopper et al., 1996; Le Page et al., 2017). In contrast, other studies find little evidence of any kin recognition (Chippindale et al., 2015; Martin & Long, 2015). In the current study, we measured the frequency of cannibalism in response to relatedness, social familiarity, starvation and density in larval *Drosophila melanogaster* and *Drosophila simulans*. Two species were used to check that cannibalism is not an anomalous behaviour exclusive to *D. melanogaster*. In addition, two complementary protocols were used to increase the robustness of our findings and check that cannibalism is not an artefact of a specific laboratory setting. We predicted that larval cannibalism rates would (a) be higher among unrelated than related individuals, (b) be higher among unfamiliar than familiar individuals, (c) increase in response to starvation and (d) increase in response to high density.

2 | MATERIALS AND METHODS

2.1 | Choice trials in *D. melanogaster*

In the first experiment, we used choice trials to determine whether cannibalism in *D. melanogaster* was affected by relatedness, social familiarity and/or the abundance of conspecifics surrounding a victim. From here onwards, we use insights from other kin recognition studies and define familiar individuals as those that have emerged and developed in close proximity to one another (Carazo et al., 2015;

Le Page et al., 2017). We differentiated between families using a GFP-Moesin (GFP-moe) transgene (Edwards et al., 1997), which makes larvae synthesise a fluorescent protein. We backcrossed GFP-moe into the wild-type outbred Dahomey genetic background for five generations. Both wild-type Dahomey and the backcrossed GFP-moe families (hereon 'WT' and 'GFP' respectively) were maintained in culture on Lewis medium at 20°C for 19 months prior to experimentation.

To produce larvae for choice trials, a virgin female and a male of the same family were removed from the stock population and paired in vials containing Lewis medium. Pairs were left to mate and lay eggs for 5 days before pairs were moved to a separate vial where females continued to lay eggs. Throughout the rest of the paper, the first vial that the adult pairs were transferred to will be referred to as the 'early' vials, and the vials the adult pairs were transferred to after 5 days will be referred to as 'late vials'. Second-instar larvae are known to preferentially cannibalise third-instar larvae over other second-instar larvae in *D. melanogaster* (Vijendravarma et al., 2013). Thus, in our choice trials, third-instar larvae are selected as victims and second-instar larvae are selected as potential cannibals. The 'late vials' from which the second-instar larvae were picked contained no yeast, this served to starve the larvae of protein and induce cannibalism.

For the relatedness trials, a third-instar victim from the WT and GFP families was selected from their respective 'early' vials and each was secured by being impaled on equally sized entomological pins at either end of a 55-mm diameter Petri dish (15 mm from the centreline) containing agar. Previous work has shown that cannibals are preferentially attracted to injured over non-injured victims (Kakeya & Takahashi, 2021; Vijendravarma et al., 2013); thus, pinning the larvae likely sped up the onset of cannibalism. Also, pinning the victims in this way ensured that they remained equidistant from the centre of the dish. After pinning the victims, 10 s instar larvae from each family were collected from the 'late' vials and placed in the centre of the Petri dish. After 1 hr, we recorded the number of larvae from each family within 5 mm of each victim and used this number as a measure of cannibalism. Throughout the experiments, partially cannibalised individuals were observed repeatedly (Le Page, S., pers. obs.). In addition, in a study by Vijendravarma et al. (2013), the number of conspecifics that aggregated around a victim was positively associated with cannibalism frequency. Thus, although this measure is indirect, it is likely that the number of individuals aggregating around a victim is a reliable measure of cannibalism. As mentioned earlier, visible fluorescent proteins were present in larvae from the GFP family, but not the WT family; thus, families could be identified by sight (Figure 1a). Previous work suggests that social familiarity between larvae is required for kin detection in adult *Drosophila* (Le Page et al., 2017). Because the cannibals and victims were collected from 'early' and 'late' vials, respectively, victims did not emerge in the same vials as cannibals and were therefore equally unfamiliar to cannibals from both families, and thus, any effects are likely to be due to parentally inherited traits rather than social familiarity.

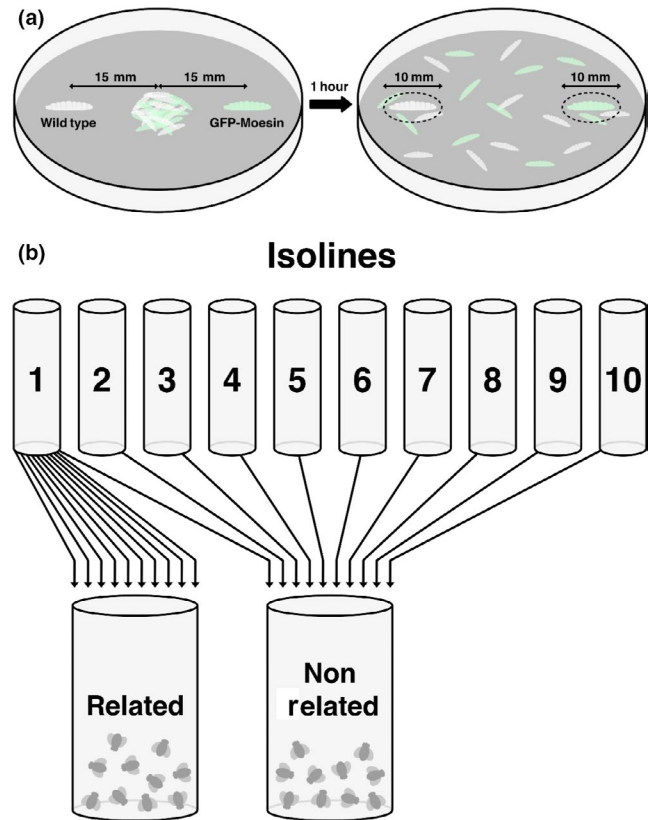


FIGURE 1 (a) In the *Drosophila melanogaster* relatedness choice trials, 20 s instar larvae from the WT and GFP families were placed at the centre of a Petri dish. A single third-instar larva from the WT and GFP was pinned at either end of the Petri dish. After 1 hr, the number of larvae from each family within a 5-mm radius of each third-instar larva were counted. (b) In the *Drosophila simulans* mixed isolate trials, related treatments were created by allowing 10 females from a single isolate to lay eggs for 65 hr in a single vial (i.e. related vials contained females exclusively from isolate 1, 2, ..., or 10). Non-related treatments were created by taking a single female from each of the 10 isolines and allowing them to lay for 65 hr in a single vial

In the choice tests measuring the effect of social familiarity on cannibalism frequency only WT larvae were used, meaning both victims were full siblings to each other and to the cannibals. To generate a familiar victim, third-instar larvae were transferred from the 'early' vial to a 'late' vial before larvae emerged in the 'late' vial. This ensured that larvae emerged in the late vial in the presence of one of the victims and would therefore be familiar with that victim. 48 hr after introducing the third-instar larvae to the 'late' vial, 20 s instar cannibals were taken from the 'late' vial, allowing us to use the third-instar larvae from the 'early' vials as unfamiliar victims. As before, 20 cannibals were placed in the centre of the Petri dish and the two victims were secured using entomological pins at 15 mm either side of the centreline. After 1 hr, we recorded the number of larvae within 5 mm of each victim.

We also performed a choice trial to test whether the number and relatedness of conspecifics already cannibalising a victim affected victim choice; that is, do cannibals prefer to co-cannibalise with

other cannibals of the same family? We placed 10 larvae from one WT family and 10 larvae from one GFP family in the centre of the Petri dish. This meant that larvae within families were related and familiar, and larvae across families were unrelated and unfamiliar. The two victims came from a large outbred Dahomey population and were therefore unrelated and unfamiliar to both families. As before, victims were secured using entomological pins at 15 mm either side of the centreline of a Petri dish with agar substrate. The number of larvae from each family within a 5-mm radius of each victim was recorded at 1, 2, 3 and 6 hr after the beginning of the trial. For all of the choice experiments, only mobile third-instar larvae of a similar size were selected as victims; this was to minimise the probability of developmental bias leading to erroneous results. All experiments were carried out at 25°C.

2.2 | Mixed isoline trials in *D. simulans*

For our second experiment, we used *D. simulans* to test whether cannibalism rates were greater in high versus low relatedness environments. Ten pre-existing *D. simulans* isolines obtained from locations in the United States, Madagascar and Croatia were used. Each isoline was established by inbreeding the offspring of a single wild caught female to produce a line of highly genetically similar individuals (David et al., 2005). Flies were kept at 22°C and reared on maize-sugar-yeast agar medium. To create the related treatment, 10 females from the same isoline were removed from the main stock and placed into individual vials to create an average relatedness among larvae of 1. In the non-related treatment, one female from each of the 10 separate isolines was taken from the main stock and placed together into individual vials to create an average relatedness of ≈ 0.1 (Figure 1a). Females from both treatments were then given 65 hr to oviposit before being removed from the vials. Note that an average relatedness of 0.1 in the non-related vials would only be achieved if all females from the different isolines laid an equal number of eggs that hatched at equal rates. We attempt to achieve this by giving all females the same amount of time to lay, but average relatedness in the non-related vials may have varied. In both treatments, females laid in vials containing 3 ml of the aforementioned agar medium with the yeast removed. Removing yeast is known to increase starvation and encourage cannibalism in *Drosophila melanogaster* larvae (Vijendravarma et al., 2013). By allocating adult females to lay in treatment vials, we removed any potential confounding by social familiarity, as all larvae would have emerged together regardless of whether they were in the related or non-related treatment. Additionally, unlike *D. melanogaster*, *D. simulans* females are not known to prefer the outer perimeter of a food surface over the middle (Chess & Ringo, 1985). Thus, female *D. simulans* are likely to distribute their eggs homogeneously across the food surface. This will likely provide consistent inter-brood social familiarity by accommodating inter-brood larval mixing early in development. All experiments were carried out at 22°C.

After 71 hr, the food surface of each of the vials was photographed using a Canon EOS 60D attached to a Leica microscope via a custom-made microscope-SLR adapter. Magnification was kept constant at 4 \times (objective) throughout the experiment to ensure that the surface area being photographed remained constant. Each vial was photographed three times at 10-min intervals; this was repeated every 24 hr for 12 days. In each image, we counted the number of detached mouthparts—because the sclerotized mouthparts are not eaten during cannibalism (Fisher A.M., pers. obs.) and used the number of detached mouthparts as a measure of cannibalism (see Figure S1 for an example image). Moreover, mouthparts that were left behind as a result of moulting are identifiable by them being attached to a dried-out exoskeleton. We refer to detached mouthparts as ‘instances of cannibalism’ for the sake of readability; however, we acknowledge that this is not a direct measure of cannibalism and may be prone to some level of error.

Density is known to effect cannibalism rates in other species (Claessen et al., 2004; Ricker, 1954) and was likely to vary naturally between vials. As such, we recorded the number of living larvae (dead larvae are identifiable by necrotised tissue) in each image and used this as a measure of density. Vijendravarma et al. (2013) found that cannibalism was more likely to occur in *D. melanogaster* between second (attackers) and third (victims) instar larvae, so the number of larvae that had reached third instar was also recorded. As each vial was photographed three times daily, we calculated the mean of the three counts for each of our measures (cannibalism, density and number of third instars) and used these mean values for statistical analysis.

2.3 | Statistical analysis

2.3.1 | *D. melanogaster* choice trials

We used separate non-parametric paired Wilcoxon tests to compare: (a) The number of larvae that chose to cannibalise-related versus non-related victims per plate ($n = 63$); (b) the number of larvae that chose to cannibalise the familiar and unfamiliar victim per plate ($n = 53$); (c) the number of cannibals per plate that were from the GFP or WT isoline; and (d) the relative number of GFP and WT larvae cannibalising a specific victim. All of the count data that were analysed in this way were taken 1 hr after the start of each trial.

A generalised linear mixed effects model (GLMM) with Poisson error correction was used to analyse the effect of time on the level of skew (|side 1—side 2|) between the number of cannibals at each victim per plate. The model included time and time² (to check for nonlinear trends) as the fixed effects. Because we were taking repeated measures of the same plates at different time intervals, plate ID was included in the model as a random effect. Stepwise likelihood ratio tests were used to determine the minimal adequate model.

2.3.2 | *D. simulans* mixed isoline trials

The number of detached mouthparts that were counted in each vial was cumulative over time. However, as we were only counting mouthparts on the surface of the food substrate, counts were not a direct measure of the total number of cannibalistic events in the vial (as some instances of cannibalism may have taken place below the surface); thus, we assume the number of visible instances of cannibalism correlated with total cannibalism. To minimise potential error associated with this indirect measure, cannibalism rate was measured for each of the related ($n = 30$) and non-related ($n = 30$) vials by taking the rate of increase in mouthparts over the 12-day experimental period. This allowed us to generate our measure of cannibalism based on 12 data points rather than a single count. The best slope estimates were calculated using a generalised linear mixed effects model (GLMM) that modelled mouthpart counts as a function of time (days) for each vial (see Supporting Information for slope estimates of each vial). These overall cannibalism rates were then analysed as a function of treatment (related vs. unrelated), mean density per vial, the mean proportion of third instars per vial and their pairwise interactions.

Cannibalism rates will likely depend on the larval density in a given vial. It was thus important to test for potential differences in density across the two relatedness treatments. To this end, we fitted the number of larvae (density) as a function of time, treatment, their interaction, a quadratic term for time (to test for a nonlinear effect) and vial specific slope and intercept using a GLMM with Poisson error and an exponential link function.

Vial-specific changes in larval density over time can be viewed in the Supporting Information. For both models, we used stepwise likelihood ratio tests to find the minimal adequate model. All statistics were calculated using R version 3.6.1; GLMMs were conducted using the LME4 package in R (Doran et al., 2007); and p values were calculated the LMERTEST package in R (Kuznetsova et al., 2017).

3 | RESULTS

3.1 | *D. melanogaster* choice trials

Choice trials observing the effect of relatedness on cannibalism showed that, after 1 hr, a higher number of *D. melanogaster* larvae chose to cannibalise unrelated over related individuals (Figure 2a: $df = 62$, $V = 304.5$, $p = 0.014$). However, in the choice trials measuring the effect of social familiarity on larval cannibalism, we found no evidence that larval *D. melanogaster* preferred to cannibalise unfamiliar over familiar individuals after 1 hr (Figure 2b: $df = 52$, $V = 247$, $p = 0.101$).

In the co-cannibalism trials, the number of larvae that chose to cannibalise a victim was not significantly different between the WT and GFP *D. melanogaster* strains ($df = 48$, $V = 202.5$, $p = 0.528$). Moreover, the number of WT cannibalising a victim was not different from the number of GFP larvae cannibalising the same victim ($df = 97$, $V = 73$, $p = 0.363$). This implies that *D. melanogaster* larvae do not preferentially cluster with related individuals for the purposes of cannibalism. The minimal adequate model for explaining variation in skew between the number of cannibals aggregating around the victims at either side of the petri dish included time as the only fixed effect. Thus, the difference between the number of cannibalistic larvae increased over time between the two possible victims (Figure 3: $df = 194$, $z = 5.18$, $p < 0.001$). This suggests that larvae are more attracted to victims surrounded by a higher number of conspecifics.

3.2 | *D. simulans* mixed isoline trials

The minimal adequate model revealed that cannibalism rates in each *D. simulans* vial are best explained by all three fixed effects (treatment, larval density and the proportion of third-instar larvae) as well as the interaction between density and treatment. Overall,

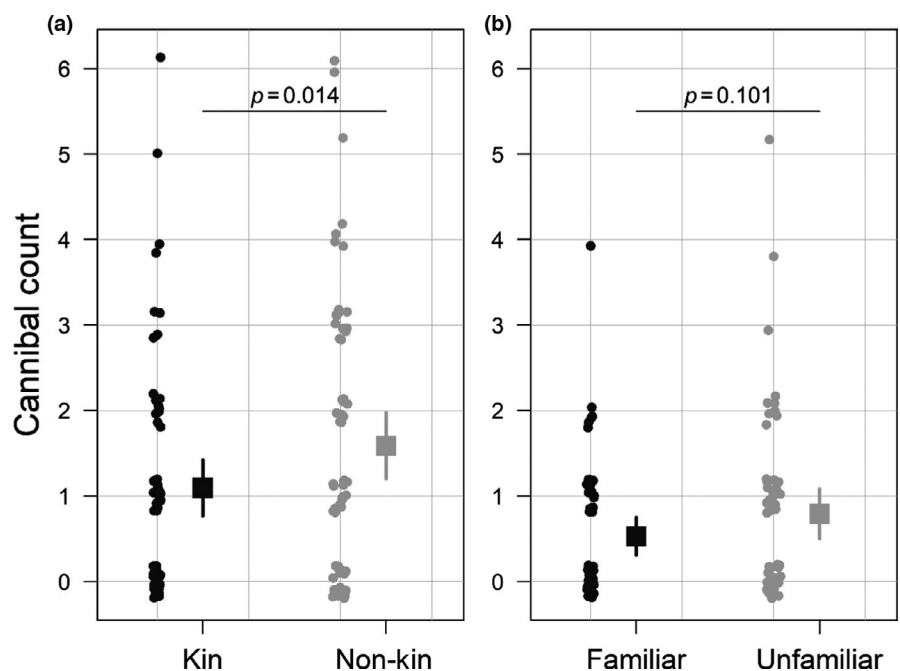


FIGURE 2 (a) More larvae were counted aggregating around non-related than related victims in *Drosophila melanogaster* choice trials. (b) The number of larvae aggregating around a socially unfamiliar victim was non-significantly higher than the number aggregating around a socially familiar victim in *Drosophila melanogaster*. Mean and 95% confidence intervals are shown. Raw data have been jittered to increase visibility

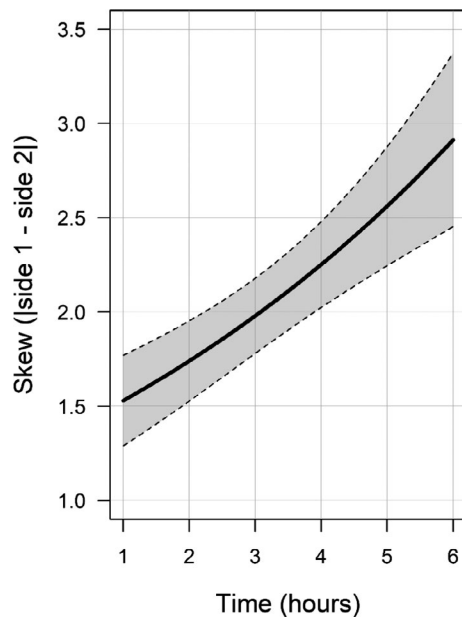


FIGURE 3 Over time, the difference between the number of cannibalistic larvae increased between the two possible larval victims. Data from *Drosophila melanogaster*. Shaded area outlines the 95% confidence interval

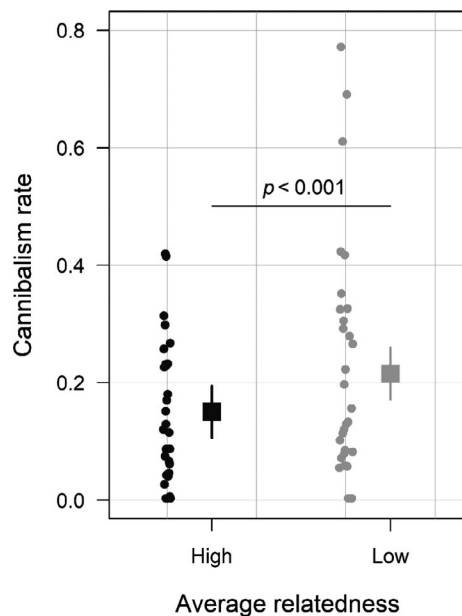


FIGURE 4 Cannibalism rate is significantly higher among larval *Drosophila simulans* in a low relatedness environment compared to a high relatedness environment. Mean and 95% confidence intervals shown. Raw data has been jittered

cannibalism rate was significantly higher in vials formed of larvae from 10 different isolines (non-related vials) compared to vials where all larvae were from the same isolate (related vials) (Figure 4: $df = 55$, $t = 9.69$, $p < 0.001$). Also, density dependence was significantly stronger in non-related than in related vials (Figure 5a, significant interaction, $df = 55$, $z = 3.05$, $p = 0.004$). However, the overall effect of density over both treatments was non-significant ($df = 55$,

$z = 1.49$, $p = 0.142$). Finally, the proportion of third-instar larvae was significantly positively associated with cannibalism rate (Figure 5b: $df = 55$, $t = 2.03$, $p = 0.047$).

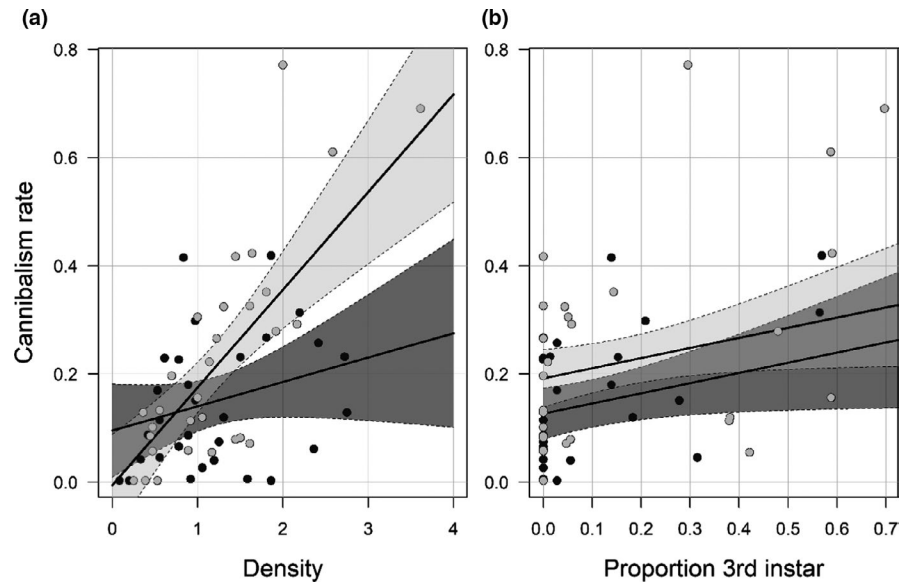
The minimal adequate model revealed that variation in larval density was best explained when the effect of relatedness was dropped from the analysis suggesting that average density was not significantly different between the kin and non-kin vials. Nor were there treatment-specific effects on density over time. However, the minimal adequate model detected a significant nonlinear decrease in larval density over time ($df = 57$, $t = -12.86$, $p < 0.001$).

4 | DISCUSSION

In this study, we provide evidence of the ability to assess conspecific relatedness in larval *Drosophila melanogaster* and *Drosophila simulans*. We show that in both species, cannibalism frequency was significantly higher between unrelated individuals than between related individuals (Figure 2a; Figure 4), even after controlling for social familiarity. In *D. melanogaster*, we show that social familiarity does not reduce larval cannibalism rates. We have also shown that density, a major driver of cannibalism in other species, is more strongly positively associated with cannibalism rates among non-related individuals than related individuals in *D. simulans* (Figure 5a). Cannibalism rate in *D. simulans* had a positive linear relationship with time (Figure S2) despite a reduction in density over time (Figure S3). This implies that hunting effort may increase in response to prolonged starvation. The ratio of third- to second-instar larvae in a vial was also positively associated with cannibalism rate in *D. simulans*. This indicates that high rates of cannibalism may be able to reduce development time under nutritional stress, or that cannibalism is more likely to occur between second- and third-instar larvae. Over time, *D. melanogaster* preferred to cannibalise victims that were being attacked by a larger number of conspecifics (Figure 3), implying that conspecific cues may also affect cannibalism frequency.

Species vary in how they assess relatedness. In some cases, indirect measures of relatedness are used to approximate which individuals are kin. For example, there is evidence from several fish species which suggests that individuals which are reared together tend to form shoals; thus, association with kin is a by-product of close proximity during early life stages (Griffiths & Magurran, 1999; Ward & Hart, 2003). Alternatively, some species directly assess their relatedness to other individuals by detecting phenotypic traits that are strongly linked to specific genes or genotypes (Keller & Ross, 1998; Lizé et al., 2006). In both our *D. melanogaster* and *D. simulans* experiment, we controlled for differences in social familiarity across treatments (see Section 2). In addition, our results explicitly show that social familiarity does not affect the frequency of cannibalism in *D. melanogaster* (however, a p -value of 0.101 suggests that an effect may have been detected with a larger sample). This is in contrast with studies observing kin recognition in adult *Drosophila*, where there is strong evidence that familiarity is used to determine relatedness (Carazo et al., 2015; Le Page et al., 2017). As such, it appears that

FIGURE 5 Cannibalism rates among *Drosophila simulans* larvae as function of larval density and the proportion of third instar. (a) Density had a more positive association with cannibalism rates among non-related (light grey) larvae than related (dark grey) larvae; (b) the proportion of third instar larvae in the vial was positively associated with cannibalism rate. 95% confidence intervals used



larvae of both species are able to directly assess relatedness using parentally inherited phenotypic cues. These findings are consistent with those of Khodaei and Long (2020), who showed that *Drosophila melanogaster* larvae were more likely to cannibalise eggs of unrelated individuals over the eggs of kin. Being able to directly assess relatedness may be important for species that are likely to encounter a mixture of related and non-related individuals early in their lifecycle. Previous evidence suggests that female *Drosophila* prefer to lay eggs on patches where other females have not laid previously (Chess & Ringo, 1985). However, females may have no choice but to share oviposition sites if suitable locations are scarce due to high population densities or poor habitat quality. Thus, environmental factors may influence the average genetic relatedness on food patches, selecting for larvae that can use kin recognition to behave optimally under a range of scenarios affecting average relatedness.

Identifying the exact cues *Drosophila* larvae use for recognition is beyond the scope of this study. However, there is evidence that a range of heritable traits such as genetically linked phenotypic traits, maternal effects and gut microbiota may be used to assess relatedness in other species (Lizé et al., 2014; Queller et al., 2003). It is also possible that cannibalistic tendencies vary between lineages. Thus, in species where larvae from the same family emerge synchronously and in close proximity to one another, larvae from a more cannibalistic family may be under strong selection to evolve anti-cannibalism adaptations to avoid being eaten by their siblings. This would create a correlation between the tendency to cannibalise and the ability to evade being cannibalised. Therefore, cannibalism rates may simply differ between related and non-related individuals because it is 'easier' for larvae from highly cannibalistic families to prey on individuals from less cannibalistic families. Although this effect has not yet been shown, it cannot be discounted as a possible explanation for differences in cannibalism rates observed between kin and non-kin.

Cannibalism rate is known to increase with population density in several groups including insects (Hopper et al., 1996; Via, 1999), fishes (Pereira et al., 2017) and amphibians (Pizzatto & Shine, 2008;

Wildy et al., 2001). Increased cannibalism rates in response to high densities could have evolved as a means of reducing competition for food, or it may simply be that opportunities to cannibalise increase with density. In this study, we found that population density in *D. simulans* was more positively associated with cannibalism rates among unrelated than related larvae (Figure 5a). Because of the disparity in how individuals in the two treatments responded to population density, we infer that on average, the fitness benefits of cannibalising at high densities are smaller than the costs associated with killing kin in *D. simulans*. However, there may be certain scenarios in which a lack of kin cannibalism in response to high densities of related larvae creates an evolutionary trap in which individuals behave in a way that is maladaptive. For example, because smaller food patches are less able to sustain multiple *Drosophila* broods, fewer females are likely to oviposit on small patches (Chess & Ringo, 1985). Therefore, smaller food patches are likely to be associated with higher average relatedness. Whereas density-induced nutritional stress on larger patches may be partially offset by opportunities to cannibalise non-related individuals, the suppression of cannibalism on small patches may lead to higher levels of starvation. However, there is evidence to suggest that larval density decreases with patch size in both *D. melanogaster* and *D. simulans* (Mitsui & Kimura, 2000). As such, females may have evolved mechanisms to avoid frequent oviposition on small patches so that food patches populated almost exclusively by kin do not experience high densities.

Many species use conspecific abundance as an indicator of food availability and/or patch quality (Grendelmeier et al., 2016; Rudolf & Rödel, 2005). We have shown that over time, *Drosophila melanogaster* larvae become increasingly attracted to victims that are being attacked by a higher number of conspecifics (Figure 3). It has previously been shown that *Drosophila* spp. engage in cooperative foraging to increase feeding efficiency when feeding on non-conspecific food sources (Dombrovski et al., 2017). As such, it could be that *Drosophila* also feed more efficiently in groups when cannibalising. Furthermore, as population density increases, larvae are likely to

have more opportunities to co-cannibalise with large number of larvae. This may be one of the mechanisms driving higher cannibalism rates at high densities in *Drosophila*.

In our co-cannibalism choice trials using *D. melanogaster*, we found that the abundance of larvae from the wild-type and GFP-Moesin families was not significantly different within feeding aggregations, suggesting that individuals do not preferentially form foraging groups with kin. This result could be considered surprising, given that one might expect individuals to avoid aggregating with non-kin who are likely to cannibalise them. Conversely, one might expect individuals from different families to mix when starved to increase their opportunities to cannibalise without harming kin. Furthermore, our result is inconsistent with the findings of Khodaei and Long (2019) who found that *D. melanogaster* larvae establish feeding groups more frequently with kin over non-kin, suggesting a preference for co-foraging with kin. However, the study by Khodaei and Long (2019) is different from the current study in several ways. Namely, larvae were not protein starved and formed foraging groups for the purposes of feeding on fly medium and not conspecifics. Also, individuals in the Khodaei and Long (2019) study were provided with a spatially continuous food source rather than two distinct food sources separated by distance (Figure 1a). As such, the fact that the results from these two studies differ suggests the formation of kin selected foraging groups may be, at least partially, determined by the extent of larval starvation and the spatial distribution of food resources.

One potential caveat of any behavioural study using lab-reared *Drosophila* is that the observed behaviours may be artefacts of adaptation to a lab environment rather than natural. Although it is impossible to dismiss the potential for a lab environment to select for cannibalistic individuals, cannibalism has been observed repeatedly across *Drosophila* spp. (Bhattacharyya, 2015; Kakeya & Takahashi, 2021; Vijendravarma et al., 2013). Also, several of these observations were of individuals that had been collected from the wild only weeks before experimentation (Bhattacharyya, 2015). Thus, individuals are unlikely to have adapted to lab conditions in such a short timeframe. Collectively, this suggests that cannibalism is a natural behaviour and not merely a product of lab rearing.

In this study, we advance the current understanding of the environmental drivers of cannibalism, and how these drivers interact to affect cannibalism frequency and the strength of kin selection. We have shown that the cannibalism of non-kin is preferred over the cannibalism of kin, and that high relatedness is likely to be assessed directly using parentally inherited phenotypic traits, rather than indirect cues such as social familiarity. We also show that relatedness reduces the likelihood that individuals will engage in cannibalistic behaviour when conspecific density is high. This could create an evolutionary trap in which related larvae cannot mitigate the effect of increased competition for food in high-density scenarios. Moreover, we have shown that in addition to relatedness cues, conspecific cues may affect the frequency of cannibalism. As density increases, conspecific cues may become an increasingly influential signal used by *Drosophila* to detect potential victims. Cannibalism

can be a significant source of mortality in natural populations and can negatively impact population viability under certain ecological scenarios. By showing that the effect of relatedness on cannibalism is modulated by population density, our work illustrates the complexity of the link between cannibalism rate and species ecology. Future research measuring cannibalism rates in response to multiple environmental factors would provide a more holistic understanding of the drivers of cannibalism in nature. Also, given the relative ease with which *Drosophila* spp. (particularly *D. melanogaster*) can be genetically manipulated, future studies could use cannibalism to expose the genetic mechanisms that underpin kin recognition.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed towards conceiving the idea for the project; S.L.P. designed and carried out the choice trial experiments; A.M.F. designed and carried out the mixed isoline experiments; A.M.F. and A.M. analysed the data; A.M.F. wrote the manuscript. All authors contributed towards refining the manuscript for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.rv15dv48f> (Fisher et al., 2021).

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