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# Offspring Dependency Is Not Reflected in the Social Immunity of Three Burying Beetle Species

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## ABSTRACT

Offspring dependency on parental care varies significantly across species, often driven by parental food provisioning and protection from predation. However, an often-overlooked factor is protection against pathogens through the exhibition of social immunity. However, if and how parental protection from microbial pressure shapes and scales with offspring dependency remains poorly understood. Here, we investigate both personal and social immunity in three species of *Nicrophorus* burying beetles—a model system known for elaborate parental care and social immunity. These species differ markedly in their offspring's ability to survive without parental care, ranging from independence to complete dependence on post-hatching care. We measured personal and social immunity in adult female and male beetles before, during, and outside of reproductive bouts. Our results show that species with more dependent offspring do not necessarily invest more heavily in social immunity, whereas reproductive state affected immunity differently across species. These findings emphasize the complexity of parent-offspring coadaptation, and the need for further study of the evolutionary trajectory from facultative to obligate family associations.

## 1 | Introduction

Family life is a well-researched phenomenon found in a plethora of taxa in the animal kingdom (Klug and Bonsall 2010). Parents and their offspring form temporary units which enhances the offspring's survival and development—benefits largely conveyed through parental care. Parental care is defined as any trait that evolved and/or is maintained because it provides benefits to offspring (Smiseth et al. 2012). During family life, parents mitigate harsh environmental conditions by providing different types of post-hatching or post-natal care, for example, nest building, food provisioning, and defense against predators, parasites and pathogens (Clutton-Brock 1991; Trumbo 1996; Royle et al. 2012), shifting selective pressures acting on offspring away from mere survival towards taking maximum advantage of the care provided by their parents. Importantly, this care comes at costs to parents, resulting in parent-offspring conflict (Klug and Bonsall 2010) as fitness benefits differ and the two parties favor different outcomes during the selection process (Janzen and Warner 2009). Although it is often in the parent's evolutionary interest to finish the current reproductive effort and save resources for the next, offspring fitness usually increases with prolonged and more intensive parental care (Smiseth et al. 2012). Through continued transference of key traits and behavior to their parents, such as foraging or immunity, offspring fitness and/or survival becomes increasingly specialized and dependent on the care they receive, leading to a co-evolution of offspring demands and parents' resource generosity (Kölliker et al. 2005). For example, early nymphal stages of social cockroaches possess specialized mouthparts that help with the uptake of liquid food provided by their mothers when offspring are still dependent on parental care (Costa 2006). Similarly, begging behavior in larvae of the burying beetle *Nicrophorus vespilloides* ceases as offspring develop and become less dependent on their parents' care (Smiseth et al. 2003). Offspring outsourcing of key life-history traits and abilities is not limited to resource acquisition, however: young of *Forficula auricularia* earwigs and *N. vespilloides* have been shown to adjust the expression of key immune genes to the presence of a caring mother (Ziadie et al. 2019; Körner et al. 2020).

Offspring traits and parental care may mutually reinforce each other towards even more parental investment into offspring and absolute (i.e., obligate) offspring dependency on care (Gardner and Smiseth 2011; Capodeanu-Nägler et al. 2016). Building on this presumption, obligate parental care may be more likely to evolve if parents are more efficient at a given task—such as obtaining food, defending a resource, or providing protection from microbes and parasites—than the offspring on their own (Smiseth et al. 2003; Gardner and Smiseth 2011). Of all the benefits parental care can provide in such a way, food provisioning and protection are considered the most widely observed forms, with plentiful evidence supporting their pivotal role in the evolution of obligate care (Smiseth et al. 2012; Capodeanu-Nägler et al. 2016; Prang et al. 2025). In social groups such as families, protection against pathogens and parasites may be as important as warding off predators (Côté and Poulinb 1995; Korb and Heinze 2016). However, the role of immunity and parental protection from microbial pressure—that is, social immunity—in the evolution of obligate care from a facultative state is severely understudied (Meunier 2015; Van Meyel et al. 2018).

Social immunity refers to a collection of behavioral and physical traits that are expressed not for the individual's benefit but for the benefits they provide to other homospecific group members (Cremer et al. 2007, 2018; Cotter and Kilner 2010a; Meunier 2015; He et al. 2018; Jones et al. 2018; Van Meyel et al. 2018). This phenomenon likely represents an important driver in the evolution towards more complex forms of sociality and the maintenance of social structures (Cremer et al. 2007, 2018), with more and more evidence suggesting social immunity as an integral factor in the evolution of subsocial species as well (Van Meyel et al. 2018). For instance, antimicrobial components in feces of earwigs and cockroaches may greatly alter the microbial pressure on the developing offspring (Rosengaus et al. 2013; Diehl et al. 2015) and facilitate prolonged family life and social evolution (Jackson and Hart 2009). A more striking example can be found in the family life of *Nicrophorus* burying beetles, during which parents care for their offspring while they develop on a vertebrate carcass the parents have monopolized (Pukowski 1933; Scott 1998; Potticary et al. 2024). *Nicrophorus* parents not only feed predigested carrion to the larvae but also continually coat the ephemeral carcass resource during pre- and post-hatching care with antimicrobial exudates, reducing the overall microbial load while also transferring a beneficial microbiome to their offspring. Oral exudates may be produced by salivary glands that produce antimicrobial compounds, as seen in termites (Lamberty et al. 2001), whereas anal exudates may stem from an abdominal gland, whose secretions served a defensive functions originally (Dettner 1993; Degenkolb et al. 2011). Although these behaviors have not explicitly been shown to reduce larval infection risk or pathogen exposure in the laboratory, they are well-known to increase offspring performance and reduce their expression of personal immunity (Pukowski 1933; Hoback et al. 2004; Cotter and Kilner 2010a; Degenkolb et al. 2011; Shukla, Plata, et al. 2018; Shukla, Vogel, et al. 2018; Ziadie et al. 2019).

Intriguingly, burying beetle offspring exhibit a broad spectrum of dependency on post-hatching parental care, ranging from facultative to obligate (Capodeanu-Nägler et al. 2016). *Nicrophorus orbicollis* larvae are highly dependent on the parental care they receive, and while they do not lack the ability to self-feed, they cannot survive on the carcass resource in absence of parental care (Scott and Traniello 1990; Trumbo 1992). This stands in stark contrast to larvae of *Nicrophorus pustulatus* which are able to self-feed on the carcass and even survive better in absence of post-hatching care in the laboratory (Kim et al. 2011; Capodeanu-Nägler et al. 2016; Capodeanu-Nägler, Prang, et al. 2018), indicating post-hatching care benefits that may derive from guarding and resource monopolization behaviors but do not yield benefits in controlled experimental conditions, a phenomenon also observed in earwigs (Thesing et al. 2015). In the middle of this spectrum, *N. vespilloides* larvae greatly benefit from the post-hatching parental care they receive but are not dependent on it, as they are able to survive and develop to adulthood without it, showcasing a facultative dependency on their parents' care (Eggert et al. 1998; Müller et al. 2025). Continuous post-hatching parental care may represent a relatively new evolutionary development in *Nicrophorus* spp. that evolved after pre-hatching care, brood guarding, and carcass preparation (Trumbo 1992). Although *Nicrophorus* species differ in post-hatching care and how larvae depend on it, they all engage in pre-hatching care by preparing and burying the carcass in anticipation of the larvae, yet under laboratory conditions, larvae of all species are able to survive on unprepared carcasses for at least 24 h (Eggert et al. 1998; Capodeanu-Nägler et al. 2016; Trumbo 2017; Trumbo and Sikes 2021).

Intriguingly, the mechanisms that drive offspring (in)dependency in this genus remain somewhat unclear: while nutritional dependency (Capodeanu-Nägler et al. 2016; Capodeanu-Nägler, Prang, et al. 2018; Prang et al. 2025) and transfer of microbial symbionts (Shukla, Vogel, et al. 2018; Brinkrolf et al. 2021; Körner et al. 2023) are likely candidates, the possible role of parental social immunity during pre- and post-hatching care is understudied. In this study, we investigated if offspring dependency aligns with parental investment into social immunity. To this end, we measured key immune parameters in parental pairs of three burying beetle species—*N. pustulatus*, *N. vespilloides*, and *N. orbicollis*—at distinct stages of their reproductive cycle (non-breeding, pre-hatching care, and post-hatching care) to investigate changes in personal and external immunity outside of and during pre- and post-hatching parental care. In line with previous studies, we expected that internal and external immunity increase in breeding compared to non-breeding females in all three species (Steiger et al. 2011; Reavey, Warnock, et al. 2014; Jacobs et al. 2016). If larval independence is at least partially driven by increased parental exudate application during pre-hatching care, we also expected shifts between pre- and post-hatching care in parents of independent larvae. Conversely, we expected that the more dependent species show fewer external immunity changes between pre- and post-hatching care phases. Across species, and in line with literature, we expected internal immunity to trade off with external immunity when breeding (Cotter et al. 2013).

## 2 | Materials and Methods

### 2.1 | Beetle Husbandry

*Nicrophorus vespilloides* beetles were collected near Bayreuth, Germany (49°55′15.6″ N, 11°34′19.2″ E), whereas *N. pustulatus* and *N. orbicollis* were collected near Lexington city, Illinois, USA (40°39′57′′ N, 88°53′49′′ W). All beetles were kept in a climate chamber at 20°C with a 16:8 h light:dark cycle.

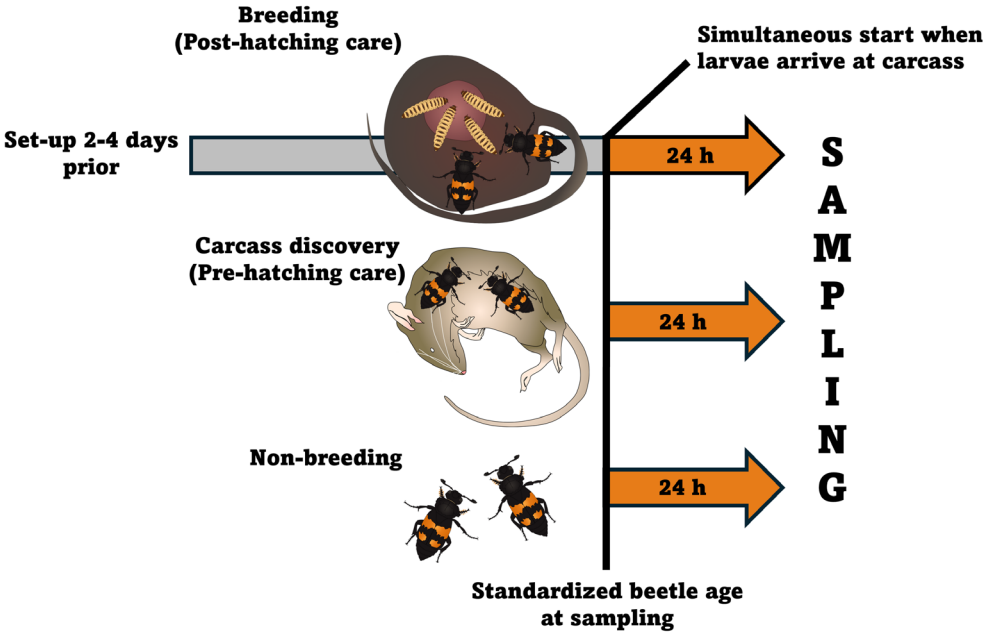


**FIGURE 1** | Experimental design, detailing how pairs of beetles were set up and when treatments were started. In order to standardize beetle age during sampling, some treatments were started earlier than others: the breeding condition requires successful mating and carcass preparation, whereas carcass discovery takes much less and non-breeding no preparation time for the beetles. The duration of pre-hatching care differs between species; see Section 2.

## 2.2 | Experimental Design

During this study, we used beetles approximately 20 days old (precise eclosion date only up to ~48 h accuracy) of the species *N. vespilloides* ($n = 110$, 55 females, 55 males), *N. pustulatus* ($n = 126$, 63 females, 63 males), and *N. orbicollis* ($n = 124$, 62 females, 62 males) and investigated them during three distinct phases of their reproductive cycle (treatments): non-breeding (not providing care), carcass discovery (pre-hatching care), and breeding (post-hatching care). Samples of all treatments were staggered over a period of 3 days to make sampling feasible, with individuals properly randomized across the 3 days (with the same number of samples across treatments each day). Given the sampling volume and time required, the three species were not done simultaneously but in sequence (*N. orbicollis*, then *N. vespilloides*, and finally *N. pustulatus*). Photometric assays were not done sequentially but with all species evenly distributed across plates.

For each sample, a pair of beetles was placed into a plastic box ($10 \times 10 \times 6$ cm) halfway filled with coconut peat (tropic-shop.de). For beetles in the breeding phase, a mouse carcass ($23.21 \pm 1.0$ g) was then added to each pair before transferring them to a dark climate chamber (20°C), where the pair started to prepare the carcass and breed. Two to four days later, depending on the species (*N. pustulatus* = 3 days, *N. vespilloides* = 2 days, *N. orbicollis* = 4 days) (Capodeanu-Nägler et al. 2016), boxes were checked for oviposition of eggs. As soon as eggs were observed, eggs and parents were separated by transferring the parents and the prepared carcass to a new box with coconut peat. This allowed us to control the time when hatched larvae arrived at the carcass. When larvae started to hatch, usually a day after separation, they were returned to their parents. On the day larvae were reunited with their parents, the carcass discovery and non-breeding treatment were started (using different pairs of beetles) to standardize the age of beetles across treatments (Figure 1). We deliberately did not standardize brood size as the number of offspring is known to vary between the species (Capodeanu-Nägler et al. 2016), and parental investment into social and personal immunity might be influenced by this manipulation.

For the carcass discovery treatment, a pair of beetles and a freshly thawed mouse carcass ($23.01 \pm 1.3$ g) were placed in coconut peat filled boxes. They were then placed in a dark climate chamber (20°C). Set-up for the non-breeding phase was similar, but we added small slices of a baby mouse carcass. Although we did not measure the exact weight of each slice, the baby mouse carcasses, on average, weigh $1.9 \pm 0.3$ g and were cut into three roughly equal parts. The small size of carrion-like food does not induce breeding but facilitates the production of oral and anal exudates (Creighton, pers. comm.). If pairs of the non-breeding treatment started to exhibit signs of pre-hatching care or egg laying, they were excluded from further analysis. Beetle pairs of the breeding phase were sampled 24 h after larvae were added, whereas beetle pairs of carcass discovery and non-breeding phase were sampled 24 h after they were set-up. This way we could sample all beetles at the same age (after pupation). We collected anal and oral exudates of the adult beetles to measure social immunity in form of exudate lytic activity, as well as hemolymph samples to measure personal immunity in form of phenoloxidase activity. Additionally, during sampling, pronotum width of every individual was measured using a caliper.

## 2.3 | Collection of Oral and Anal Exudates

Oral and anal exudates were collected in 5 μL glass capillaries (Hirschmann, ringcaps) from mouth and abdomen. Collected

exudates, at minimum 1 μL, were then diluted 1:10 with phosphate-buffered saline (PBS) and stored at −80°C until further use in later assays. We noted the volume extracted for each individual, but exudates could not be collected from each beetle used in our study. To collect exudates, beetles were gently picked up and held in hand, whereas carefully blowing air onto their antennae. In most cases, this agitates the beetle enough that they release exudates from mouth and/or abdomen, which can then be collected. This method ensures that beetles release exudates on their own, making it less likely that other fluids are mixed in the sample. This procedure is well established with the *Nicrophorus* model (Cotter et al. 2010; Miller et al. 2019). The volume of secreted exudates was measured and noted for later analysis. Note that we did not repeatedly sample individual beetles; hence our data are of limited use to make a robust statement on the total amount of exudates produced by the beetles. For the analysis of lytic activity, we required at least 1 μL of exudate; all samples of less than 1 μL exudate had to be excluded from the lytic activity analysis.

### 2.4 | Hemolymph Extraction

Hemolymph was always extracted after exudates were collected. Beetles were first anaesthetized with $CO_2$ and then their soft part of the cuticle between their pro- and mesothorax (beneath the pronotum) was carefully pierced with a sterilized dissection needle. Emerging hemolymph formed a droplet on the cuticle of the beetle and was collected using a 5 μL glass capillary (Hirschmann, ringcaps). We noted the volume extracted of each individual. For the phenoloxidase assays, at least 2 μL of hemolymph was required from each beetle, which were then diluted 1:5 with MOPS buffer (3-Morpholinopropane sulfonic acid, 5 mM with added protease inhibitor) and frozen at −80°C until further use.

### 2.5 | Exudate Lytic Activity Assay

To measure the lytic activity of collected anal and oral exudates as a measurement of social immunity, we added 10 μL of previously thawed diluted exudate sample to each well of a 96-well plate before adding 90 μL of *Micrococcus luteus* solution (3 mg/10 mL PBS). Negative controls included 10 μL of PBS instead of exudate sample, whereas positive controls included 10 μL of lysozyme solution (1 mg/10 mL PBS). Lytic activity was measured using a microplate reader (Synergy H1 Microplatereader; Biotek) by measuring the optical density of the *M. luteus* solution at 600 nm for 2 h, every 2 min. During later analysis, we examined activity curves of $V_{max}$ measurements and excluded curves with an $R^2$-value (goodness of fit) of less than 0.8, as well as any samples whose activity curves were disjointed or fractured.

### 2.6 | Hemolymph Phenoloxidase Activity Assay

In order to measure phenoloxidase activity in the hemolymph as a measurement of personal immunity, hemolymph samples were thawed on ice while adding 10 μL of double-distilled water to each well of a 96-well plate. Ten microliter of thawed sample were then added to each well as 10 μL 5 mM MOPS buffer (no protease inhibitor). At last, we added 180 μL of L-DOPA (L-3,4-Dihydroxyphenylalanine) solution (39.4 mg L-DOPA/20 mL MOPS buffer (5 mM, no protease inhibitor)) and mixed the solution by carefully pipetting. The L-DOPA solution was freshly prepared for each 96-well plate by dissolving the L-DOPA and then vortexing the solution for 15 min. The L-DOPA solution was then placed in an ultrasonic bath for 3 min before sterile filtering it into a pipetting reservoir. PO activity was measured through measuring the light adsorption at 490 nm for 2 h every 2 min, using a Synergy H1 Microplatereader (Biotek). During later analysis, we excluded samples with activity curves whose $R^2$ value (goodness of fit) was less than 0.8 as well as any curves that looked disjointed or fractured.

### 2.7 | Statistical Analysis

All statistical analyses were done using R (v4.3.3; R Core Team 2024) and RStudio (Posit Team 2024), loaded with data organization aids dplyr, and tidyr (Wickham et al. 2023, 2024). To test the influence of treatment, species and sex on immune parameters, we used generalized linear mixed-effect models (GLMers) (Bates et al. 2015), with sample-IDs included as a random factor (response variable ~ treatment × species × sex + (1|ID)). Model fidelity was analyzed using the DHARMa package (Hartig 2022). To obtain *p*-values of general effects we used an Anova (type II) from the car package (Fox and Weisberg 2019). Post hoc pairwise comparisons were done through the emmeans function (Lenth et al. 2025) with Tukey adjustment. To determine directions of significant interactions, we used custom contrasts of emmeans for relevant pairwise comparisons, which were adjusted for false discovery rate. Additionally, we tested if normalized size and immune parameters do correlate through a correlation test (base R). If we found a correlation (Table S1), normalized size was included as a covariate in the models. As brood size (number of offspring) can affect parental investment, as was shown for *N. vespilloides* (Duarte et al. 2016), we accounted for this by testing the subset of beetles during the breeding and found that their immune investment (phenoloxidase or lytic activity of exudates) was not affected by the number of offspring on the carcass (Table S2).

Due to low sample size, models fitted with oral exudate lytic activity and differences in volume as response variables did not converge. In these cases, we used a Kruskal–Wallis test, followed by Dunn's test of multiple comparisons (Ogle et al. 2025), with Benjamini–Hochberg false discovery rate. Figures were created using packages ggplot2 (Wickham 2016) and RColorBrewer (Neuwirth 2022).

## 3 | Results

### 3.1 | Effects on Personal Immunity

Phenoloxidase activity was affected by the reproductive phases of beetles ($\chi^2 = 6.059_2$, $p$-value = 0.048, Table 1) and the normalized size of beetles ($\chi^2 = 6.195_1$, $p$-value = 0.013, Table 1), as phenoloxidase (PO) activity correlated positively with the normalized size of beetles (Table S1). However, PO activity was not affected by species ($\chi^2 = 0.325_2$, $p$-value = 0.850, Table 1), sex

($\chi^2 = 0.256_1$, $p$-value = 0.593, Table 1) nor by an interaction of all factors ($\chi^2 = 6.451_4$, $p$-value = 0.168, Table 1). However, subsequent post hoc comparisons did not show any differences between the reproductive phases (Table 2; Figure 2A).

## 3.2 | Effects on Social Immunity

### 3.2.1 | Anal Exudates

Anal exudate lytic activity differed between species ($\chi^2 = 22.207_2$, $p$-value < 0.001, Table 1) and between the reproductive phase beetles were in ($\chi^2 = 12.706_2$, $p$-value = 0.002, Table 1) but was not affected by sex ($\chi^2 = 1.065_1$, $p$-value = 0.151, Table 1) or by an interaction of the factors ($\chi^2 = 5.753_4$, $p$-value = 0.168, Table 1). Post hoc testing revealed that the anal exudate lytic activity of *N. pustulatus* was lower than those of *N. orbicollis* and *N. vespilloides* (Table 2; Figure 2B), whereas there was no difference between *N. orbicollis* and *N. vespilloides* (Table 2). In all three species, post hoc testing showed that lytic activity was higher during the breeding phase compared to the non-breeding phase (Table 2; Figure 2B), whereas there was no detectable difference between lytic activity of anal exudates that were collected from beetles during carcass discovery compared to samples from breeding and non-breeding beetles (Table 2; Figure 2B).

**TABLE 1** | Results of GLMers and Kruskal–Wallis test to test for differences in immune parameters between *Nicrophorus* spp. and the different phases of their reproductive cycle.

| Generalized linear mixed-effects model | Predictor | $\chi^2$ | df | $p$ |
|---|---|---|---|---|
| Phenoloxidase activity | Treatment | 6.059 | 2 | **0.048** |
| | Species | 0.325 | 2 | 0.850 |
| | Sex | 0.256 | 1 | 0.593 |
| | Normalized size | 6.195 | 1 | **0.013** |
| | Treatment × species | 6.276 | 4 | 0.179 |
| | Treatment × sex | 4.678 | 2 | 0.096 |
| | Species × sex | 2.682 | 2 | 0.262 |
| | Treatment × species × sex | 6.451 | 4 | 0.168 |
| Anal exudate activity | Treatment | 12.706 | 2 | **0.002** |
| | Species | 22.207 | 2 | **< 0.001** |
| | Sex | 1.065 | 1 | 0.151 |
| | Treatment × species | 0.337 | 4 | 0.987 |
| | Treatment × sex | 2.172 | 2 | 0.338 |
| | Species × sex | 1.173 | 2 | 0.556 |
| | Treatment × species × sex | 5.753 | 4 | 0.218 |
| **Kruskal–Wallis test** | **Predictor** | $\chi^2$ | **df** | $p$ |
| Oral exudate activity | Treatment | 11.981 | 2 | **0.003** |
| | Species | 8.655 | 2 | **0.013** |
| | Sex | 0.909 | 1 | 0.340 |

*Note:* Statistically significant $p$-values are highlighted in bold.

When investigating the effects of species and reproductive phase on the volume of anal exudates, we found that the volume was affected by an interaction of the reproductive phase and the species ($\chi^2 = 10.821_4$, $p$-value = 0.029, Table 3), as well as by the reproductive phase ($\chi^2 = 17.845_2$, $p$-value < 0.001, Table 3) and species alone ($\chi^2 = 118.066_2$, $p$-value < 0.001, Table 3). Additionally, the volume of anal exudates was affected by the normalized size of the beetles ($\chi^2 = 24.547_1$, $p$-value < 0.001, Table 3), as beetle size and anal exudate volume correlated positively (Table S1). Post hoc testing revealed that *N. orbicollis* beetles produced higher volumes of anal exudates during carcass discovery compared to non-breeding and breeding beetles (Table 4; Figure 3A), whereas the volume of non-breeding and breeding *N. orbicollis* beetles did not differ (Table 4; Figure 3A). *N. pustulatus* beetles' anal exudate volume is increased during breeding and carcass discovery compared to non-breeding beetles, whereas the volumes of beetles during breeding and carcass discovery did not differ (Table 4; Figure 3A). The volume of anal exudates of *N. vespilloides* was not affected by the reproductive phase the beetles were in at the time of sampling (Table 4; Figure 3A). During breeding and non-breeding, the anal exudate volumes of *N. pustulatus* beetles were higher than those of *N. orbicollis* and *N. vespilloides* beetles (Table 4; Figure 3A), whereas the volume did not differ between *N. vespilloides* and *N. orbicollis* (Table 4; Figure 3A). However, during carcass discovery, *N. pustulatus* beetles again showed the highest volume of anal exudates compared to *N. orbicollis* and *N. vespilloides* beetles (Table 4; Figure 3A), whereas *N. orbicollis* showed an increased volume of exudates compared to *N. vespilloides* during this phase of their reproductive cycle (Table 4; Figure 3A).

### 3.2.2 | Oral Exudates

Oral exudate activity was affected by the reproductive phase beetles were in at the time of sampling ($\chi^2 = 11.981_2$, $p$-value = 0.003, Table 1) and between the species ($\chi^2 = 8.655_2$, $p$-value = 0.013, Table 1) while sex did not affect the lytic activity ($\chi^2 = 0.909_1$, $p$-value = 0.340, Table 1). Post hoc testing revealed that the lytic activity of oral exudate during the non-breeding phase was lower than during the breeding phase of their reproductive cycle, whereas there was no difference to the carcass discovery phase (Table 5; Figure 2C). *N. orbicollis* did not differ in their lytic activity compared to the other two species, but oral exudates of *N. vespilloides* showed a higher lytic activity than *N. pustulatus* (Table 5; Figure 2C). When investigating the oral exudate volume of beetles during their reproductive cycle, we found that oral exudate volume was affected by sex ($\chi^2 = 7.9464_1$, $p$-value = 0.005, Table 3) and by the species ($\chi^2 = 49.585_2$, $p$-value < 0.001, Table 3) but not by the treatment ($\chi^2 = 4.963_2$, $p$-value = 0.084, Table 3). Across all species, males produced a lower oral exudate volume than females (Figure 3B),

**TABLE 2** | Estimated marginal means with "Tukey" adjustment and pairwise comparisons for immune parameters by beetles' reproductive phase and species.

| Estimated marginal means | Contrasts of treatment | Estimate | SE | df | *t*-ratio | *p* |
|---|---|---|---|---|---|---|
| Phenoloxidase activity | Breeding vs. Carcass discovery | −2.46 | 1.16 | 157 | −2.120 | 0.089 |
| | Breeding vs. Non-breeding | −2.85 | 1.22 | 170 | −2.346 | 0.052 |
| | Carcass discovery vs. Non-breeding | −0.39 | 1.22 | 167 | −0.321 | 0.945 |
| Anal exudate activity | Breeding vs. carcass discovery | −0.415 | 0.211 | 127 | −1.965 | 0.125 |
| | Breeding vs. non-breeding | −0.814 | 0.232 | 157 | −3.503 | **0.001** |
| | Carcass discovery vs. non-breeding | −0.399 | 0.229 | 153 | −1.746 | 0.192 |
| **Estimated marginal means** | **Contrasts of species** | **Estimate** | **SE** | **df** | ***t*-ratio** | ***p*** |
| Anal exudate activity | *N. orbicollis* vs. *N. pustulatus* | −0.623 | 0.218 | 141 | −2.855 | **0.014** |
| | *N. orbicollis* vs. *N. pustulatus* | 0.404 | 0.234 | 151 | 1.722 | 0.201 |
| | *N. pustulatus* vs. *N. vespilloides* | 1.027 | 0.220 | 148 | 4.672 | **< 0.001** |

*Note:* Statistically significant *p*-values are highlighted in bold.

whereas post hoc testing revealed that the oral exudate volume of *N. orbicollis* was lower than that of *N. vespilloides* or *N. pustulatus* (Table 5; Figure 3B), whereas there was no difference in volume between the latter two (Table 5; Figure 3B).

## 4 | Discussion

Parental care is a milestone in the evolution of sociality and allows offspring to allocate resources to their development as they transfer key functions, like defence against pathogens, over to their parents, possibly increasing the dependency on their parents' care (Kölliker et al. 2005). To test the hypothesis that parents take over progressively more communal immune defence with increasing offspring dependency, we measured personal and external immune parameters of parents during different phases of their reproductive cycle in three *Nicrophorus* species which differ in their expression of and their offspring's dependency on parental care (Capodeanu-Nägler et al. 2016).

During the course of our experiment, we found that the personal immunity (phenoloxidase activity; PO) of all three *Nicrophorus* species was affected by the different phases of their reproductive cycle but did not differ between the sexes. As subsequent post hoc testing did not reveal any differences between the three stages, we may speculate after visual inspection of the data that *Nicrophorus* beetles upregulate their PO activity upon discovering carcass, whereas their PO activity decreases during the first 24 h of post-hatching care (breeding). Upregulation of personal immunity capabilities may be a necessary step to monopolize the carcass as beetles come in close contact with carcasses' potentially dangerous microbiome (Shukla, Plata, et al. 2018). A similar pattern has been reported in *N. vespilloides*, as PO activity first increases and then decreases the longer beetles stay with the carcass (Cotter and Kilner 2010b) while no such pattern was found in *N. orbicollis* (Steiger et al. 2011). It could be argued that females should invest more into immunity than males to maximize their fitness, as their reproductive effort is much higher (Rolff 2002), as seen in several other species (Kurtz et al. 2000; Rolff 2001; Schwarzenbach et al. 2005; Gershman et al. 2010). Depending on resource and partner availability, a more phenotypically plastic approach might be favored (McKean and Nunney 2005). Cotter et al. (2013) argue that *N. vespilloides* females should invest less into their personal immunity than males, as they gain less fitness benefits from a longer lifespan; however, we found no such sex differences in our study. Since personal and social immunity may trade off (Cotter et al. 2013), beetles that are actively caring should express a lower personal immunity during that time, instead focusing on manipulating the carcass resource by application of antimicrobial exudates.

Examining the lytic activity of the species' anal and oral exudates, as a means of social immunity, showed that, whereas all three *Nicrophorus* species may differ in their offspring dependency, they still share certain similarities. In all species, we could observe an upregulation of anal exudate lytic activity towards the breeding phase of their reproductive cycle. Antimicrobial properties of anal exudates probably represent an evolutionary new development in *Nicrophorus* spp., and their ancestral functions are most likely a defensive repellant for other insects and/or predators (Degenkolb et al. 2011; Lindstedt et al. 2017), as defensive glands are common in their close relatives, Silphini (Dettner 1993), which never provide direct or indirect parental care. Anal exudates of *N. orbicollis* are modified during breeding to be less toxic for their offspring (Trumbo and Sardi 2026). In *N. vespilloides*, whose offspring actively contribute to carcass preservation (Arce et al. 2013; Reavey, Warnock, et al. 2014), lytic activity increases during the breeding phase (Cotter and Kilner 2010b; Arce et al. 2012), as the expression of *Lys6*, a lysozyme encoding gene, peaks during the time of larval hatching (Palmer et al. 2016). A similar mechanism might be at play for the obligately dependent *N. orbicollis*, who also shows an upregulation of lytic activity from the moment of egg laying to active parental care (Steiger et al. 2011), and the post-hatch care-independent *N. pustulatus*. The latter is a particularly interesting case, as a previous study did not find any antibacterial activity in *N. pustulatus*' anal exudates (Hoback et al. 2004), potentially due to their suspected brood parasitism (Trumbo 1994; Ratcliffe 1996), although more recent research suggests instead that *N. pustulatus* uses snake eggs as a (potentially preferred)

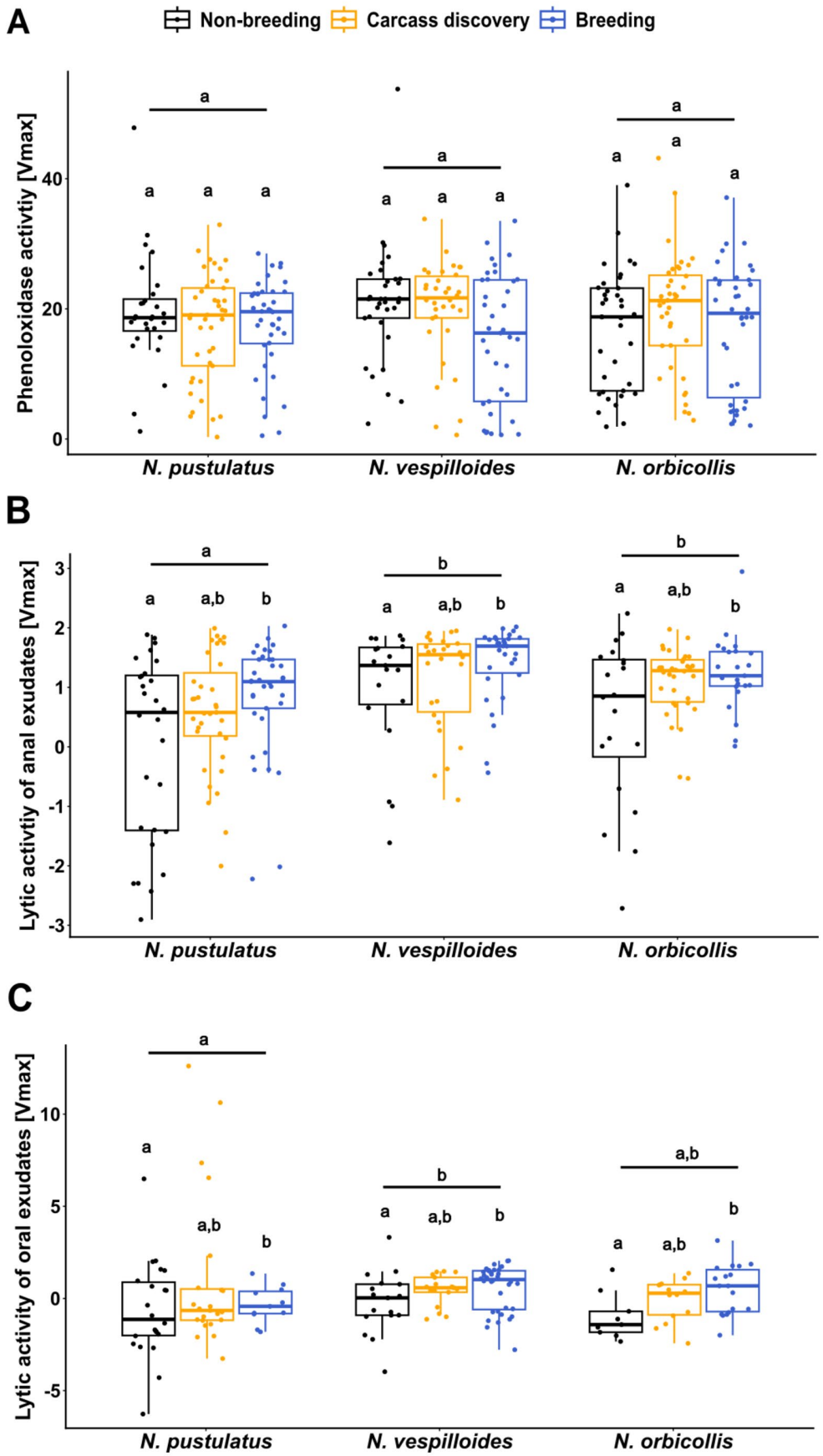


**FIGURE 2** | Legend on next page.

**FIGURE 2** | Boxplots showcasing how key immune parameters behave during three distinct stages of *Nicrophorus* spp. reproductive cycle (non-breeding [NB], carcass discovery [CD], and breeding [BR]). (A) shows the phenoloxidase activity of hemolymph samples (personal immunity) of *N. pustulatus* ($n = 109$, NB = 28, CD = 41, BR = 20), of *N. vespilloides* ($n = 105$, NB = 33, CD = 34, BR = 38) and of *N. orbicollis* ($n = 115$, NB = 37, CD = 40, BR = 38) during the different stages of their reproductive cycle. Note that while there was an effect of reproductive phase on phenoloxidase activity in the GLMer, the following post hoc test showed no difference between treatments. (B) Shows the lytic activity of anal exudates samples (external immunity) of *N. pustulatus* ($n = 100$, NB = 28, CD = 35, BR = 37), or *N. vespilloides* ($n = 75$, NB = 19, CD = 26, BR = 30) and of *N. orbicollis* ($n = 77$, NB = 20, CD = 34, BR = 73) during the different stages of their reproductive cycle. (C) Shows the lytic activity of oral exudate samples (external immunity) of *N. pustulatus* ($n = 56$, NB = 22, CD = 23, BR = 11), of *N. vespilloides* ($n = 70$, NB = 17, CD = 17, BR = 36) and of *N. orbicollis* ($n = 41$, NB = 9, CD = 14, BR = 18). Boxplots show median (horizontal line), 25% and 75% quartile (boxes) and 1.5 × interquartile range (whiskers). Statistical differences are indicated by letters ($p$-value ≤ 0.05).

**TABLE 3** | Results of GLMers and Kruskal–Wallis test to test for differences in anal and oral exudate volume between *Nicrophorus* spp. and the different phases of their reproductive cycle.

| Generalized linear mixed-effects model | Predictor | $\chi^2$ | df | $p$ |
|---|---|---|---|---|
| Anal exudate volume | Treatment | 17.845 | 2 | **< 0.001** |
| | Species | 118.066 | 2 | **< 0.001** |
| | Sex | 0.271 | 1 | 0.575 |
| | Normalized size | 24.547 | 1 | **< 0.001** |
| | Treatment × species | 10.821 | 4 | **0.029** |
| | Treatment × sex | 3.886 | 2 | 0.059 |
| | Species × sex | 1.352 | 2 | 0.437 |
| | Treatment × species × sex | 2.350 | 4 | 0.807 |
| **Kruskal–Wallis test** | **Predictor** | $\chi^2$ | **df** | $p$ |
| Oral exudate volume | Treatment | 4.963 | 2 | 0.084 |
| | Species | 49.585 | 2 | **< 0.001** |
| | Sex | 7.946 | 1 | **0.005** |

*Note:* Statistically significant $p$-values are highlighted in bold.

breeding resource (Smith et al. 2007). Out of the three species we tested, we observed the lowest lytic activity in anal exudates of *N. pustulatus*. Considering the use of this alternative breeding resource, the microbiome of *N. pustulatus* does not differ compared to other *Nicrophorus* spp. (Kaltenpoth and Steiger 2014), but the use of this alternative breeding resource could explain the absence/reduced activity, as (snake) eggs contain lysozyme (Phillips 1967; Thammasirirak et al. 2006), leading to a reduced selection pressure to combat microbes. However, it is important to keep in mind that this, just like any other ecological differences between the tested species, highlights the limitations of a species comparison done on three data points such as this.

In addition to how the reproductive cycle affects the lytic activity of exudates, we were also interested in whether the volume of secreted exudates differs between the species and certain phases of reproduction. We observed that *N. pustulatus* secreted the highest volumes out of all three species investigated and showed an increased volume during the carcass discovery of their reproductive cycle. *N. vespilloides* beetles, which did not adjust their volume during any phase of the reproductive cycle, and *N. orbicollis*, who did show an increased volume after discovering a carcass and during breeding, did not otherwise differ in the volume of anal exudates. Taking the differences in lytic activity between the species into account, one could argue that the increased volume of *N. pustulatus* is a compensatory response to their low lytic activity, whereas *N. vespilloides* and *N. orbicollis*, which show a much higher lytic activity, can justify applying less but more potent exudate to the carcass. As our study design did not include exhaustive sampling of exudates (e.g., sampling individuals beetles multiple times per day, or more persistently to obtain a sample from every beetle), we cannot make definitive statements about the overall volume of exudates *Nicrophorus* beetles can produce The question that emerges is whether high exudate sample volumes result from beetles producing and constantly applying more exudates, or whether they occur because beetles apply little to no secretions to the carcass. Our data does not offer a conclusive answer to this question, but we provide preliminary evidence that *Nicrophorus* spp. beetles likely differ in external immune strategies under similar conditions.

Analysis of oral exudates did reveal that all three tested *Nicrophorus* species upregulate the lytic activity of their oral exudates towards the breeding phase of their reproductive cycle, whereas *N. vespilloides* showed the highest lytic activity out of the three species and *N. pustulatus* the lowest. Although oral and anal exudates are both applied to the carcass directly (Pukowski 1933), potentially in an effort to transfer the parental microbiome to their offspring (Shukla, Vogel, et al. 2018, 201), the main function of oral secretions lies in transferring pre-digested carcass to the carcass (Capodeanu-Nägler, Eggert, et al. 2018; Trumbo and Sardi 2026). When investigating the volume of oral exudates secreted by the beetles, we found that the obligately dependent *N. orbicollis* had the lowest volume, whereas the other less dependent species did not differ between them. In all species, females showed higher volumes of oral exudates. *N. orbicollis* offspring relies heavily on parental provisioning of food and is not able to self-feed on the carcass resource during the first 24 h, potentially due to their un-serrated mandibles (Benowitz et al. 2018; Capodeanu-Nägler, Prang, et al. 2018). We thus speculate that the higher volume of females across all three tested species implies that they feed the larvae more than males as many studies have shown that during

**TABLE 4** | Estimated marginal means with "false discovery rate" adjustment and pairwise comparisons for anal exudate volume by beetles' reproductive phase and species.

| Estimated marginal means | Custom contrasts of biological significance | Estimate | SE | df | Lower CL | Upper CL | *t*-ratio | *p* |
|---|---|---|---|---|---|---|---|---|
| Anal exudate volume | *N. orbicollis* (Breeding) vs. *N. orbicollis* (Carcass discovery) | −0.415 | 0.120 | 171 | −0.778 | −0.052 | −3.474 | **0.001** |
| | *N. orbicollis* (Breeding) vs. *N. orbicollis* (Non-breeding) | −0.041 | 0.120 | 172 | −0.405 | 0.323 | −0.345 | 0.815 |
| | *N. orbicollis* (Carcass discovery) vs. *N. orbicollis* (Non-breeding) | 0.457 | 0.118 | 172 | 0.097 | 0.816 | 3.856 | **<0.001** |
| | *N. pustulatus* (Breeding) vs. *N. pustulatus* (Carcass discovery) | −0.158 | 0.118 | 171 | −0.517 | 0.201 | −1.339 | 0.274 |
| | *N. pustulatus* (Breeding) vs. *N. pustulatus* (Non-breeding) | 0.251 | 0.118 | 171 | −0.107 | 0.610 | 2.127 | 0.057 |
| | *N. pustulatus* (carcass discovery) vs. *N. pustulatus* (Non-breeding) | 0.410 | 0.118 | 170 | 0.052 | 0.767 | 3.477 | **0.001** |
| | *N. vespilloides* (Breeding) vs. *N. vespilloides* (Carcass discovery) | −0.036 | 0.124 | 170 | −0.413 | 0.340 | −0.293 | 0.815 |
| | *N. vespilloides* (Breeding) vs. *N. vespilloides* (Non-breeding) | −0.042 | 0.126 | 170 | −0.425 | 0.340 | −0.336 | 0.815 |
| | *N. vespilloides* (Carcass discovery) vs. *N. vespilloides* (Non-breeding) | −0.006 | 0.129 | 170 | −0.398 | 0.386 | −0.047 | 0.963 |
| | *N. orbicollis* (Breeding) vs. *N. pustulatus* (Breeding) | −0.744 | 0.119 | 170 | −1.106 | −0.382 | −6.236 | **<0.001** |
| | *N. orbicollis* (Breeding) vs. *N. vespilloides* (Breeding) | 0.038 | 0.121 | 170 | −0.329 | 0.405 | 0.315 | 0.815 |
| | *N. pustulatus* (Breeding) vs. *N. vespilloides* (Breeding) | 0.782 | 0.119 | 170 | 0.419 | 1.144 | 6.548 | **<0.001** |
| | *N. orbicollis* (Carcass discovery) vs. *N. orbicollis* (Carcass discovery) | −0.487 | 0.118 | 170 | −0.844 | −0.129 | −4.133 | **<0.001** |
| | *N. orbicollis* (Carcass discovery) vs. *N. orbicollis* (Carcass discovery) | 0.417 | 0.123 | 170 | 0.045 | 0.789 | 3.400 | **0.002** |
| | *N. orbicollis* (Carcass discovery) vs. *N. orbicollis* (Carcass discovery) | 0.904 | 0.123 | 170 | 0.532 | 1.276 | 7.370 | **<0.001** |
| | *N. orbicollis* (Non-breeding) vs. *N. orbicollis* (Non-breeding) | −0.534 | 0.118 | 172 | −0.893 | −0.175 | −4.510 | **<0.001** |
| | *N. orbicollis* (Non-breeding) vs. *N. orbicollis* (Non-breeding) | −0.046 | 0.125 | 172 | −0.425 | 0.334 | −0.365 | 0.815 |
| | *N. orbicollis* (Non-breeding) vs. *N. orbicollis* (Non-breeding) | 0.488 | 0.125 | 170 | 0.110 | 0.866 | 3.919 | **<0.001** |

*Note:* Statistically significant *p*-values are highlighted in bold.

biparental care, burying beetle females expend more energy and provide more direct care (e.g., feeding larvae with regurgitated carrion) than males who focus on brood guarding and carcass maintenance (Fetherston et al. 1990; Scott 1990; Smiseth and Moore 2004; Creighton et al. 2015; Körner et al. 2026; Müller et al. 2026, manuscript under review).

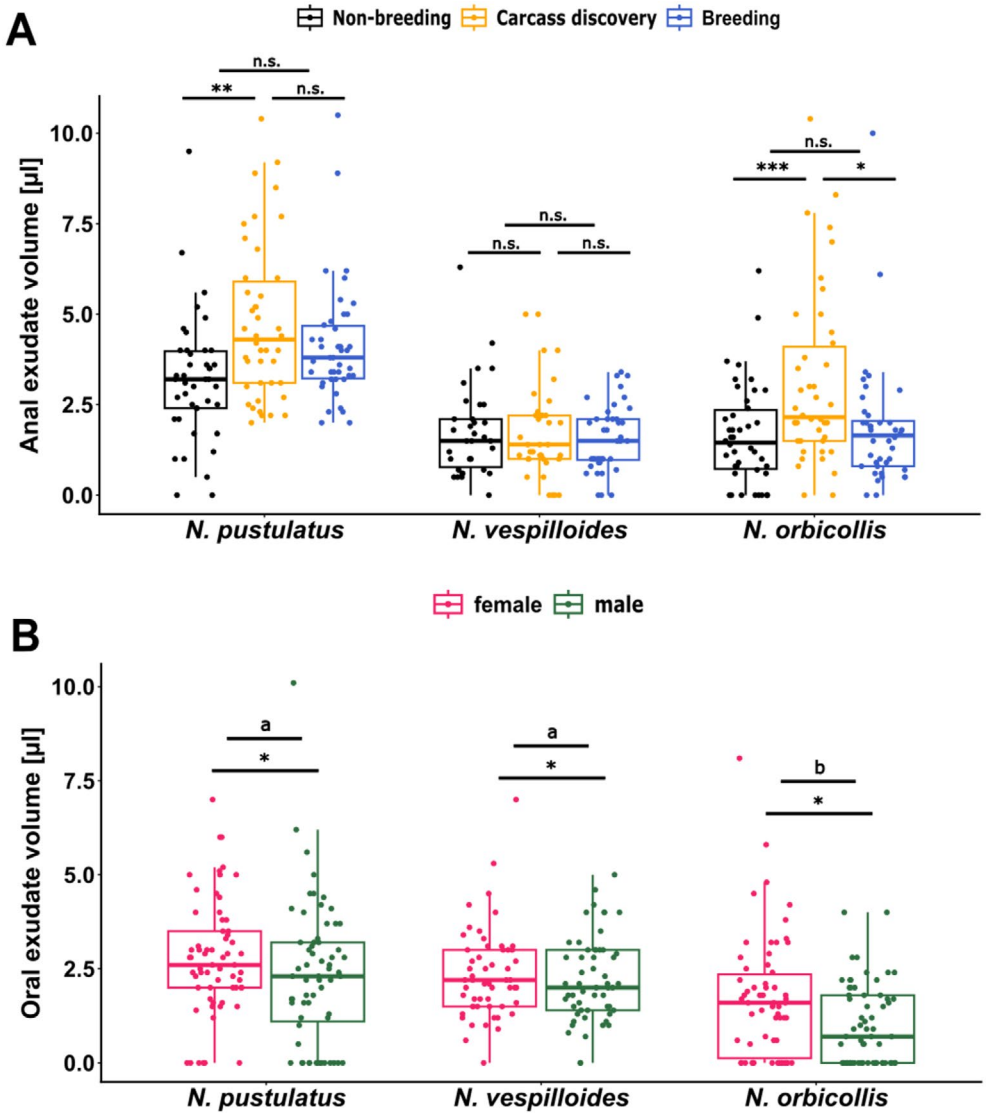


**FIGURE 3** | Box plots comparing the volumes of anal and oral exudes of three *Nicrophorus* spp. (A) Shows how the volume of anal exudates is affected by three distinct stages of the reproductive cycle (non-breeding [NB], carcass discovery [CD], and breeding [BR]) in *N. pustulatus* ($n = 126$, NB = 42, CD = 42, BR = 42), in *N. vespilloides* ($n = 110$, NB = 34, CD = 36, BR = 40) and in *N. orbicollis* ($n = 124$, NB = 42, CD = 42, BR = 40). (B) Shows differences in the volume of oral exudates between the sexes in *N. pustulatus* ($n = 126$, female = 63, male = 63), in *N. vespilloides* ($n = 110$, female = 55, male = 55) and in *N. orbicollis* ($n = 124$, female = 62, male = 62). Boxplots show median (horizontal line), 25% and 75% quartile (boxes) and 1.5 × interquartile range (whiskers). Statistical differences are indicated by asterisks and letters ($p$-value ≤ 0.05; * < 0.05; ** ≤ 0.01; *** ≤ 0.001).

Despite the tested species' stark differences in offspring dependency, they share significant behavioral and ecological similarities. We provide results that, at least partially, confirm our hypothesis that varying offspring dependency of three *Nicrophorus* species may be partially reflected in their social immunity strategies. We are, however, aware of the limitations that are imposed by such a species comparison, as our sample size is very limited to only three species. Still, we argue that species comparisons such as this have their merit and help to understand how closely related species differ in respect to their behavioral ecology and their evolution. *Nicrophorus* beetle have proven to be suitable for such studies (Hoback et al. 2004; Benowitz et al. 2016, 2018; Capodeanu-Nägler et al. 2016; Capodeanu-Nägler, Eggert, et al. 2018; Trumbo and Sikes 2021; Prang et al. 2022). Trumbo and Sikes (2021) hypothesized that carcass monopolization and concealment was an essential step in the evolution of parental care in the *Nicrophorus* genus and may represent a relatively new evolutionary development as their sister genus *Ptomascopus* does not exhibit any burial or care behavior (Choe and Crespi 1997). Anal and oral exudates not only help with control of the carcass microbiome and the resource's preparation but also allow for vertical microbiome transfer which further aids in the conservation of the carcass nursery (Wang and Rozen 2017; Shukla, Vogel, et al. 2018). However, as with any model system, the comparability of results greatly depends on standardized methods. For example, differences in personal and social immunity between reproductive stages in *N. orbicollis* shown in Steiger et al. (2011) were not replicated here since we did not measure PO using an encapsulation assay, did not challenge beetle immunity with the nylon implant before exudate collection, and measured immunity 24 h earlier (Steiger et al. 2011). Going forward, it may be prudent to attempt

**TABLE 5** | Dunn's test with "Benjamini–Hochberg" adjustment for pairwise comparisons, following significant Kruskal–Wallis tests, for the oral exudate activity and volume.

| Dunn's pairwise comparison | Comparisons | *Z*-score | *p* (unadjusted) | *p* (adjusted) |
|---|---|---|---|---|
| Oral exudate activity | Breeding vs. carcass discovery | −1.682 | 0.093 | 0.093 |
| | Breeding vs. non-breeding | −3.457 | 0.001 | **0.002** |
| | Carcass discovery vs. non-breeding | −1.755 | 0.079 | 0.119 |
| | *N. orbicollis* vs. *N. pustulatus* | −1.061 | 0.289 | 0.289 |
| | *N. orbicollis* vs. *N. vespilloides* | 1.548 | 0.122 | 0.182 |
| | *N. pustulatus* vs. *N. vespilloides* | 2.914 | 0.004 | **0.011** |
| Oral exudate volume | *N. orbicollis* vs. *N. pustulatus* | −6.604 | < 0.001 | **< 0.001** |
| | *N. orbicollis* vs. *N. vespilloides* | −5.350 | < 0.001 | **< 0.001** |
| | *N. pustulatus* vs. *N. vespilloides* | 1.032 | 0.302 | 0.302 |

*Note:* Statistically significant *p*-values are highlighted in bold.

to obtain more consistent results using standardized methodology or more comparable metrics such as gene expression.

We ultimately did not find stark contrasts in the beetles' investment into social immunity during pre- and post-hatching care that clearly align with differences in offspring dependency across the three species we investigated. Given the involvement of offspring in *Nicrophorus* social immunity (Arce et al. 2013; Reavey, Beare, and Cotter 2014; Duarte et al. 2016), future studies should investigate the opposite side of the equation and determine whether offspring with varying dependency alter their own immune investment more or less in accordance with their reliance on parental care, for example by measuring larval personal and social immunity, and gene expression.

### Author Contributions

**Maximilian Körner:** conceptualization, methodology, formal analysis, resources, writing – original draft, writing – review and editing, supervision, project administration, funding acquisition, validation. **Laura Cherpion:** methodology, validation, investigation, data curation. **Leon Müller:** formal analysis, writing – original draft, data curation, writing – review and editing, visualization.


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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

All data required to replicate analyses in this manuscript will be available for download via a public repository under repository https://doi.org/10.5281/zenodo.19333565 upon acceptance of the manuscript.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Pearson's product–moment correlation test to investigate if phenoloxidase activity, lytic activity of exudates and their volume correlate with normalized beetle size. **Table S2:** Results of GLMers and Kruskal–Wallis test to test for an influence of brood size on their personal and social immunity in a subset of breeding *Nicrophorus* spp. Statistically significant *p*-values are highlighted in bold.