Author for correspondence:
Ahva L. Potticary
e-mail: ahva.potticary@uga.edu

THE ROYAL SOCIETY PUBLISHING

takeout gene expression is associated with temporal kin recognition

Ahva L. Potticary, Elizabeth C. McKinney, Patricia J. Moore and Allen J. Moore

Department of Entomology, University of Georgia, Athens, GA 30602, USA

ALP, 0000-0002-1157-5315; PJM, 0000-0001-9802-7217; AJM, 0000-0002-1498-3322

A key component of parental care is avoiding killing and eating one's own offspring. Many organisms commit infanticide but switch to parental care when their own offspring are expected, known as temporal kin recognition. It is unclear why such types of indirect kin recognition are so common across taxa. One possibility is that temporal kin recognition may evolve through alteration of simple mechanisms, such as co-opting mechanisms that influence the regulation of timing and feeding in other contexts. Here, we determine whether *takeout*, a gene implicated in coordinating feeding, influences temporal kin recognition in *Nicrophorus orbicollis*. We found that *takeout* expression was not associated with non-parental feeding changes resulting from hunger, or a general transition to the full parental care repertoire. However, beetles that accepted and provided care to their offspring had a higher *takeout* expression than beetles that committed infanticide. Together, these data support the idea that the evolution of temporal kin recognition may be enabled by co-option of mechanisms that integrate feeding behaviour in other contexts.

1. Introduction

Parenting is expensive, and thus a variety of mechanisms to avoid caring for unrelated offspring have evolved. Many organisms commit infanticide of conspecific young but switch to parental care at the time when their own young would be expected, known as temporal kin recognition [1–4]. The time lag between the cue that 'starts the clock' and the behavioural shift to parental care can be quite long. For example, male mice are infanticidal until 18–20 days after ejaculation, after which they show parental care to young [4]. Temporal kin recognition often works in the absence of conspecific phenotypic cues; indeed, parents presented with young at the correct time and place will even accept and feed young of other species (e.g. [3,5,6]). Temporal kin recognition is a short-term

biological timing problem—that is, it can occur over several days, is reversible and not seasonal—and has been linked to circadian rhythms across taxa [4,7]. For this reason, it has been suggested that the mechanisms that regulate feeding and timing in other contexts are co-opted to enable the evolution of temporal-based kin recognition [8].

Here, we investigate whether *takeout*, a gene known to be associated with coordinating feeding behaviour through an association with circadian rhythms [9,10], has been co-opted to facilitate temporal kin recognition in the burying beetle *Nicrophorus orbicollis*. Two RNA-seq studies of different species of burying beetle comparing gene expression in a parenting state with a non-parenting state identify *takeout* as one of the genes differentially expressed [11,12]. Thus, *takeout* appears to play some role in burying beetle parenting although the transcriptomic studies could not identify the specific function given the broad comparison. In insects, *takeout* gene expression is involved in pathways which convey temporal and nutritional information, like starvation, to influence feeding activity [9], has been linked to attraction or aversion to chemosensory cues [13], and is often located in areas that correspond to chemosensory functions such as the antennae [14]. *takeout* mRNA can cycle in the absence of light in *Drosophila* [15], although *takeout* can also be influenced by light and dark cues [16]. We hypothesized that because parenting requires a switch from eating offspring to feeding offspring, and because this switch occurs at a very specific time in burying beetles, this switch will involve *takeout*.

Burying beetles require small animal carcasses to breed, and once a suitable carcass is located, the female lays eggs in the soil nearby and the parents bury and prepare the carcass as a food resource for their offspring [17]. Upon hatching, larvae crawl to the carcass where they are fed regurgitated carrion by the parents. Burying beetles identify their larvae temporally; parents of both sexes will commit infanticide of any larvae that arrive too early, and will only accept and feed larvae that arrive at the time where their own offspring can be expected [1,7]. However, parents will accept any larvae that arrive at the correct time, even larvae of other burying beetle species [18]. Temporal kin recognition in *Nicrophorus* can be manipulated by changing light schedules [7]; thus, the mechanism that influences the timing of temporal kin recognition is still sensitive to photic inputs despite *Nicrophorus* breeding underground, which hints at co-option of mechanisms involved in daily cycling. Intriguingly, the embryonic periods or time to larval acceptance for many *Nicrophorus* occur in increments of approximately 24 h; for example, larvae are accepted at approximately 48 h post egg-laying in *Nicrophorus mexicanus* and *N. vespilloides* [7,19], 72 h in *N. quadripunctatus* [20], and 96 h in *N. orbicollis* (this study). Some burying beetles only bury the carcass under the leaf litter, like *N. vespilloides*, while others such as *N. orbicollis* bury the carcass multiple centimetres below the soil surface [17,21]. For these reasons, mechanisms involved in both feeding and timing, that can cycle in the absence of light cues, may be particularly likely to enable the evolution of temporal kin recognition in *Nicrophorus* [8].

Our hypothesis that *takeout* has been co-opted to influence temporal kin recognition makes several predictions. First, *takeout* should be associated with non-parental contexts that have been previously linked to *takeout* in other species. Second, *takeout* should not be associated with parental care in general, such as preparation of the carcass. Third, *takeout* expression should differ between parents that accept larvae and those that commit infanticide independently of any role in non-parental contexts or in general parenting. To test these predictions, we first experimentally induce hunger in beetles to determine the effect of food deprivation on *takeout* expression. We next measure *takeout* expression during the development of parenting behaviour. We then collect parents both as they show natural variation in temporal kin recognition and experimentally manipulate when parents expect their larvae to arrive to determine whether *takeout* expression differs between parents that accept or commit infanticide. We find that *takeout* expression is associated with the likelihood that parents will accept their larvae but find little evidence for an association between *takeout* and non-parental roles or the development of parenting.

2. Material and methods

2.1. Insect colony and husbandry

Nicrophorus orbicollis used in this study were part of an outbred colony that we supplemented regularly with beetles from Whitehall Forest in Athens, GA [22]. We designed housing with the goal of reducing stress and maximizing the animals' welfare. We complied with the regulations in the USA for experiments on invertebrates. We maintained all virgin beetles in an incubator (Percival, model no. I41VLC8, Perry, IA, USA) at $22 \pm 1°C$ on a 14 : 10 hour light : dark cycle to simulate summer breeding conditions. All individuals used for this study were bred in the laboratory to ensure that parentage was

known and that age, and rearing conditions were standardized. We housed beetles individually at larval dispersal in 9 cm diameter by 4 cm deep circular compostable deli containers (EP-RDP5, Eco-Products) filled with approximately 2.5 cm of potting soil (Performance Organics All Purpose In-Ground Soil, Miracle-Gro). Once we placed larvae into individual containers, they had no further social interactions with other burying beetles until we allocated them into the behavioural or non-parental treatments. This species is nocturnal and buries breeding carcasses multiple centimetres below the soil surface. As we did not provide the beetles sufficient soil to completely bury the carcass, we moved beetles that were allocated to the parental treatments (development of parenting and temporal kin recognition) to an incubator under constant darkness to simulate underground breeding. Beetles in the non-parental treatment (hunger versus fed) were kept in the same incubator as other virgins. We defined individuals as age Day 1 on the day of eclosion. We fed beetles ad libitum organic ground beef twice a week following ecolsion. For all treatments, we randomly assigned individuals Day 13–19 to treatments, and for parental contexts, we randomly mated focal virgins aged post-eclosion with a non-relative.

2.2. Gene identification

We used Geneious Prime (v. 2022.1.1) to blast the *Nicrophorus orbicollis* transcriptome [23] for putative *takeout* homologues of *Drosophila melanogaster* (Q9VBV3) and *Nicrophorus vespilloides* (XP_017773976). To visualize protein conservation across *takeout* copies, we aligned protein sequences from *D. melanogaster* [9], *N. vespilloides*, *Locusta migratoria* [13], *Reticulitermes speratus* [24], *Bombyx mori* and *Anopheles gambiae* [25], and using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and produced plots with Javalview [26].

2.3. *takeout* expression in non-parental contexts

To test the hypothesis that *takeout* is associated with hunger in non-parental contexts, we experimentally manipulated food availability for female and male beetles. We selected virgin beetles Day 13–19 post-eclosion and randomly allocated them to a hunger treatment (no feeding for one week), and a fed treatment (ad libitum feeding for one week). The goal of this manipulation was to induce hunger without resulting in starvation or mortality. We selected a week for the length of the manipulation because *Nicrophorus* beetles can survive for multiple weeks without food [27], and body mass loss can occur in *N. orbicollis* over short periods of time from similar periods of withholding food [28,29]. We conducted this experiment with $n = 14$ females and males in each treatment, although three female beetles died during the experiment ($n = 2$ 'hunger' females and $n = 1$ 'fed' female).

2.4. *takeout* expression and parenting

2.4.1. *takeout* and the development of parenting

To test the hypothesis that *takeout* is associated with the development of parenting, defined as the days elapsed since egg-laying and specifying the period where parents transition from a non-parenting state to a state when larvae may be accepted, we collected female and male beetles at 0 (eggs laid), and then at 24, 48, 72 and 96 h post-egg laying, which is the approximate time that larvae should first appear at the carcass. We mated and allocated pairs to breeding boxes with approximately 2 cm of potting soil. We checked breeding boxes every hour from 8.00 to 17.00 for eggs; we only included set-ups where the breeding box was observed with no eggs one hour and with at least one fertile egg the next hour. After eggs were laid, we allocated individuals to treatments (0, 24, 48, 72 and 96) and collected beetles relative to the time that their eggs were first observed (e.g. if eggs were first observed at 9.00, adults in the 24 h treatment were collected at 9.00 the subsequent day). We collected $n = 10$ of both sexes for each treatment.

2.4.2. *takeout* and temporal kin recognition

2.4.2.1. Manipulation of larval arrival time

To test the hypothesis that *takeout* specifically influences the transition from infanticide to acceptance and feeding of larvae, we performed an experiment where we manipulated the temporal cues used by parents to anticipate offspring arrival to sample adults as they committed infanticide of the larvae or accepted and fed offspring. After we paired beetles with a mouse (20–23 g) and observed eggs, we moved the focal female and the brood ball to a new breeding box and placed a small piece of beef in

the original breeding box with the eggs (first batch) to attract the larvae and checked for hatch 1–3 times a day. We kept the focal male separate from the female and brood ball for 24 h and then returned the male to the breeding box with the female. Parents were separated and reunited to 'restart the clock' on temporal kin recognition, because separating and reuniting the parents results in a renewal of mating behaviour and often results in the female laying a new batch of eggs (second batch). As a result, the pairs' own larvae from the first batch arrive before parents expected them based on the most recent bout of mating and egg-laying.

After reuniting the male and female, we again checked breeding boxes daily for eggs. We provided the breeding pair with 10 larvae, at least 5 of which were their offspring from the first clutch of eggs and were supplemented with same-age larvae from other clutches if insufficient numbers were available from the focal pair's clutch, as *N. orbicollis* cannot identify their own larvae [7,18]. Individuals were presented with larvae and observed based upon the time of day that their larvae hatched, and there were no differences in the time of day that individuals were collected between individuals that showed acceptance or infanticide behaviour. All observations were conducted by a single observer. Following the addition of larvae, we observed the focal individuals for at least 30 min to detect acceptance or rejection of larvae following established protocols [30]. If the focal individuals did not respond in this time, pairs were checked every 30 min to identify the onset of acceptance or infanticide. In some cases, one parent would accept larvae while the other parent committed infanticide; in these cases, the male and female were sampled and categorized separately according to the phenotype that they exhibited. Focal individuals categorized as 'infanticide' were collected while the focal individual was attacking/eating larvae. If the parents did not eat the larvae within an hour of larval placement, the carcass was checked every 15–30 min until aggression towards larvae was observed. Focal individuals that were categorized as 'accept' were collected immediately after the parent made antennal contact with the larvae and a feeding event was observed. This manipulation often led to a discrepancy in the temporal cues used by males versus females—for example, if the female did not lay a second batch of eggs—as females base their window of larval acceptance on when eggs are laid [7], and males likely base their window on timing and frequency of female acceptance of mating (A.L.P., personal observation). Thus, to reach an adequate sample size, we removed the female after the first batch of eggs were laid, which increased the likelihood that males would accept larvae ($n = 13$ setups). We collected $n = 20$ for both sexes for both behaviours (infanticide and accept).

2.4.2.2. Natural variation in temporal kin recognition

We took advantage of natural variation in temporal kin recognition to test the hypothesis that variation in acceptance or infanticide of larvae is associated with variation in *takeout*. To do this, we followed the methods above but altered the procedure such that the focal beetles were provided larvae at the correct time. Specifically, after we detected eggs, we allowed the pair to remain in the breeding box for 24–36 h to ensure that egg-laying was completed. Then, the focal individual was moved with the brood ball to a new breeding box to ensure that the larvae did not arrive at the carcass naturally, and the non-focal parent was removed. Once we detected larvae and/or 96 h had elapsed since we detected the first fertile egg in the original breeding box, we provided focal parent with a mixed brood of five larvae to determine whether parents accepted or committed infanticide using the same behavioural methods described above. However, this manipulation also resulted in abandonment, where the parent ceased attending to the carcass, which resulted in significant decay and fungal growth on the carcass, and/or the parent failing to feed larvae despite contact. As both infanticide and abandonment both constitute a lack of parental care towards larvae, we categorized infanticide and abandonment as rejection. After observation of acceptance or rejection of larvae (including both infanticide and abandonment), we collected the heads of focal individuals to determine how natural variation in temporal kin recognition was associated with *takeout* gene expression. We collected $n = 19$ females and $n = 25$ males for this experiment.

2.5. Gene expression analysis

We collected samples by removing the beetles' heads with dissecting scissors and then immediately flash froze them in liquid nitrogen [31]. We then stored samples at −80°C until RNA extraction. Samples were homogenized in liquid nitrogen using a mortar and pestle (cat. no. 60310, CoorsTek, Golden, CO, USA). Following homogenization, we extracted RNA from each sample using Qiagen's RNAeasy Lipid Tissue Mini-Kit (cat. no. 74106) following [31]. We also treated samples with DNase I (Qiagen) on the column according to manufacturer's instructions to help ensure minimal genomic DNA contamination. We quantified RNA using a Qubit 2.0 Fluorometer (Invitrogen Corporation, Carlsbad, CA, USA) using the

RNA Broad Range protocols per manufacturer's instructions. We synthesized cDNA using oligo dT and random hexamers with Quanta Biosciences qScript reverse transcriptase master mix (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's instructions from 500 ng total RNA. RNA was stored at −80°C and cDNA was stored at −20°C.

We performed quantitative real-time PCR (qRT-PCR) using primers from the PCR-validated consensus sequences for our gene of interest and an endogenous control gene (*gapdh*) following methods of Cunningham *et al.* [32] and using transcriptome of Benowitz *et al.* [33]. We performed qRT-PCR with Roche LightCycler 480 SYBR I Green Master Mix using a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA) following the manufacture's protocol with triple technical replicates of 10 µl reactions. Primers were at a working concentration of 1.33 µmol l^{-1}. Annealing temperature was 60°C during the amplification cycles. We established the stability of the endogenous reference gene by determining that *gapdh* did not vary across the sexes or experimental treatments. Additional information suggested by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, including primer validations, can be found in appendix A.

2.6. Statistical analyses

All statistical analyses were performed using JMP PRO (v. 16.0.0, http://jmp.com) and all figures were produced in SigmaPlot (v. 14.5, http://www.sigmaplot.co.uk). Results are means ± s.e. We used the $\Delta\Delta C_T$ method [34] to convert raw expression data to normalized relative expression values. For all analyses, we used the treatment group that occurred earliest as the comparison group. Females and males were analysed together for all experiments, as previous research has found sex-biased expression of *takeout* [35,36] and thus there is an *a priori* expectation for an interaction between *takeout* and sex. All statistical tests conducted in this study were two-tailed, as there was no *a priori* expectation for the direction of associations between variables. We analysed the manipulation of fed versus hungry beetles, the development of parenting, and the experimental manipulation of infanticide versus acceptance using two-way ANOVA with sex and behaviour (infanticide versus accept) as factors. We analysed *takeout* expression in the experiment of natural variation in infanticide versus reject using *t*-tests; for this experiment, only male samples were included for molecular and statistical analyses as only two females rejected larvae when larvae were presented at the correct time.

3. Results

3.1. Gene identification

Plots illustrating sequence homology between *N. orbicollis*, *N. vespilloides*, *L. migratoria*, *R. speratus*, *D. melanogaster* and *A. gambiae* indicate moderate conservation between the different species (figure 1). The *N. orbicollis takeout* studied here has a per cent identity of 25.22% with the *D. melanogaster takeout*. This is comparable to the reported per cent identities for *takeout* genes with the *D. melanogaster takeout* across species, including *N. vespilloides* (25.74%), *L. migratoria* (24.47%), *R. speratus* (RsTO1: 25.74%, RsT02: 24.69%) and *A. gambiae* (AgTOL1: 24.08%, AgT02: 40.0%). We uploaded sequences to GenBank under accession number GGAA01005642.1.

3.2. *takeout* expression in non-parental contexts

There was no statistically significant difference in *takeout* expression between beetles that were fed versus hungry ($F_{1,50} = 0.28$, $p = 0.60$), the sexes ($F_{1,50} = 1.74$, $p = 0.19$), nor was there an interaction between treatment and sex ($F_{1,50} = 1.40$, $p = 0.24$; figure 2*a*).

3.3. *takeout* expression and parenting

We did not find an association between *takeout* and the development of parenting. *takeout* expression was not associated with the development of parenting ($F_{4,94} = 0.43$, $p = 0.79$), sex of the beetle ($F_{1,94} = 1.1.52$, $p = 0.22$), and there was no interaction between development of parenting and sex ($F_{4,94} = 2.12$, $p = 0.20$; figure 2*b*).

takeout was associated with temporal kin recognition in both females and males. In the experimental manipulation of larval arrival time, parents that accepted their larvae had higher *takeout* gene expression
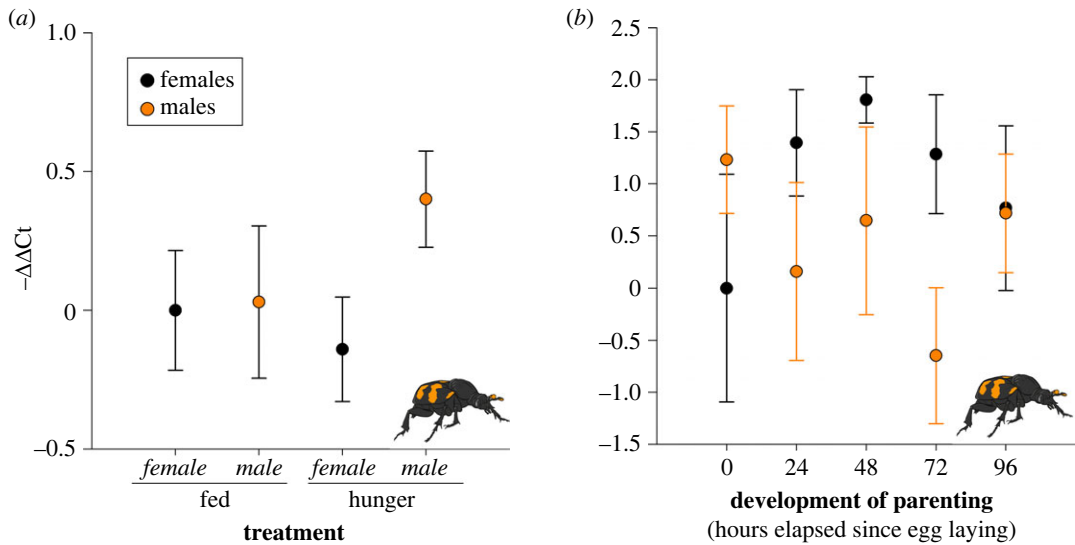
Figure 1. Amino acid alignment of takeout family. Amino acid alignment of members of *takeout* family in *N. vespilloides*, *N. orbicollis*, *L. migratoria*, *R. speratus*, *D. melanogaster* and *A. gambiae*. Shaded regions represent greater than 40% similarity among sequences.

than beetles that committed infanticide of their own larvae ($F_{1,76} = 5.03$, $p = 0.03$; figure 3a), and females generally showed higher *takeout* expression than males ($F_{1,76} = 6.04$, $p = 0.02$), but there was not a statistically significant interaction between behaviour (infanticide versus accept) and sex of the carer on *takeout* expression ($F_{1,76} = 0.25$, $p = 0.62$). We found a similar pattern in the natural variation of accept versus reject; males that accepted had higher *takeout* expression than males that rejected their larvae ($t_{1,23} = -2.12$, $p = 0.05$; figure 3b). There was an insufficient number of female beetles that naturally rejected their larvae to analyse. Male beetles showed more variation in accept versus reject when they were presented with larvae at the correct time (males: $n = 15$ accept versus $n = 10$ reject) than females ($n = 17$ accept versus $n = 2$ reject).

4. Discussion

Evolution of complex behaviours like temporal kin recognition can involve the co-option of behavioural precursors [37,38] and thus the mechanisms that produce them [39,40]. Following this logic, we hypothesized that the gene *takeout*, which has been shown to be involved in timing and feeding [9,10,13–15,25,36], may have been co-opted to enable the evolution of temporal kin recognition. We found that the version of *takeout* we examined was associated with the likelihood that both females and males would accept larvae, such that beetles with higher *takeout* expression were more likely to accept larvae. Somewhat contrary to our *a priori* expectations, *takeout* was not associated with hunger or a transition to the full behavioural repertoire associated with parenting.

Co-option as an evolutionary process predicts that pre-existing mechanisms are transferred to a different context for a new function [38,41]. For this reason, it was surprising that *takeout*, which has been shown to integrate timing with feeding [9], was associated with temporal kin recognition but not hunger state in *N. orbicollis*. One explanation is that a different form of *takeout* influences feeding in *N. orbicollis*. While our data support placing the gene we examined in the *takeout* family, *takeout* typically has multiple copies [36]. Another possibility is that location of *takeout* expression in the brain may influence the role of *takeout* in hunger versus temporal kin recognition more so than relative expression itself, although future research would be needed to test this idea. Alternatively, it is possible that our methods were not sufficient to indicate an association between hunger and *takeout*. This seems unlikely, as similar procedures successfully induced starvation and reduced body mass in *Nicrophorus* and other comparably sized beetles [28,29,42] and removing food produced variation in *takeout* mRNA within ten hours in *Drosophila* [9]. We also did not find an association between *takeout* and the development of parenting, i.e. days elapsed since egg laying, suggesting that *takeout* is not associated with parental behaviour generally but is more specific to the acceptance of larvae. These data suggest that the version of *takeout* we examined may be associated with a specific role limited to temporal kin recognition, consistent with the specificity it has with timing of feeding.

Parents varied both in their likelihood of accepting larvae that showed up at the correct time and in *takeout* expression. Female and male parents often differ in how precise they are in discriminating their

Figure 2. *takeout* is not associated with non-parental contexts or the development of parenting. Y-axis represents the relative gene expression ($-\Delta\Delta Ct$) for *takeout*. Black circles represent females, while orange circles represent males. There was no association between *takeout* and whether beetles were (*a*) well-fed or hungry (females: $n = 13$ fed, $n = 12$ hungry; males: $n = 14$), nor (*b*) with the development of parenting in either female or male beetles ($n = 10$ for each treatment). Results are mean $\pm$ s.e.
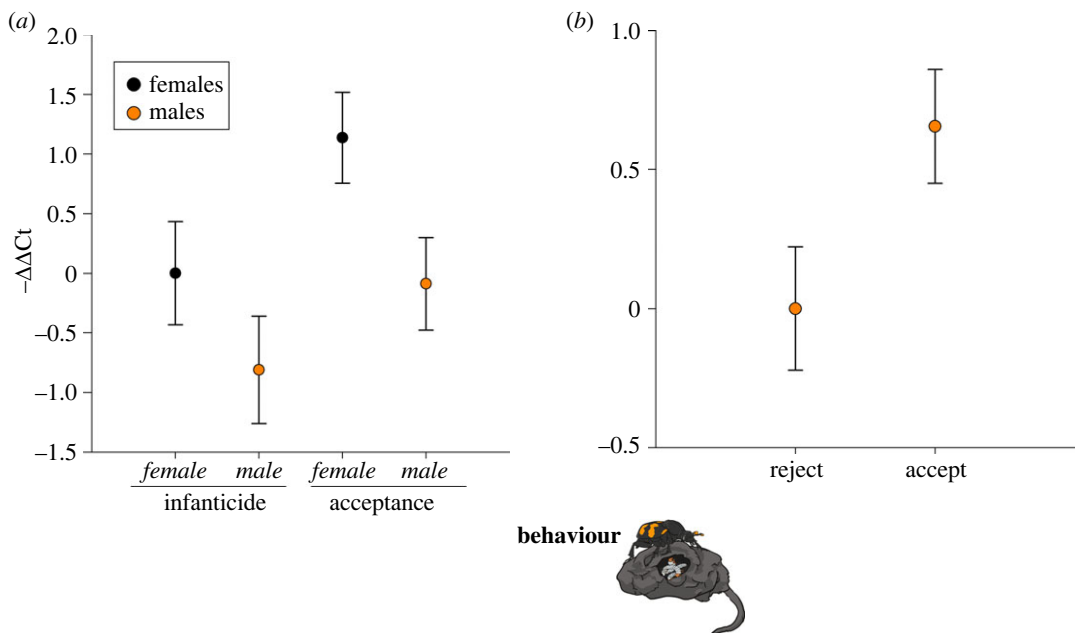
Figure 3. *takeout* is associated with temporal kin recognition. Y-axis represents the relative gene expression ($-\Delta\Delta Ct$) for *takeout*. Black circles represent females, while orange circles represent males. (*a*) Female and male parents that accepted their larvae had higher *takeout* than beetles that committed infanticide, and females generally showed higher *takeout* expression than males ($n = 20$ for each behaviour). (*b*) Males showed natural variation in infanticide when presented with larvae at the correct time, and for these males, those that accepted their larvae also showed higher *takeout* expression than those that rejected their larvae ($n = 10$ reject, $n = 15$ accept). Results are mean $\pm$ s.e.

offspring from others across taxonomic groups, from vertebrates to invertebrates [43,44], and it is an open question why the sexes differ in this regard. On a proximate level, females and males often use different cues to 'start the clock' for temporal kin recognition, which can produce an asymmetry in information such that females have equal or more precise information on when to transition to parenting than males do [3,45]. For example, female mice experience physiological changes over pregnancy, while in males, mating and social interactions with the female influence the likelihood males will transition to a non-infanticidal state [4,46–49]. Since males often have less reliable information about when offspring should arrive, this may lead to greater variation in acceptance. On a more evolutionary scale, it is a common

pattern that females show more canalized care, whereas males are more environmentally sensitive in their care behaviour, and this pattern is also apparent in *Nicrophorus* species [50,51].

We also found that *takeout* expression differed between the females and males in the analysis of parents that accept versus commit infanticide of their larvae. It is intriguing that *takeout* is associated with temporal kin recognition for both sexes, despite the likely scenario that females and males use different cues to initiate the transition to accepting offspring. *takeout* often has male-biased effects on social behaviours like courtship in *Drosophila*, with sex-specific expression in the fat body surrounding the brain in males but not females [35,36,52]. Non-sex-specific effects of *takeout* are generally associated with timing and feeding [10,15,25,53–55], where *takeout* expression often occurs in chemosensory centres like the antennae [9,35]. Guo *et al.* [13] found that *takeout* expression in antennae and hind leg of both sexes was involved in modulating attraction and repulsion to conspecifics through regulation of peripheral olfactory sensitivity. Since we found an association between *takeout* and temporal kin recognition in both sexes, it is an intriguing question for future research where and how *takeout* expression is associated with transitions in behaviour between females and males.

Ethologists have long predicted that complex behaviours can evolve through co-option of pre-existing behavioural precursors [37,38]. We have shown an association between a gene that influences timing and feeding in other contexts with temporal kin recognition. Given that we did not see an association between hunger states and the general transition to a parenting state, this study raises many questions about the mechanisms by which *takeout* influences temporal kin recognition and its evolutionary history.

Appendix A

Additional information suggested in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines not already provided in this paper.

Quantitative real-time PCR (qRT-PCR) primer sequences

takeout:

(1) forward: GGGACATGGTCCAGCAAATA
(2) reverse: GCATAATCGGTACCTCCCTTT

gapdh:

(1) forward: CGACTACATGGTGTACCTGTTC
(2) reverse: TGCCGTTGACGATGAGTTT

Primers were manufactured by Integrated DNA Technology (IDT, Coralville, IA, USA) and purified with IDT's standard desalting technique.

qRT-PCR validation

Primer efficiency:

takeout: $E = 1.96$, $r^2 = 0.9993$

gapdh: $E = 1.95$, $r^2 = 0.9996$

References

1. Müller JK, Eggert A-K. 1990 Time-dependent shifts between infanticidal and parental behavior in female burying beetles a mechanism of indirect mother-offspring recognition. *Behav. Ecol. Sociobiol.* **27**, 11–16. (doi:10.1007/BF00183307)

2. Beecher MD, Beecher IM, Hahn S. 1981 Parent-offspring recognition in bank swallows (*Riparia riparia*): II. Development and acoustic basis. *Anim. Behav.* **29**, 95–101.

3. Elwood RW. 1994 Temporal-based kinship recognition: a switch in time saves mine. *Behav. Process.* **33**, 15–24. (doi:10.1016/0376-6357(94)90057-4)

4. Perrigo G, Belvin L, Vom Saal FS. 1992 Time and sex in the male mouse: temporal regulation of infanticide and parental behavior. *Chronobiol. Int.* **9**, 421–433. (doi:10.3109/07420529209064554)

5. Benowitz KM, Moody KJ, Moore AJ. 2015 Are species differences in maternal effects arising from maternal care adaptive? *J. Evol. Biol.* **28**, 503–509. (doi:10.1111/jeb.12573)

6. Davies N. 2000 *Cuckoos, cowbirds and other cheats*. London, UK: T. & A.D. Poyser.

7. Oldekop JA, Smiseth PT, Piggins HD, Moore AJ. 2007 Adaptive switch from infanticide to parental care: how do beetles time their behaviour? *J. Evol. Biol.* **20**, 1998–2004. (doi:10.1111/j.1420-9101.2007.01364.x)

8. Moore AJ, Székely T, Komdeur J. 2010 Prospects for research in social behaviour: systems biology meets behaviour. In *Social behaviour: genes, ecology and evolution* (eds T Székely, AJ Moore, J Komdeur). Cambridge, UK: Cambridge University Press.

9. Sarov-Blat L, So WV, Liu L, Rosbash M. 2000 The *Drosophila takeout* gene is a novel molecular link between circadian rhythms and feeding behavior. *Cell* **101**, 647–656. (doi:10.1016/S0092-8674(00)80876-4)

10. Meunier N, Belgacem YH, Martin JR. 2007 Regulation of feeding behaviour and locomotor activity by takeout in *Drosophila*. *J. Exp. Biol.* **210**, 1424–1434. (doi:10.1242/jeb.02755)

11. Parker DJ, Cunningham CB, Walling CA, Stamper CE, Head ML, Roy-Zokan EM, McKinney EC, Ritchie MG, Moore AJ. 2015 Transcriptomes of parents identify parenting strategies and sexual conflict in a subsocial beetle. *Nat. Commun.* **6**, 8449. (doi:10.1038/ncomms9449)

12. Moss JB, Cunningham CB, McKinney EC, Moore AJ. 2022 Gene expression underlying parenting and being parented shows limited plasticity in response to different ambient temperatures. *Mol. Ecol.* **31**, 5326–5338. (doi:10.1111/mec.16649)

13. Guo W, Wang X, Ma Z, Xue L, Han J, Yu D, Kang L. 2011 *CSP* and *Takeout* genes modulate the switch between attraction and repulsion during behavioral phase change in the migratory locust. *PLoS Genet.* **7**, e1001291. (doi:10.1371/journal.pgen.1001291)

14. Fujikawa K, Seno K, Ozaki M. 2006 A novel takeout-like protein expressed in the taste and olfactory organs of the blowfly, *Phormia regina*. *FEBS J.* **273**, 4311–4321.

15. So WV, Sarov-Blat L, Kotarski CK, McDonald MJ, Allada R, Rosbash M. 2000 *takeout*, a novel Drosophila gene under circadian clock transcriptional regulation. *Mol. Cell. Biol.* **20**, 6935–6944. (doi:10.1128/MCB.20.18.6935-6944.2000)

16. Benito J, Hoxha V, Lama C, Lazareva AA, Ferveur JF, Hardin PE, Dauwalder B. 2010 The circadian output gene takeout is regulated by Pdp1epsilon. *Proc. Natl Acad. Sci. USA* **107**, 2544–2549. (doi:10.1073/pnas.0906422107)

17. Scott M. 1998 The ecology and behavior of burying beetles. *Annu. Rev. Entomol.* **43**, 595–618. (doi:10.1146/annurev.ento.43.1.595)

18. Benowitz KM, McKinney EC, Moore AJ. 2016 Difference in parenting in two species of burying beetle, *Nicrophorus orbicollis* and *Nicrophorus vespilloides*. *J. Ethol.* **34**, 315–319. (doi:10.1007/s10164-016-0477-5)

19. Anduaga S. 2009 Reproductive biology of *Nicrophorus mexicanus* Matthews (Coleoptera: Silphidae). *Coleopt. Bull.* **63**, 173–178.

20. Takata M, Hayashi S, Thomas CE, Koyama S. 2015 The proximate cause of asynchronous hatching in the burying beetle *Nicrophorus quadripunctatus*. *J. Ethol.* **33**, 197–203. (doi:10.1007/s10164-015-0431-y)

21. Wilson DS, Fudge J. 1984 Burying beetles: intraspecific interactions and reproductive success in the field. *Ecol. Entomol.* **9**, 195–203. (doi:10.1111/j.1365-2311.1984.tb00715.x)

22. Potticary AL, Otto HW, McHugh JV, Moore AJ. 2023 Spatiotemporal variation in the competitive environment, with implications for how climate change may affect a species with parental care. *Ecol. Evol.* **13**, e9972. (doi:10.1002/ece3.9972)

23. Won HI *et al.* 2018 *De novo* assembly of the burying beetle *Nicrophorus orbicollis* (Coleoptera: Silphidae) transcriptome across developmental stages with identification of key immune transcripts. *J. Genomics* **6**, 41–52. (doi:10.7150/jgen.24228)

24. Fujiwara K, Karasawa A, Hanada T, Tobo M, Kaneko T, Usui M, Maekawa K. 2023 Caste-specific expressions and diverse roles of takeout genes in the termite *Reticulitermes speratus*. *Sci. Rep.* **13**, 8422. (doi:10.1038/s41598-023-35524-7)

25. Saito K, Su ZH, Emi A, Mita K, Takeda M, Fujiwara Y. 2006 Cloning and expression analysis of takeout/JHBP family genes of silkworm, *Bombyx mori*. *Insect Mol. Biol.* **15**, 245–251. (doi:10.1111/j.1365-2583.2006.00612.x)

26. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009 Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189–1191. (doi:10.1093/bioinformatics/btp033)

27. Steiger S, Richter K, Müller JK, Eggert A-K. 2007 Maternal nutritional condition and genetic differentiation affect brood size and offspring body size in *Nicrophorus*. *Zoology* **110**, 360–368. (doi:10.1016/j.zool.2007.06.001)

28. Trumbo ST, Xhihani E. 2015 Mass–size relationships, starvation and recovery in an engorging feeder. *Physiol. Entomol.* **40**, 257–263. (doi:10.1111/phen.12110)

29. Trumbo ST, Robinson GE. 2004 Nutrition, hormones and life history in burying beetles. *J. Insect Physiol.* **50**, 383–391. (doi:10.1016/j.jinsphys.2004.01.008)

30. Potticary AL, Cunningham CB, McKinney EC, Moore P, Belay A, Moore AJ. In press. Insect homolog of oxytocin/vasopressin associated with parenting of males but not females in a subsocial beetle. *Evolution*. (doi:10.1093/evolut/qpad113)

31. Roy-Zokan EM, Cunningham CB, Hebb LE, McKinney EC, Moore AJ. 2015 Vitellogenin and vitellogenin receptor gene expression is associated with male and female parenting in a subsocial insect. *Proc. R. Soc. B* **282**, 20150787. (doi:10.1098/rspb.2015.0787)

32. Cunningham CB, Douthit MK, Moore AJ. 2014 Octopaminergic gene expression and flexible social behaviour in the subsocial burying beetle *Nicrophorus vespilloides*. *Insect Mol. Biol.* **23**, 391–404. (doi:10.1111/imb.12090)

33. Benowitz KM, McKinney EC, Cunningham CB, Moore AJ. 2017 Relating quantitative variation within a behavior to variation in transcription. *Evolution* **71**, 1999–2009. (doi:10.1111/evo.13273)

34. Livak KJ, Schmittgen TD. 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* **25**, 402–408. (doi:10.1006/meth.2001.1262)

35. Dauwalder B, Tsujimoto S, Moss J, Mattox W. 2002 The *Drosophila takeout* gene is regulated by the somatic sex-determination pathway and affects male courtship behavior. *Genes Dev.* **16**, 2879–2892. (doi:10.1101/gad.1010302)

36. Vanaphan N, Dauwalder B, Zufall RA. 2012 Diversification of takeout, a male-biased gene family in *Drosophila*. *Gene* **491**, 142–148. (doi:10.1016/j.gene.2011.10.003)

37. Tallamy DW. 1984 Insect parental care. *BioSci* **34**, 20–24. (doi:10.2307/1309421)

38. West-Eberhard M. 2003 *Developmental plasticity and evolution*. New York, NY: Oxford University Press.

39. Cunningham CB, Badgett MJ, Meagher RB, Orlando R, Moore AJ. 2017 Ethological principles predict the neuropeptides co-opted to influence parenting. *Nat. Commun.* **8**, 14225. (doi:10.1038/ncomms14225)

40. Moore AJ, Benowitz KM. 2019 From phenotype to genotype: the precursor hypothesis predicts genetic influences that facilitate transitions in social behavior. *Curr. Opin. Insect Sci.* **34**, 91–96. (doi:10.1016/j.cois.2019.04.007)

41. Plachetzki DC, Oakley TH. 2007 Key transitions during the evolution of animal phototransduction: novelty, 'tree-thinking,' co-option, and co-duplication. *Integr. Comp. Biol.* **47**, 759–769. (doi:10.1093/icb/icm050)

42. Auerswald L, Gäde G. 2000 Metabolic changes in the African fruit beetle, *Pachnoda sinuata*,

during starvation. *J. Insect Physiol.* **46**, 343–351. (doi:10.1016/S0022-1910(99)00187-0)

43. Pinxten R, Eens M, Verheyen RF. 1991 Responses of male starlings to experimental intraspecific brood parasitism. *Anim. Behav.* **42**, 1028–1030. (doi:10.1016/S0003-3472(05)80159-9)

44. Ringler E, Pašukonis A, Ringler M, Huber L. 2016 Sex-specific offspring discrimination reflects respective risks and costs of misdirected care in a poison frog. *Anim. Behav.* **114**, 173–179. (doi:10.1016/j.anbehav.2016.02.008)

45. Davies N, Hatchwell B, Robson T, Burke T. 1992 Paternity and parental effort in dunnocks *Prunella modularis*: how good are male chick-feeding rules? *Anim. Behav.* **43**, 729–745. (doi:10.1016/S0003-3472(05)80197-6)

46. Elwood RW, Kennedy HF. 1991 Selectivity in paternal and infanticidal responses by male mice: effects of relatedness, location, and previous sexual partners. *Behav. Neural Biol.* **56**, 129–147. (doi:10.1016/0163-1047(91)90568-b)

47. Perrigo G, Bryant WC, vom Saal FS. 1990 A unique neural timing system prevents male mice from harming their own offspring. *Anim.*

Behav. **39**, 535–539. (doi:10.1016/S0003-3472(05)80419-1)

48. Mei L, Yan R, Yin L, Sullivan RM, Lin D. 2023 Antagonistic circuits mediating infanticide and maternal care in female mice. *Nature* **618**, 1006–1016. (doi:10.1038/s41586-023-06147-9)

49. Elwood RW, Stolzenberg DS. 2020 Flipping the parental switch: from killing to caring in male mammals. *Anim. Behav.* **165**, 133–142. (doi:10.1016/j.anbehav.2020.05.001)

50. Smiseth PT, Dawson C, Varley E, Moore AJ. 2005 How do caring parents respond to mate loss? Differential response by males and females. *Anim. Behav.* **69**, 551–559. (doi:10.1016/j.anbehav.2004.06.004)

51. Benowitz KM, Moore AJ. 2016 Biparental care is predominant and beneficial to parents in the burying beetle *Nicrophorus orbicollis* (Coleoptera: Silphidae). *Biol. J. Linn. Soc.* **119**, 1082–1088. (doi:10.1111/bij.12830)

52. Lazareva AA, Roman G, Mattox W, Hardin PE, Dauwalder B. 2007 A role for the adult fat body in *Drosophila* male courtship behavior. *PLoS*

Genet. **3**, e16. (doi:10.1371/journal.pgen.0030016)

53. Smith G, Macias-Muñoz A, Briscoe AD. 2016 Gene duplication and gene expression changes play a role in the evolution of candidate pollen feeding genes in *Heliconius* butterflies. *Genome Biol. Evol.* **8**, 2581–2596. (doi:10.1093/gbe/evw180)

54. Bauer J, Antosh M, Chang C, Schorl C, Kolli S, Neretti N, Helfand SL. 2010 Comparative transcriptional profiling identifies takeout as a gene that regulates life span. *Aging* **2**, 298–310. (doi:10.18632/aging.100146)

55. McDonald MJ, Rosbash M. 2001 Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* **107**, 567–578. (doi:10.1016/s0092-8674(01)00545-1)

56. Potticary AL, McKinney EC, Moore PJ, Moore AJ. 2023 Data from: *takeout* gene expression is associated with temporal kin recognition. Dryad Digital Repository. (doi:10.5061/dryad.kd51c5bbv)

57. Potticary AL, McKinney EC, Moore PJ, Moore AJ. 2023 *takeout* gene expression is associated with temporal kin recognition. Figshare. (doi:10.6084/m9.figshare.c.6778102)