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PHEROMONE COMMUNICATION IN FEATHER-FEEDING WING LICE (INSECTA: PHTHIRAPTERA)

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ABSTRACT

Pheromone communication is central to the life history of insect parasites. Determining how pheromones affect parasite behavior can provide insights into host–parasite interactions and suggest novel avenues for parasite control. Lice infest thousands of bird and mammal species and feed on the host's feathers or blood. Despite the pervasiveness of lice in wild populations and the costs they exact on livestock and poultry industries, little is known about pheromone communication in this diverse group. Here, we test for pheromone communication in the wing lice (*Columbicola columbae*) of Rock Doves (*Columba livia*). Wing lice spend the majority of their lives on bird flight feathers where they hide from host preening by inserting their bodies between coarse feather barbs. To feed, wing lice must migrate to bird body regions where they consume the insulating barbs of contour feathers. We first show that wing lice readily form aggregations on flight feathers. Next, using a Y-tube olfactometer, we demonstrate that wing lice use pheromone communication to move toward groups of nearby conspecifics. This pheromone is likely an aggregation pheromone, as wing lice only produce the pheromone when placed on flight feathers. Finally, we found that when forced to choose between groups of male and female lice, male lice move toward male groups and females toward female groups, suggesting the use of multiple pheromones. Ongoing work aims to determine the chemical identity and function of these pheromones.

Lice are a diverse group of nearly 5,000 species of ectoparasitic insects that infest birds and mammals. These permanent parasites cement their eggs to the host's feathers or hair and spend their entire lives on the host where they consume blood or insulating feathers (Marshall, 1981). Louse infestations reduce host fitness in natural populations (Clayton et al., 2016) and cause economic losses in livestock and poultry production (Levot, 2000; Lehane, 2005; Mishra et al., 2017).

Like other insect systems, chemical communication is presumably central to the life history of lice. For example, human head lice (*Pediculus humanus capitis*) are known to respond to host-generated volatiles (Wigglesworth, 1941; Galassi et al., 2018; Adjemian et al., 2022), which likely helps them better exploit hosts. However, only a handful of studies has examined the possibility of pheromone communication between lice. Early work showed that human body lice (*Pediculus humanus humanus*) are attracted to the feces of conspecifics (Wigglesworth, 1941). Later, Mumcuoglu et al. (1986) found that head lice are attracted to ammonium compounds found in their feces. Attraction to low concentrations of ammonia is widespread in insects (Vulpe et al., 2021), and ammonium compounds are often used in insect traps (Lasa and Williams, 2021). More recently, Galassi et al. (2020) found that head lice are attracted to recently fed lice (that have defecated) and not starved lice (that have not defecated), consistent with pheromones originating in their feces. They additionally identified several volatile compounds from the feces that could potentially act as pheromones. Aside from these studies, the role of pheromones in governing important behaviors such as aggregation and mating remains poorly understood.

Here, we test for pheromone communication in feather-feeding wing lice (*Columbicola columbae*) that infest Rock Doves (*Columba livia*). Wing lice are small ectoparasites (~2 mm long) that spend their entire life span on the host where they feed on the insulating barbules of body feathers (Marshall, 1981). The consumption of insulating feathers increases thermal conductance (Booth et al., 1993), which negatively affects host survival and reduces the frequency of mating displays in infested birds (Clayton, 1990; Clayton et al., 1999).

Birds combat wing lice primarily by preening (Clayton et al., 2010). To avoid preening, wing lice insert their elongated bodies between the coarse barbs of flight feathers (Fig. 1; Bush et al., 2006) and spend >90% of their lives on these feathers (Bush and Malenke, 2008). Of interest, wing lice often insert between the same or nearby feather barbs, forming small groups (Fig. 1; C. W. Harbison, pers. obs.). This observation led us to examine wing louse distribution in more detail to determine whether they

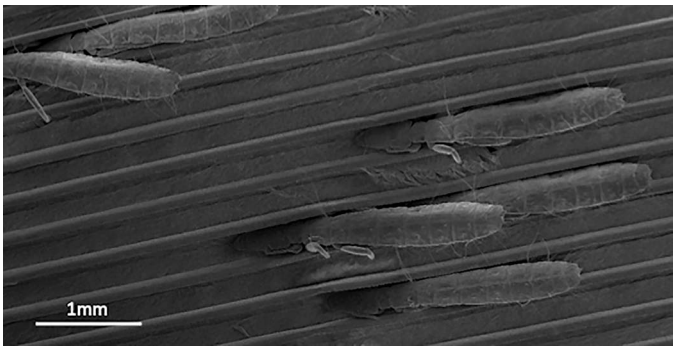

Figure 1. Scanning electron micrograph of a typical aggregation of wing lice (*Columbicola columbae*) found inserted between the barbs of a Rock Dove flight feather (*Columba livia*).

form aggregations and to test for the possibility that pheromone communication can facilitate aggregation behavior.

We first confirm that wing lice display a clustered, nonrandom distribution on flight feathers, suggesting the use of an aggregation pheromone. Next, we experimentally confirm that lice use volatile pheromones to move toward aggregations of nearby male and female lice and are capable of distinguishing between volatiles released by male and female aggregations.

MATERIALS AND METHODS

Louse distribution on flight feathers

To determine the distribution of wing lice on flight feathers, we conducted a brief visual examination of the flight feathers of 13 birds. Wing lice spend most of their time with their bodies inserted between the coarse feather barbs of the wings and tail to hide from host preening (Bush and Malenke, 2008). During the visual examination, each flight feather was divided into 3 regions (proximal, central, and distal), and the number of lice on each feather region was recorded. This was done for the 20 primary remiges, 20 secondary remiges, and 12 tail rectrices for a total of 156 flight feather regions per bird (2,028 total regions for the 13 birds examined). Because of the short duration of visual examination (~2–3 min per bird) and the relatively slow movement of lice, there was little chance that lice could move between feather regions or adjacent feathers during the examination. Additionally, most of lice remained motionless and stayed inserted between feather barbs during the examination. Morisita's index of aggregation (Dale et al., 2002) was calculated for each bird to determine whether louse distribution on the 156 flight feather regions examined was randomly distributed or clustered. One-way analysis of variance (ANOVA) with posthoc Tukey honestly significant difference was used to compare the number of lice found on proximal, central, and distal regions of flight feathers.

To determine the sex ratio of louse aggregations, 66 aggregations of lice were removed from the birds after the visual examination was completed and the sex of all lice was recorded. We compared the sex ratio of each aggregation to that of a 50:50 female (F):male (M) ratio using chi-square tests.

All birds were housed in Siena's animal facilities and were provided food and water ad libitum. To help maintain louse cultures, bird beaks were fitted with C-shaped bits that prevent efficient preening and increase louse loads (Clayton et al., 1999). All procedures were approved by Siena College's Institutional Animal Care and Use Committee, protocol Harbison-Biol499-1.

In vitro testing for pheromone communication in lice

To determine whether wing lice can detect and move toward nearby conspecifics, we used a glass Y-tube olfactometer (5-cm main tube splitting into 2 6-cm-long arms) where lice chose between an airstream with louse scent in 1 arm and a control stream of air in the opposite arm (Fig. 2). Before reaching the Y-tube, the air was humidified using a deionized H_2O bubbler, purified by passing through a chamber with activated charcoal, and split into 2 streams that then passed through the test chambers. The 'experimental' chamber contained male or female target lice (n = 20 per chamber) that were placed on wing feathers, body feathers, or in an empty chamber. Control chambers contained only wing feathers, only body feathers, or remained empty. All feathers were obtained from louse-free birds to eliminate the chance of any pre-existing louse volatiles contaminating feathers. We additionally ran trials with an empty chamber vs. a chamber with feathers to determine whether lice were attracted to the scent of feathers and control trials where both chambers remained empty.

For each trial, an individual louse was placed at the base of the Y and was given 5 min to walk up the apparatus and choose a side (n = 60 lice per treatment). A choice was recorded when the louse moved 1.5 cm up an arm. Those that did not choose within

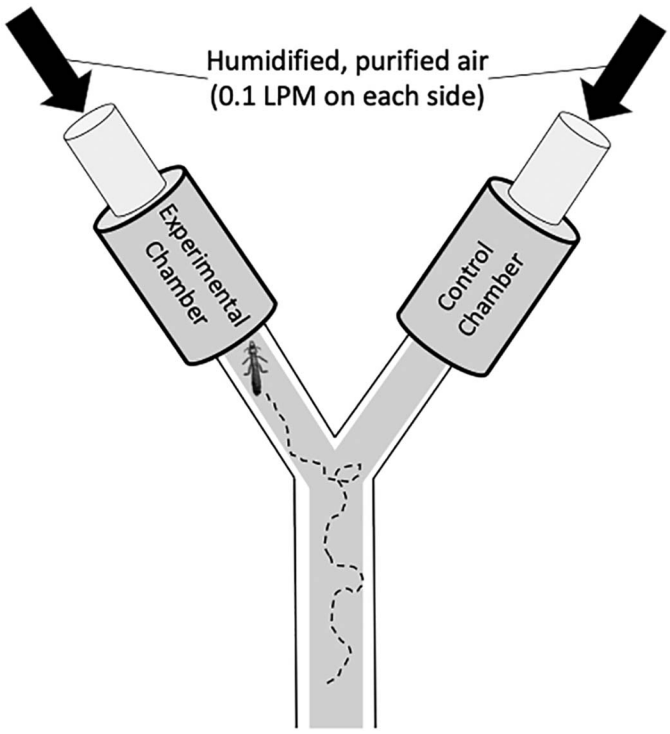

Figure 2. Experimental setup for the Y-tube olfactometer. Humidified and purified air was sent through the apparatus at 0.1 L/min. For each trial, an individual louse was placed on a cardboard track and a choice was recorded when it traveled 1.5 cm up either arm. The experimental chamber contained groups of wing lice placed in an empty chamber, on body feathers, or on wing feathers. The control chamber was empty or contained body or wing feathers, respectively.

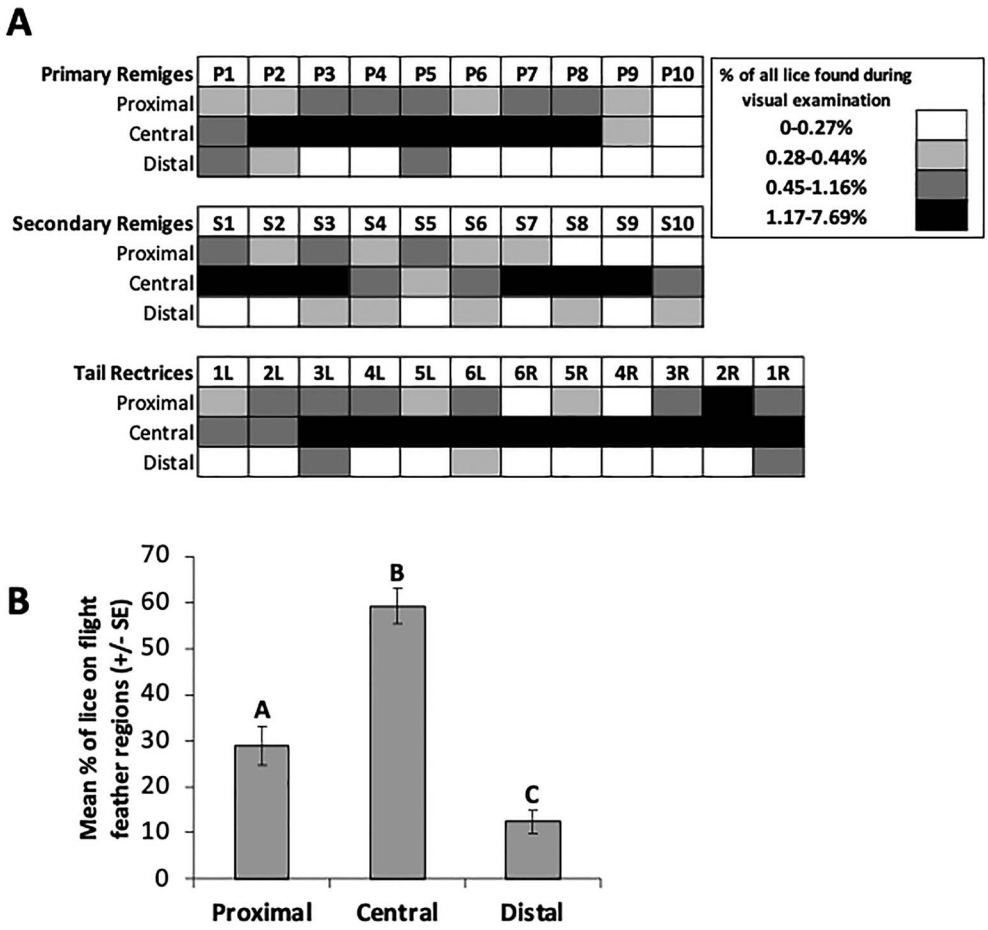

Figure 3. Location of all wing lice (*Columbicola columbae*) found on Rock Dove (*Columba livia*) wing and tail feathers. Each flight feather was divided into 3 equal regions (proximal, central, and distal) and the position of all wing lice was recorded during a 2- to 3-min visual examination of each bird (n = 13 birds). (**A**) Percentage of all lice found in each region on all wing feathers (remiges) and tail feathers (rectrices). (**B**) Mean number of lice (±SE) found on proximal, central, and distal regions of flight feathers. Lice showed a significant preference for the central regions of wing and tail feathers (different letters represent a significant difference between means; analysis of variance with posthoc Tukey–Kramer tests).

5 min were excluded from the analysis. Because of the inability of lice to walk on glass, we added a Y-shaped cardboard track to the apparatus. After 5 trials, a new track was used and we switched the side of the experimental and control airstreams.

RESULTS

Louse distribution on flight feathers

The flight feathers of 13 Rock Doves were examined to determine the distribution pattern of wing lice. Every flight feather was split into 3 equally sized regions (proximal, central, distal) and louse abundance in each region was recorded for the 20 primary remiges, 20 secondary remiges, and 12 tail rectrices of each bird. Wing lice were not distributed uniformly across these regions: 466 regions (23%) harbored lice, whereas 1,571 (77%) were louse free (Fig. 3A). Additionally, wing lice were not distributed equally between proximal, central, and distal feather regions (Fig. 3B; ANOVA, $F = 43.1$, df = 2, $P < 0.0001$). Central feather regions harbored, on average (±SE), 57.1 (± 0.04%) of all lice recorded, which was over 4 times the percentage of lice found on distal feather regions (11.6 ± 0.02%; Tukey–Kramer, $P < 0.0001$), and over twice that of proximal regions (31.8 ± 0.04%; Tukey-Kramer, $P < 0.0001$).

Wing lice showed a strong propensity to group within feather regions. Of the feather regions with lice, 51.3% harbored aggregations of ≥3 lice, whereas 48.7% harbored 1–2 lice. Aggregations in 'inhabited' regions averaged (±SE) 5.3 ± 0.2 lice, with as many as 62 lice found on a single feather region (Fig. 4). Wing louse aggregations were found on the wings and tail of each bird examined. The mean number of lice (±SE) found within regions inhabited by lice was significantly greater on remiges (6.5 ± 0.84) as compared with rectrices (3.8 ± 0.12; *t*-test, $P < 0.001$).

We used Morisita's index of aggregation to determine whether wing louse distribution across the wings and tail feathers of each bird differed from a random distribution. For all 13 birds examined, wing louse distributions were significantly different from random, indicating that they form aggregations on flight feathers (Morisita's index, $\chi^2 = 326–2{,}809$; $P < 0.0001$ for all comparisons). We additionally restricted our data to include only central feather regions where most of the lice were located. Twelve of the 13 birds showed significant clumping of lice within central regions (Morisita's index, $\chi^2 = 88–743$; $P < 0.01$ for all comparisons), whereas 1 bird showed a nonsignificant trend (Moristia's index,

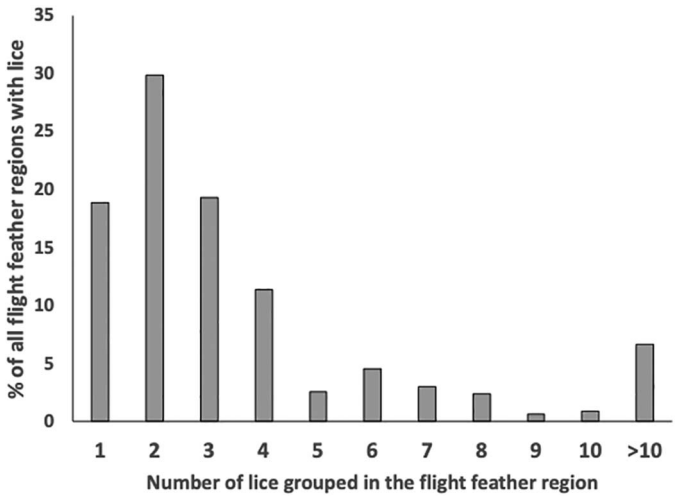

Figure 4. Histogram showing the distribution of louse group sizes (*Columbicola columbae*) on Rock Dove (*Columba livia*) flight feather regions. Each feather was divided into 3 regions and louse distribution was recorded on each bird using a 2- to 3-min visual examination (n = 13 birds). Only feather regions containing lice are included (n = 466 regions of 2,028 examined). Over 50% of all inhabited feather regions harbored ≥3 lice.

$\chi^2 = 66$, $P = 0.07$). Louse aggregations on a feather were regularly mirrored by a similar aggregation on an adjacent feather and at a similar distance from the base of each feather. When feathers were kept in a natural position, the adjacent groups were stacked so louse groups lay nearly on top of each other—this similar distribution on adjacent feathers would be expected if pheromones were used to initiate aggregation.

To determine the sex ratio of louse aggregations, we haphazardly removed 66 aggregations of ≥3 lice from birds and recorded their sex. The size of aggregations examined ranged from 3 to 33 lice, with an average size (±SE) of 6.5 ± 0.6 lice. Nearly all of the 432 lice collected were adults; only 4 nymphs were found and were not used to determine sex ratios. Overall, 56.6% of the lice were male and 43.4% female, which did not differ from a 50:50 M:F ratio (χ^2 test, $P = 0.16$). Sixty-two of the 66 aggregations collected had an M:F ratio that did not differ significantly from a 50:50 ratio (χ^2 test, $P > 0.05$). Fifty-seven of the aggregations contained both males and females; 6 contained only females and 3 contained only males.

Louse abundance on each bird was estimated using the visual examination method of Clayton and Drown (2001), in which loads from 1 wing and the tail are used to accurately predict total abundance. Estimated louse abundance ranged from 585.8 to 2,845.5 lice per bird, with an average (±SE) of 1,252.8 (±172.5) lice, which is within the range of louse loads found on wild birds (Harbison et al., 2008).

In vitro testing for pheromone communication in lice

A Y-tube olfactometer was used to test whether individual lice could detect and move toward or away from volatiles released by male and female target lice (Fig. 2). Male wing lice showed a strong preference for volatiles released by both female target lice placed on wing feathers and target males placed on wing feathers, with 85.4% and 90.7%, respectively, of male lice moving toward target lice (Table I; $\chi^2 = 26.9$, $P < 0.0001$ toward females; $\chi^2 = 22.0$, $P < 0.0001$ toward males). However, males showed no significant preference for target lice when female (64.3%; $\chi^2 = 0.2$, $P = 0.64$) and male lice (44.4%; $\chi^2 = 1.1$, $P = 0.29$) were placed on body feathers. Additionally, males showed no significant preference for the target lice when female (56.8%; $\chi^2 = 0.2$, $P = 0.68$) and male target lice (52.2%; $\chi^2 = 0.8$, $P = 0.37$) were placed in empty chambers.

Female wing lice followed a similar pattern as males and showed a strong preference for volatiles released by target females placed on wing feathers (Table I; 79.6% $\chi^2 = 19.0$, $P < 0.0001$) and target males placed on wing feathers (87.8%; $\chi^2 = 26.5$, $P < 0.0001$). Females showed no significant preference for the target lice when female (45.8%; $\chi^2 = 0.2$, $P = 0.68$) and male target lice (54.5%; $\chi^2 = 0.4$, $P = 0.55$) were placed on body feathers. Additionally, females showed no significant preference for the target

Table I. Percentage of wing lice (*Columbicola columbae*) that chose the experimental or control arm while crawling up the Y-tube olfactometer. Data were compared with a 50:50 ratio using chi-square tests (n = 60 lice per treatment).

Sex of test subjects	Contents of experimental chamber	Contents of control chamber	% Choosing experimental arm	% Choosing control arm	*P*
Female	Females on wing feathers	Wing feathers	79.6	20.4	<0.0001
Female	Males on wing feathers	Wing feathers	87.8	12.2	<0.0001
Female	Females on body feathers	Body feathers	45.8	54.2	0.68
Female	Males on body feathers	Body feathers	54.5	45.5	0.55
Female	Females only	Empty	57.3	42.7	0.25
Female	Males only	Empty	55.9	44.1	0.49
Male	Females on wing feathers	Wing feathers	85.4	14.6	<0.0001
Male	Males on wing feathers	Wing feathers	90.7	9.3	<0.0001
Male	Females on body feathers	Body feathers	64.3	35.7	0.64
Male	Males on body feathers	Body feathers	44.4	55.6	0.29
Male	Females only	Empty	56.8	43.2	0.68
Male	Males only	Empty	52.2	47.8	0.37
Female	Males on wing feathers	Females on wing feathers	25.5	74.5	<0.001
Male	Males on wing feathers	Females on wing feathers	77.8	22.2	<0.0001
Female	Wing feathers	Empty	59.3	40.7	0.44
Male	Wing feathers	Empty	38.5	61.5	0.24
Male and female	Empty	Empty	41.2	58.8	0.63

lice when female (57.3%; $\chi^2 = 1.3$, $P = 0.25$) and male target lice (55.9%; $\chi^2 = 0.5$, $P = 0.49$) were placed in empty chambers.

To determine whether lice can distinguish between volatiles released by each sex, wing lice chose between an arm containing target females and an arm containing target males. All target lice were placed on wing feathers to promote pheromone production. Females showed a strong and significant preference for the female lice (74.5%; $\chi^2 = 11.3$, $P < 0.001$) and males showed a strong preference for the male lice (77.8%; $\chi^2 = 16.7$, $P < 0.0001$).

Both male (38.5%; $\chi^2 = 1.4$, $P = 0.24$ and female lice (59.3%; $\chi^2 = 0.6$, $P = 0.44$) showed no significant preference for chambers containing only wing feathers vs. empty chambers, indicating that lice were not attracted to volatile chemicals from host feathers. Finally, as an additional control, we presented male and female lice with 2 empty chambers and saw no preference for either arm (41% left, 59% right; $\chi^2 = 0.2$, $P = 0.63$).

DISCUSSION

We first documented that wing lice readily congregate in groups on flight feathers. Next, we showed that individual wing lice are attracted to pheromones released by both male and female lice. However, wing lice only produced pheromones when placed on flight feathers where they also form aggregations. Additionally, lice exhibited strong sex-specific responses when forced to choose between volatiles from male or female groups, suggesting that multiple pheromones are used to communicate. Ongoing studies aim to determine the chemical identity of the pheromones used and their impacts on louse behaviors.

After a thorough visual examination of 13 birds, we found that wing lice were not distributed randomly throughout flight feathers. Instead, most wing lice found (51%) were grouped near one another on a subset of the available feather regions. Only 22.9% (n = 466) of the 2,028 flight feather regions examined harbored wing lice, with lice inhabiting 20.7% of all available proximal, 37.4% of available central, and 10.8% of available distal feather regions. Louse aggregations ranged from 3 to 62 lice within a single feather region (group size averaged 5.3 lice ± 0.2 SE). Lice were commonly found inserted linearly 'head to abdomen' within the same interbarb space, and aggregations would often appear on adjacent feathers and at the same distance from the body. The clustered distribution of wing lice and the proximity of wing louse groups on adjacent feathers suggest the use of an aggregation pheromone.

Y-tube olfactometer results showed clear evidence of pheromone communication, with significant numbers of lice moving up the arm toward the volatile compound(s) emitted by groups of target lice. However, target lice only appeared to produce pheromones when placed on flight feathers, where they also readily form aggregations. Female lice moved toward female (79.6% of all trials) and male target lice (87.8%) when they were placed on wing feathers, but showed no significant preference for either arm when target lice were placed on body feathers or in empty chambers. Male lice showed a similar pattern and moved toward female (85.4%) and male target lice (90.7%) when they were placed on wing feathers, but showed no significant preference for either arm when target lice were placed on body feathers or in empty chambers. Further behavioral tests are needed to confirm whether these results indicate the use of an aggregation pheromone.

Many insects are known to form aggregations. What benefits might aggregation provide wing lice? One possibility is that aggregations enable efficient mate-finding. Mating is known to occur on flight feathers (Villa et al. 2019) and it is primarily adults that congregate on flight feathers (juvenile instars are more widely distributed on the bird [Nelson and Murray, 1971]). Additionally, 86% of the aggregations we examined contained both females and males. Lice are very small (~2 mm long) and forming mixed-sex aggregations likely helps them overcome the challenge of locating a mate on the much larger host.

Another possibility is that aggregating may reduce predation via dilution effects. In this scenario, larger aggregations would help reduce predation rates via the increased protection of centrally located individuals or because of limitations in predation caused by predator handling time (Wertheim et al., 2005). Preening is a bird's main defense against lice (Clayton et al., 2010) and it is certainly feasible that aggregating may dilute its impact. Alternatively, Villa et al. (2016) showed that preening is an inducible defense in pigeons; infested pigeons increased preening rates by roughly a third as compared with louse-free birds. Thus, it is also possible that larger louse aggregations may cause birds to increase preening rates, which could reduce the benefits of aggregating. Additionally, larger louse aggregations may be more conspicuous to birds, which almost certainly use their vision to detect lice while preening (Bush et al., 2010). Thus, larger aggregations may face greater predation pressure as compared with smaller groups or solitary lice. Further work is needed to clarify the impact of preening on louse aggregation behavior.

Aggregations are also known to protect from desiccation by reducing the amount of surface area exposed to the air or by creating a more humid microclimate (Wertheim et al., 2005). This has been demonstrated in several systems: water loss was significantly reduced in male dust mite clusters (*Dermatophagoides farina*) as compared with solitary mites (Glass et al., 1988), and aggregating is known to reduce desiccation in woodlice (Broly et al., 2014). Desiccation is of particular concern for feather-feeding lice, as they use a water vapor uptake system to extract moisture from ambient air. Rudolph (1983) showed that this system begins to fail at a relative humidity (RH) near 45–50%, and Moyer et al. (2002) found that wing lice can be eliminated from birds kept in a low-humidity environment (<40% RH) after only 6 wk. They further show that louse loads on wild dove species are significantly reduced in populations from Arizona (arid) as compared with populations in more humid climates. If aggregation does prevent desiccation in wing lice, we would predict that aggregation behavior would be more prevalent in arid as compared with humid environments.

Finally, multiple factors may act synergistically to promote aggregation behavior. For example, aggregation has been shown to prevent desiccation in Collembola species and increase the probability that females will pick up a deposited male spermatophore (Wertheim et al., 2005).

We also found that wing lice produce sex-specific pheromones. Male and female lice readily moved toward either sex when the opposite chamber was louse free. However, when forced to choose between groups of male and female target lice, 77.8% of males moved toward male groups and 74.5% of females moved toward female groups. One possibility is that wing lice produce postnuptial pheromones that repel the opposite sex, which is well documented in other insect systems. For example, Xu et al.

(2014) found that males of the parasitoid wasp, *Cotesia glomerata*, are attracted to virgin females but show no attraction to, and are even repelled by, mated females. Females, in turn, were repelled by mated males. However, pheromones that repel the opposite sex would presumably lead to sex-specific aggregations on flight feathers, and we found that nearly all aggregations contained both males and females. One possibility is that a general aggregation pheromone would promote mixed-sex aggregations, and a separate pheromone(s) would help identify unmated individuals within these groups, thus leading to more efficient mate finding.

In summary, we show that wing lice readily form mixed-sex aggregations on flight feathers. We further show that pheromone communication is used to locate and move toward nearby wing lice, which may explain the formation of these aggregations. Additionally, wing lice appear to produce sex-specific pheromones that enable them to distinguish between and respond to individuals of each sex. Previous work has shown that ammonium compounds from louse feces attract conspecifics (Wigglesworth, 1941; Mumcuoglu et al., 1986; Galassi et al., 2020). In this study, wing lice were only briefly placed in the test chambers (~5 min); thus it is likely that they responded to pheromone(s) derived from sources other than their feces.

We are currently working to determine the chemical identity of the pheromones used by males and females. Lice likely respond to a limited number of pheromones; the recently assembled genome of *Columbicola columbae* only found 13 odorant receptors genes, which is greatly reduced in number as compared with free-living insects such as *Drosophila melanogaster*, which contain >150 odorant receptor genes (Baldwin-Brown et al., 2021). This reduction in odorant receptors is likely a result of their stable environment on hosts and simplified feather diet.

There is much to learn about chemical communication within this diverse group of ectoparasites. Identifying louse behaviors that are induced by pheromones will lead to a greater understanding of host–parasite interactions and possibly lead to novel solutions for controlling louse populations on humans, livestock, and poultry.

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