© 2022. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2022) 225, jeb243738. doi:10.1242/jeb.243738

RESEARCH ARTICLE

Context-dependent influence of threat on honey bee social network dynamics and brain gene expression

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ABSTRACT

Adverse social experience affects social structure by modifying the behavior of individuals, but the relationship between an individual's behavioral state and its response to adversity is poorly understood. We leveraged naturally occurring division of labor in honey bees and studied the biological embedding of environmental threat using laboratory assays and automated behavioral tracking of whole colonies. Guard bees showed low intrinsic levels of sociability compared with foragers and nurse bees, but large increases in sociability following exposure to a threat. Threat experience also modified the expression of caregiving-related genes in a brain region called the mushroom bodies. These results demonstrate that the biological embedding of environmental experience depends on an individual's societal role and, in turn, affects its future sociability.

KEY WORDS: Automated behavioral tracking, Biological embedding, *Apis mellifera*, Neurogenomics, Social insects

INTRODUCTION

Interactions between an individual and its environment affect the molecular dynamics of the brain. For social animals, this can impact the structure and dynamics of social interactions, as adverse experience is 'biologically embedded' (Hertzman, 1999) to influence future behavior via changes in brain gene expression (Alaux and Robinson, 2007; Hsu et al., 2006; Shpigler et al., 2017a; Stevenson and Schildberger, 2013; Yang et al., 2001). Although resulting behavioral modifications may adaptively anticipate future events of a similar nature (Bode et al., 2010; Traniello and Robinson, 2021), adverse experience can also produce negative behavioral consequences for both individual and society by disrupting normal physiology and development, leading to aberrant social interactions (Berens et al., 2017; Hertzman, 1999, 2012). For humans, this is especially the case in complex psychiatric conditions such as post-traumatic stress disorder (PTSD), in which even relatively brief experiences can modify subsequent behavior and gene expression throughout an individual's lifetime, with effects that potentially span generations (Dias and Ressler, 2014; Yehuda, 2002; Yehuda et al., 2009). Multilevel analyses of brain, behavior and social network dynamics would clarify our understanding of both the positive and negative aspects of biological embedding.

Our current understanding of the biological embedding of social adversity has been shaped by two key insights. First, there is strong individual variation in the effects of exposure to social adversity. This individual variation is related to genotype, internal physiology, motivation and previous experience, which interact to shape an individual's behavioral state (Boyce et al., 2012; Ellis and Boyce, 2008; Soliemanifar et al., 2018). Second, harmful effects of experiencing social adversity are mitigated to some extent by affiliative interactions with other group members (Sippel et al., 2015; Southwick and Charney, 2012). In other words, the effects of biological embedding are context-dependent. These insights highlight the importance of understanding how biological embedding is affected by an individual's current behavioral state and its sociability (i.e. the number and kind of affiliative interactions it exhibits). However, it is not known how an individual's behavioral state affects its sociability and response to social adversity at the behavioral and molecular levels.

We addressed this issue by leveraging the rich, naturally occurring division of labor in colonies of the western honey bee (*Apis mellifera*). Living in a complex eusocial society, honey bees exhibit multiple forms of division of labor (Robinson, 1992). Queens lay eggs while sterile female workers perform all non-reproductive tasks related to colony growth and development. Division of labor among workers is based on behavioral maturation: adult worker bees spend the first 2–3 weeks of their life specializing in a variety of tasks in the hive and then shift to colony defense and foraging outside for the remainder of their 4–6 week lifespan. Task specialization is influenced by individual as well as social factors, i.e. variation in genotype, physiology and experience, as well as colony demography and pheromone dynamics (Huang et al., 1994; Leoncini et al., 2004; Page et al., 2012; Robinson, 1992). Task specialization results in 'extreme' behavioral states during which a similar task repertoire is performed over periods of days to weeks. Affiliative states include nursing, which involves caring for the colony's brood and queen, and foraging, which involves collecting nectar and pollen from the local environment and then sharing it with other colony members. Agonistic states include guarding the colony and attacking intruders. Although much is known regarding the genetic (Page et al., 2012), social (Huang and Robinson, 1992, 1996; Leoncini et al., 2004; Robinson, 1992), nutritional (Ament et al., 2010; Wheeler et al., 2015), endocrine (Giray and Robinson, 1996; Hamilton et al., 2017; Huang et al., 1994) and neuromolecular (Hamilton et al., 2019; Whitfield et al.,

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Received 2 November 2021; Accepted 17 February 2022

2003; Zayed and Robinson, 2012) mechanisms that regulate honey bee division of labor, as stated above, a connection between societal role and sociability has not yet been established.

Honey bee colonies respond to threat with an intricate system that also involves division of labor and chemical communication (Breed et al., 1990; Moore et al., 1987; Nouvian et al., 2016). Guard bees that patrol the hive entrance are the first bees that respond to a threat; they attack intruders and release alarm pheromones, which trigger other bees to respond aggressively and engage the threat. Intruders that breach the entrance also provoke guard-like responses, including biting and stinging, by some bees in the hive (Hamilton et al., 2019; Shpigler et al., 2017a; Traniello et al., 2019).

Biological embedding traditionally has focused on human development, pinpointing environmental factors that influence individuals and their social groups over the course of years or even decades. Although their lifespan during spring, summer and autumn is only 4–7 weeks, recent empirical studies of honey bees have demonstrated persistent effects of early-life experience on health, behavior and gene expression (Amdam et al., 2009; Rittschof et al., 2015; Rueppell et al., 2017; Walton et al., 2021), suggesting that the biological embedding framework can be readily extended to social insects (Traniello and Robinson, 2021). We used honey bee division of labor to study the internal and external factors that influence biological embedding of threat. We determined how adverse experience – a simulated attack that poses a survival threat – affects brain gene expression and future behavior as a function of an individual's behavioral state, either agonistic or affiliative.

We studied trophallaxis as a measure of sociability with an automated behavioral tracking system that features barcodes affixed to every individual in a colony located in a glass-walled observation hive (Geffre et al., 2020; Gernat et al., 2018; Shpigler and Robinson, 2015). Trophallaxis involves the transfer of fluids containing food and signaling molecules between nestmates (Leboeuf et al., 2016) and is thought to coordinate group decision-making processes (Seeley et al., 1991). Honey bee trophallaxis interaction networks appear to facilitate the spread of information within the colony (Gernat et al., 2018) and can be used, together with physical contact and spatial proximity (Crall et al., 2018; Mersch et al., 2013; Otterstatter and Thomson, 2007), to generate subnetworks that accurately infer task specialization (Wild et al., 2021). We were thus able to explore the effect of a colony disturbance on social structure and test the hypothesis that threat experience induces context-specific changes in sociability.

We performed two experiments that involved manipulations to perturb the colony environment and track the effects on trophallaxis social behavior. In experiment I, we used physiological manipulations to disturb colony division of labor. In experiment II, we used a brief disturbance to simulate an attack on the colony. In experiment I, we also coupled whole-colony automated monitoring with laboratory assays of small groups of individuals that reflect task-related differences associated with division of labor. Comparing results from experiments I and II enabled us to determine the relationship between behavioral state and sociability in the context of a threat that provoked an aggressive response relative to a nonthreatening perturbation that does not provoke aggression.

In experiment III, to gain insights into the neuromolecular signatures associated with differences in affiliative caregiving and behavioral state as a function of exposure to social adversity, we performed gene expression analyses of the mushroom bodies (MB) of the bee brain. The MB, well known to be involved in arthropod learning and memory and multi-modal sensory integration (Menzel, 2001, 2012; Strausfeld, 2012), have been recently shown to also be involved in social responsiveness (Shpigler et al., 2017a, 2018; Traniello et al., 2019). An outline of the three experiments is given in Fig. 1.

In this study, the following hypotheses were tested: in experiment I, we tested the hypothesis that individual differences in sociability are context-specific, i.e. dependent upon an individual's task-related behavioral state; in experiment II, we tested the hypothesis that changes in sociability following threat experience also are context-specific (dependent on individual task-related behavioral state); and in experiment III, we tested the hypothesis that both threat experience and behavioral state are associated with changes in future behavior and brain gene expression. Taken together, our study examines societal role as a means of connecting the biological embedding of experience to changes in future behavior.

MATERIALS AND METHODS

Animals

Behavioral experiments took place at the University of Illinois Bee Research Facility, Urbana, IL, USA, in June–October 2017, August–September 2018 and July–August 2019. Colonies in this area are a mixture of European subspecies of *Apis mellifera* Linnaeus 1758, primarily *A. mellifera ligustica* Spinola 1806. Although seasonal influences on behavior, longevity and brain gene expression have been detected over winter months (Aurori et al., 2014; Münch et al., 2013), we do not expect that this influenced our results. Each experiment was performed within a single year and analyses were limited to age-matched bees that were 2 weeks old or younger at the completion of the experiment.

For experiment I, we used adult worker bees from colonies headed by a queen instrumentally inseminated with semen from a single drone (SDI). Owing to haplodiploidy, workers from SDI colonies have an average coefficient of relatedness of 0.75. For experiments II and III, we used adult worker bees from colonies headed by a naturally mated (NM) queen. To obtain 0- to 24-h-old bees for experiments, honeycomb frames containing pupae were removed from colonies 1 to 4 days prior to the beginning of each experiment and maintained in a dark incubator at 34°C and 50% relative humidity.

Laboratory-based behavioral assays

Laboratory-based behavioral assays were performed using groups of 9 (experiment I, in order to balance representation of three treatments) or 10 (experiment III) age-matched adult workers collected from honeycomb frames as described above and individually paint-marked with a unique color applied to the dorsal thorax (Testors Paint, Rockford, IL, USA), and transferred to a vertically oriented 100×20 mm Petri dish (Thermo Fisher Scientific, Waltham, MA, USA) containing a section of beeswax foundation (Mann Lake Ltd, Hackensack, MN, USA). Dishes were provided with a tube of honey (~1.2 ml), 50% sucrose solution (2 ml) and a pollen ball (70% pollen, 30% sucrose solution described above). Before testing, bees were allowed to acclimate at least 60 min to normal fluorescent lighting prior to conducting the behavioral assays on each day the experiment was conducted.

For experiments I and III, we utilized two well-established behavioral assays to measure either aggression or affiliative caregiving (Hamilton et al., 2019; Shpigler and Robinson, 2015; Shpigler et al., 2017a; Traniello et al., 2019). To measure aggression, groups of bees were subjected to a 5 min interaction with a foreign bee (an 'intruder') and observed for aggressive

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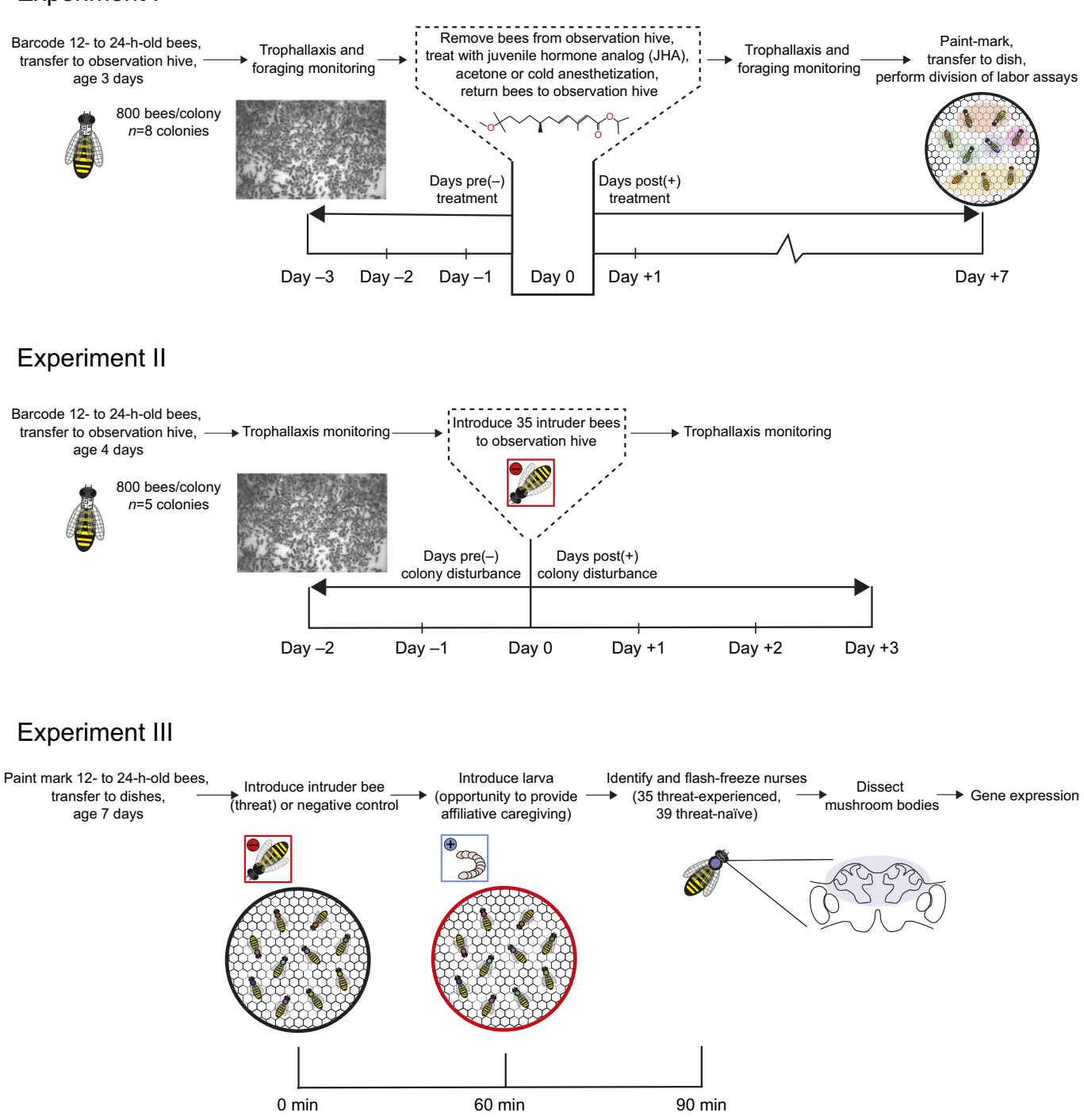

Fig. 1. Overview of experimental design and analysis. Experiment I: bees were barcoded, transferred to an observation hive, and allowed to age for 3 days prior to the first day of observation (corresponding to 3 days prior to treatment). On day 0, bees were removed and treated with juvenile hormone analog (JHA), acetone or cold anesthesia, and returned to the observation hive. At the end of the experiment, bees were removed, paint-marked and transferred to small dishes for behavioral observations. In dish cartoon (far right), colors correspond to the following behavioral states, as described in the Materials and Methods: generalist, dark green; forager, orange; nurse, purple; guard, pink; non-responder, light green; background, yellow. Experiment II: general design was similar to that of experiment I, but automated monitoring did not begin until the bees in the observation hive were allowed to age undisturbed for 4 days. On day 0, 35 intruder bees were added to the observation hive to generate a colony disturbance, which lasted ∼5 min. Resident bees were left undisturbed for the remainder of the experiment. Experiment III: bees were paint-marked, added to dishes and left undisturbed for 7 days. At 0 min on day 7, a single intruder bee or negative control was added to each dish; the intruder was removed after 5 min, regardless of vital status. Resident bees were then left undisturbed for 60 min, after which a larva was introduced for 5 min. Dishes were then left undisturbed for 30 min, after which nurses (bees that displayed affiliative caregiving toward the larva and did not respond aggressively toward the intruder in the previous assay) were collected for mushroom body gene expression analysis via qPCR. Images are not drawn to scale.

interactions such as biting and stinging. For affiliative caregiving, groups were exposed to a larva for 5 min, and we recorded instances of caregiving interactions: licking, wax-building and food provisioning. For both intruder and brood care assays, aggression and caregiving were scored according to criteria following previous studies (Hamilton et al., 2019; Shpigler et al., 2017a). Individual bees were identified as 'guards' or 'nurses' based on consistent (≥20–30 s) observations of biting and stinging or larval feeding, respectively, similar to previously used criteria (Shpigler et al., 2017a, 2018; Traniello et al., 2019).

Barcoding and setup

Observation hives containing colonies with barcoded bees were set up as previously described (Geffre et al., 2020; Gernat et al., 2018; Jones et al., 2020). For each trial (n=8 or 5 from SDI or NM colonies for experiments I and II, respectively), we used 800 bees (12–24 h old) per observation hive. In experiments that use smaller colonies (Robinson et al., 1989), or small groups of ∼10 individuals (Shpigler et al., 2018), bees of the same age take on tasks and divide the labor similarly to more typical colonies, in which labor is divided among individuals of different ages. Bees were cold-anesthetized, barcoded using a small amount of cyanoacrylate glue and a wooden applicator, and allowed to recover in a large container with comb and honey. We then carefully transferred each bee to a glass-walled observation hive containing a single honeycomb frame provisioned with honey (top 18 rows, ∼200 mg per cell, ∼330 g in total) and pollen paste (next six rows, ∼100 mg per cell, ∼45 g total; pollen paste was made from 45% honey, 45% pollen and 10% water). An SDI (experiment I) or NM (experiment II) queen was then anesthetized with carbon dioxide, barcoded and allowed to recover on the honeycomb. For experiment I, cells containing honey were covered with wax to prevent the honey from being transferred to the glass. The wax was additionally scented using 5 ml strawberry or orange food extract (McCormick & Company Inc., Hunt Valley, MD, USA) to help establish colony identity. Bees were observed remodeling the wax and accessing the honey within 24 h of the beginning of the experiment.

We then transferred observation hives to a dark room with the same ambient conditions as the laboratory-based behavioral assays (described above). Each hive was connected to the outside via a foraging port, a plastic entrance tube that we kept occluded for the first 2–3 days to prevent young bees from wandering outside before flight muscles were properly developed. On the second or third night after the colony was formed, the foraging port was opened, and bees were allowed to freely forage for the remainder of the experiment. As previously described (Geffre et al., 2020; Gernat et al., 2018), images of the entire honeycomb were captured at a rate of 1 frame s^{-1} with a Prosilica GT6600 machine vision camera (Allied Vision, Exton, PA, USA). LED lights (Smart Vision Lights, Muskegon, MI, USA) illuminated the observation hive from both the front and rear of the honeycomb with infrared light, which bees cannot see. We replaced the glass window once per day with a clean window to ensure clear image acquisition. Otherwise, the colonies were left alone except for the perturbation (experiment I) or territorial disturbance (experiment II).

Image processing and trophallaxis prediction

Hive images were processed and analyzed as in Gernat et al. (2018). Briefly, the images were resized and sharpened to improve the rate of automated barcode detection. The location and orientation of each bee within the hive was inferred based on the identifiers present on each barcode, using software developed by Gernat et al. (2018). After detection, barcodes were filtered to remove those that could not be decoded, were duplicated, moved too quickly from one image to the next (most likely owing to a spurious detection), or that corresponded to dead bees.

Trophallaxis identification was performed using a convolutional neural network (CNN) as in Gernat et al. (2020 preprint). If the CNN detected an extended proboscis between the heads of two bees, it recorded a trophallaxis event. Consecutive trophallaxis events between the same bees were combined into a single interaction, and these raw interactions were merged if they were less than 1 min apart. Merged interactions were then temporally filtered to remove potentially spurious trophallaxis detections (those <3 s or >3 min). Information pertaining to the remaining interactions (when and between whom they occurred, duration, etc.) was used to generate a series of directed networks.

Quantitative PCR

We focused our gene expression analysis on the honey bee MB, as the MB transcriptome has previously been shown to be responsive to both social threat and opportunity (Hamilton et al., 2019; Shpigler et al., 2018; Traniello et al., 2019). We performed MB dissection and RNA extraction as previously described (Lutz and Robinson, 2013). Briefly, flash-frozen bees were placed in a dry ice/ethanol bath and a small window was chipped in the frons of the head capsule. Entire heads were then placed in RNAlater-ICE Frozen Tissue Transition Solution (Thermo Fisher Scientific) overnight at 20°C. Brains were then fully dissected on wet ice, and the MB was carefully removed and stored at −80°C. RNA was extracted with the PicoPure RNA Isolation Kit (Thermo Fisher Scientific) with DNase treatment (Invitrogen, Carlsbad, CA, USA) as has been previously described (Lutz and Robinson, 2013; Shpigler et al., 2018), and quantified via a NanoDrop™ spectrophotometer (Thermo Fisher Scientific).

Quantitative PCR (qPCR) was performed according to standard protocols (Hamilton et al., 2019). cDNA synthesis was performed using Arrayscript reverse transcriptase, and *Root Cap Protein 1* (*Rcp1*) from *Arabidopsis thaliana* was used as an exogenous spike-in to monitor synthesis efficiency. qPCR was performed with SYBR Green dye on an ABI QuantStudio 6 (both Thermo Fisher Scientific), and data were analyzed in R using one-way ANOVA with blocking for colony. We tested five internal reference genes to find a suitable set for normalization: *s8*, *rps18*, *rp49*, *ef1a* and *gapdh*. Expression of both *e1fa* and *gapdh* were not significantly different between colonies (t-test, P>0.3) but slightly differed between behavioral groups (ANOVA, P<0.1), whereas the remaining genes showed no difference between behavioral groups (ANOVA, P>0.2) or colony (t-test, P>0.6). Therefore, we excluded *e1fa* and *gapdh* and normalized genes of interest to the geometric mean of *s8*, *rps18* and *rp49*.

We analyzed genes previously associated with honey bee aggression (Rittschof, 2017; Rittschof and Robinson, 2013; Shpigler et al., 2017a, 2018; Traniello et al., 2019) and/or affiliation (Shpigler et al., 2018; Traniello et al., 2019), all of which are listed with respective qPCR primers in Table S1. Two highly conserved immediate early genes (IEGs), *hormone receptor 38* (*hr38*) and *early growth response protein 1* (*egr1*), have shown similar changes associated with both aggressive and affiliative contexts (Shpigler et al., 2018; Traniello et al., 2019). *Odorant binding protein 14* (*obp14*) was one of the most strongly differentially expressed genes in the honey bee MB following a display of caregiving, but was not found to be active in aggressive contexts (Shpigler et al., 2018); its specific function outside of odor

perception remains unknown. We also examined expression levels of the canonical learning and memory biomarker *cyclic-AMP response element binding protein* (*CREB*), which was also found to be upregulated in the MB following caregiving and has been hypothesized to support long-term memory formation in honey bees (Gehring et al., 2016a,b; Shpigler et al., 2018).

Experiment I: Determine the relationship between behavioral state and sociability

Colonies were established in observation hives as described above and, after the first 3 days, automated monitoring was performed uninterrupted for three consecutive days before the perturbation, as described below, was performed. On the seventh day post-perturbation, the hive was opened and all the bees were placed in groups of nine in Petri dishes containing wax, honey and pollen. To remove/return bees, the glass observation window was replaced with a Plexiglas window that had resealable portholes, and the observation hive was placed in an area lit by an infrared LED light (Smart Vision Lights); this allowed the bees to be transferred individually to the dish via forceps with minimal disturbance (bees cannot see red light).

We used physiological manipulations in order to compare results of experiments I and II and explore the specificity of the relationship between colony division of labor, sociability and responsiveness to threat. We used a juvenile hormone analog (JHA), a treatment known to influence task specialization by accelerating behavioral maturation (Hamilton et al., 2019; Robinson, 1987). We also used cold anesthesia, which has transient effects on worker honey bee JH titers (Lin et al., 2004). We expected that these manipulations would shift behavioral state and thus allow us to measure molecular and behavioral mechanisms associated with biological embedding.

Bees were barcoded to form pairs of colonies on the same day, each sharing a common SDI colony of origin. Each colony in a pair was then assigned randomly as either a JHA- or cold-treated hive. Bees from the JHA-treated hives were cold-anesthetized in groups of three and each bee in the trio received a different treatment: one was treated topically with 200 µg per bee of the JHA methoprene dissolved in 1 µl of acetone, a dose known to attenuate the length of the brood rearing phase and induce precocious foraging (Robinson, 1987); the second bee was treated with 1 µl of acetone alone; and the third was only cold-anesthetized. This created observation hives in which one-third of the bees were treated with JHA, one-third treated with acetone and one-third cold-anesthetized. All bees in JHA-treated hives were paint-marked with a combination of two colors that corresponded to the treatment. After all the bees recovered from anesthetization, they were returned to their observation hive. Bees from cold-treated hives were cold-anesthetized in groups of three and randomly painted with a combination of two colors. Except for treatment, both observation hives in each pair were set up and handled identically. Because all of these procedures took ∼12–18 h per trial, we excluded monitoring data for the day of treatment for each replicate. In total, four JHA- and four cold-treated colonies were created.

To assess the influence of perturbation on the interaction patterns of individual bees, we generated static trophallaxis networks for each day prior to and after treatment. This allowed us to determine the general interaction properties of each individual (including the number of interactions, interaction partners and the median interaction duration) in the observation hives. We compared these properties between the JHA- and cold-treated hives, as well as between differently treated individuals within the same hives (JHA-treated, acetone-treated and untreated). We also used general interaction properties (number of interactions, number of interaction partners and median duration of interactions) of each individual to assess whether task specialization was related to sociability over the last 2 days of the observation. Because individual bees exhibited a high degree of stability in these patterns over a 48 h period, the final 2 days of observation provided an accurate measure of the bees' sociability at the time of the division of labor assays that we subsequently performed. This allowed us to capture an individual's sociability at the time of behavioral state analysis, whereas analyzing the full 10 days of monitoring data allowed us to capture changes in sociability relative to perturbation.

To identify foragers using our automated monitoring system, bee flight in and out of the hive was recorded by an entrance monitor (Geffre et al., 2020) attached to the exterior terminus of the entrance tube to which the observation hive was connected. The entrance monitor consisted of a small enclosure with a simple maze (slowing bees down to facilitate image capture) and a camera mount partitioned from the rest of the enclosure by a removable glass window. A Raspberry Pi Camera Module v1.3 (Raspberry Pi Foundation, Cambridge, UK) was installed in the enclosure and set to record .mjpg videos at a temporal resolution of 3 frames s^{-1} during the hours of 07:00 to 19:00 h CST, automatically adjusting for changes in light conditions over the course of the day.

Movies of bees exiting and returning to their hive were first converted to still images using ffmpeg, and then processed to detect barcoded bees as in Jones et al. (2020). Raw detection data were combined into incoming and outgoing 'passes' by vectorizing the bee's displacement toward or away from the hive entrance as in Geffre et al. (2020). Age at onset of foraging was predicted by adapting previously published criteria used in conjunction with automated identification via RFID tags (Hamilton et al., 2019; Tenczar et al., 2014). In honey bees, foraging is a long-term behavioral state; bees have foraging careers of up to 2 weeks (Seeley, 1986). Therefore, to be identified as a forager in our study, a bee had to have been detected taking at least six trips, with more than three trips per day for any 2 days. Honey bees also leave the colony to defecate and to learn the location of the colony prior to foraging. To limit the chances of falsely identifying pre-foraging bees as foragers, at least 25% of the bee's trips had to be made during peak foraging hours (10:00–15:00 h CST) for the bee to be identified as a forager. Similar but less stringent criteria have been validated via manual observation as providing accurate indicators of foraging behavior (Tenczar et al., 2014).

Following the automated monitoring experiment, bees were removed from their observation hives and prepared for dish-based assays in the laboratory, as described above, with nine uniquely marked bees per dish. In JHA-treated hives, an equal proportion of bees from the three treatment groups were present in each dish. Brood care assays were performed in a controlled environment room (34°C and 50% relative humidity) under ambient lighting, and aggression assays were performed in a temperature-controlled room (28°C). All bees were tested in both assays in random order; the second assay was performed 60 min after the beginning of the first assay. In total, between 30 and 46 dish assays were performed across each of the four JHA and two cold-treated hives. Bees from the remaining two cold-treated hives were not subjected to the dish assays owing to logistical constraints.

Utilizing established criteria (Hamilton et al., 2019; Shpigler et al., 2018; Traniello et al., 2019), bees were determined to be guards or nurses based on performance in each assay, whereas foragers were later identified via the automated monitoring

system. In addition, fanning, wax-building and vibration-signaling behaviors were recorded during both assays to give a more comprehensive picture of each individual's behavioral state. Bees that weakly responded to either the brood care or aggression stimulus and did not exhibit any of the behaviors mentioned above were referred to as 'baseline' bees of unknown behavioral state. Bees that completely ignored both brood care- or aggression-inducing stimuli and also did not forage were labeled as 'non-responders', as has been previously described (Shpigler et al., 2017b), and bees that exhibited two or more behavioral states (i.e. brood care, aggression and/or foraging) were labeled as 'generalists'. These data were combined with trophallaxis network information from the same bees collected via automated monitoring. Each behavioral state was represented on average in the dish assays as follows (in order of frequency): baseline, ∼0.43; forager, ∼0.16; nurse, ∼0.14; non-responder, ∼0.12; guard, ∼0.08; generalist, ∼0.08. These proportions are consistent with previous laboratory-based division of labor assays (Shpigler et al., 2017b) as well as observations in typical colonies of honey bees maintained in apiaries in the field (Johnson, 2008; Moore et al., 1987; Wilson, 1971).

Experiment II: Determine the effects of threat experience on trophallaxis sociability

As in previous studies (Shpigler et al., 2017a; Traniello et al., 2019), the colony disturbance was administered 6 days after the observation hive was first set up; at that time, the 800 resident bees were 7 days old. The day before the colony disturbance, we collected bees from a different, unrelated typical colony that either defensively postured at the colony entrance (Breed et al., 2004) or attempted to attack an investigator after the colony was struck with a brick (Avalos et al., 2020). For each replicate, we used the same colony for collecting aggressive bees. These bees, hereafter referred to as 'intruders', were immobilized on wet ice, barcoded, and a wing was clipped to prevent returning to their home colony. A thin ring of silver Testors paint was applied encircling the abdomen in order to make the intruders easy to visualize during analysis of the recorded footage. Intruders were housed in an incubator overnight in a Plexiglas container containing honey, pollen and beeswax.

The morning of the disturbance, we replaced the glass window of the observation hive with a new window that was identical except for a small half-circle cutout that allowed us to introduce bees to the observation hive via a cylindrical tube. We allowed bees to acclimate to this new window for at least 60 min, although it did not have any noticeable effect on behavior as bees had experienced regular window-changing in days prior. Next, 35 intruders were loaded into a cylindrical tube, which was then slid into the half-circle cut. We gently moved the glass such that we could introduce the intruders but resident bees could not escape and then pressed intruders through the tube onto the frame via a cardboard disc affixed to the end of a thin stick. This allowed us to rapidly introduce intruders to the host frame without disrupting the recording or physically harming either the intruders or residents. After the intruders were introduced, we replaced the glass and left the observation hive undisturbed for the remainder of the experiment, except for daily changing of the window. This experimental design allowed us to capture social interactions for several days before and after the disturbance.

To be consistent with the scoring system utilized in the dish-based assays, we analyzed hive footage for 5 min following the introduction of the intruders. We used the image processing software GIMP to analyze one image every 10 s (30 images per trial) and annotated images with behavioral responses toward the intruders (including antennation, licking, rearing, biting and stinging), which were clearly visible on the frame. Images were then passed through a custom program to read the barcode ID of individual bees, which was manually recorded along with respective behavioral displays for each image. We observed an overall decrease in biting and stinging compared with bees exposed to a territorial intruder when kept in small groups (Hamilton et al., 2019; Shpigler et al., 2017a, 2018); to our knowledge, scoring of aggression inside the hive on honeycomb rather than at the hive entrance has not been previously performed. Taking these differences into account, we adjusted our scoring system such that bees were required to be aggressive in at least three images (representing ≥30 s, similar to previous studies; Shpigler et al., 2017a,b, 2018), but we counted aggressive posturing, typically considered 'less' aggressive than biting and stinging (Hamilton et al., 2019; Shpigler et al., 2017a), toward this final score. This modification allowed us to identify the most aggressive bees while avoiding individuals that reflexively engaged in a brief display of aggression. As a control, we identified bees participating in retinue behavior (affiliative licking, grooming and feeding of the queen, also in at least three images). Similar to experiment I, remaining bees were collectively referred to as 'baseline'.

Experiment III: Determine the effects of threat experience on affiliative caregiving and caregiving-related brain gene expression

Groups of 10 age-matched bees (0–24 h old) emerging from honeycomb from two colonies (assayed separately) headed by an NM queen were maintained undisturbed for 7 days in Petri dishes containing wax, honey and pollen, randomly divided into 'threat-naïve' and 'threat-experienced' categories and sequentially subjected to two behavioral trials with a 60 min inter-trial interval. This interval has been previously demonstrated to be sufficient to trigger substantial threat-responsive changes in behavior in each trial (Shpigler et al., 2017a). Threat-naïve bees were given an inanimate object that was previously shown to induce both minimally aggressive and investigative behaviors (Traniello et al., 2019), whereas threat-experienced bees were subjected to a territorial intrusion, as described above. We excluded any individual that displayed both nursing and aggression from further analysis. These behaviors are rarely observed being performed by the same individual in a typical honey bee colony (Robinson, 1992; Shpigler et al., 2018), and removing these individuals prevented the possibility that our results would include the influence of subtle physical injury or exhaustion on affiliative caregiving.

At 30 min following the second exposure, bees were flash-frozen in liquid nitrogen and transported to a −80°C freezer where they remained until molecular analysis. We also performed a baseline control (hereafter 'control') experiment in which dishes of bees were exposed to two inanimate objects with a 60 min inter-trial interval and collected 30 min after the second exposure, to match the behavioral trials. For the molecular analysis, control bees were selected across colonies and experimental days to reduce any experimental bias.

Statistical analyses

Impact of perturbation or threat on trophallaxis network dynamics

We used trophallaxis, the liquid exchange of food by mouth between two workers, as a surrogate for social structure, and defined

an individual worker's sociability as the number of trophallaxis interactions, interaction partners or median interaction duration on a given experimental day, similar to what has been done in previous honey bee experiments (Hewlett et al., 2018a,b). Individual barcode IDs from the dish assays and forager detector (experiment I) or manual image annotations (experiment II) were used to sort bees into behavioral categories, and respective trophallaxis interactions for each day of recording were extracted, combined and fitted to a negative binomial generalized linear mixed model (GLMM). We then performed a type II Wald chi-square test to compare sociability across behavioral groups, followed by *post hoc* tests: the false discovery rate (FDR) was controlled for using the Šidák method (longitudinal planned contrasts) or a Tukey's *post hoc* test (for pairwise comparisons of aggregated data from the last 2 days of the experiment).

Effect of threat experience on future affiliative caregiving

We compared behavioral responses of threat-naïve and threat-experienced bees using linear mixed-effect models (LMMs) with previous experience as a fixed effect and housing group nested within colony as a random effect. Affiliative caregiving scores were log-transformed to meet test requirements (Rittschof, 2017; Shpigler et al., 2017a). We used the statistical package 'lme4' (Bates et al., 2015) in R (v. 3.6.1) after checking post-transformation data met test assumptions, and models were fit using maximum likelihood. Considering our high sample size, which heavily outweighed the number of model parameters, we generated *P*-values via likelihood ratio tests between full models and reduced models without fixed effects using ANOVA (Barr et al., 2013; Luke, 2017).

Gene expression: qPCR

We used one-way ANOVA followed by Tukey's *post hoc* test to explore differences between behavioral categories. Expression levels for *obp14* met test assumptions following log transformation, and all other genes met assumptions without transformation.

RESULTS

Experiment I: Differences in sociability are dependent upon task-related behavioral state

To determine sociability level, we monitored trophallaxis continuously in eight colonies housed in glass-walled observation hives in which all colony members were barcoded. We aggregated these trophallaxis interactions into a series of discrete static daily networks so that we could analyze patterns in individual sociability over time. We analyzed three metrics of sociability: (1) the number of interactions exhibited by each individual, (2) the number of interaction partners for each individual and (3) the median interaction duration for each individual. We first examined the relationship of each of these metrics to one another by comparing their mean or median values for each day of the experiment.

There was a highly significant correlation between the number of interactions and interaction partners on each day (Spearman's correlation, ρ=0.99, P<2.2e-16; Fig. 2A). By regressing these data, we found that each bee had about one to two interactions with each of its partners per day, indicating that bees generally do not exhibit strong preferences to repeatedly interact with the same individuals. There was a weaker (but still statistically significant) negative correlation between the median interaction duration and both the frequency and number of interaction partners (ρ=−0.32 and −0.35, respectively, P<2.2e-16; Fig. 2B,C). The numbers of interactions and interaction partners for each individual were highly autocorrelated (i.e. the relative rank order of bees was preserved over time) (Fig. 3A,B). By contrast, the median interaction duration fluctuated substantially over time, but appeared to stabilize in the last few days of recording, although to a lesser extent than other metrics (Fig. 3C). These results indicate that sociability is, in general, highly stable over time, with individual differences likely influenced by a combination of intrinsic and extrinsic factors.

Because our analyses result in a directed social network (Gernat et al., 2020) where the trophallaxis donor and receiver(s) are known for each interaction, we also looked for differences in the tendency to either donate or receive fluid as a finer-grained exploration of individual differences in behavior. Individuals exhibited a high degree of stability in their preference to donate or receive over time, indicating that intrinsic preferences could govern the type, number or duration of interactions demonstrated by individuals. Individual differences in these tendencies were highly correlated across interactions, interaction partners and interaction duration (Fig. S1). There was substantial individual variation in the proportion of interactions (Fig. S1A), interaction partners (Fig. S1B) and median duration (Fig. S1C) that can be ascribed to biases toward giving or receiving.

At the conclusion of the trophallaxis monitoring component of experiment I, bees were removed from observation hives, placed in Petri dishes containing wax, honey and pollen, and subjected to laboratory division of labor assays to assess behavioral state. Trophallaxis sociability and foraging activity were computed after data collection was completed. The combination of laboratory division of labor assays and in-hive sociability measurements allowed us to investigate whether the individual differences in sociability reported above could be partially explained by division of labor; that is, whether sociability varies in a context-dependent manner that relates to current occupation. We did this by taking advantage of the finding that individuals maintain highly stable trophallaxis dynamics over 48 h periods, as noted above. We averaged the number of interactions, interaction partners or median interaction duration over the final 2 days of automated monitoring to represent an accurate measure of sociability at the time of sampling, and then removed the bees from their hive in order to assess task specialization in laboratory behavioral assays. Guards and nurses were individuals that consistently showed territorial aggression or affiliative larval care, respectively (Shpigler et al., 2018; Traniello et al., 2019). Foragers were identified via a hive entrance monitor (Geffre et al., 2020) as individuals that made consistent foraging trips during peak foraging hours, as described in the Materials and Methods.

We found substantial differences in the levels of trophallaxis sociability for individuals showing different task specializations (Fig. 4A–C), and these differences were significant for all three sociability metrics (GLMM, interactions: $\chi^2_{51,526}$=90.72, P<2e-16; interaction partners: $\chi^2_{51,526}$=77.72, P<2.6e-15; interaction duration $\chi^2_{51,526}$=20.63, P<1e-4). 'Generalists' (individuals that performed a combination of nursing, guarding and foraging behavior), foragers and nurses showed higher levels of trophallaxis sociability (number of interactions and interaction partners) whereas non-responders (bees that did not respond to stimuli eliciting the performance of nursing, guarding, or foraging) and guards showed lower levels of sociability (Fig. 4A,B). 'Baseline' bees, defined as individuals that weakly displayed territorial aggression and/or affiliative caregiving, also showed lower levels of sociability.

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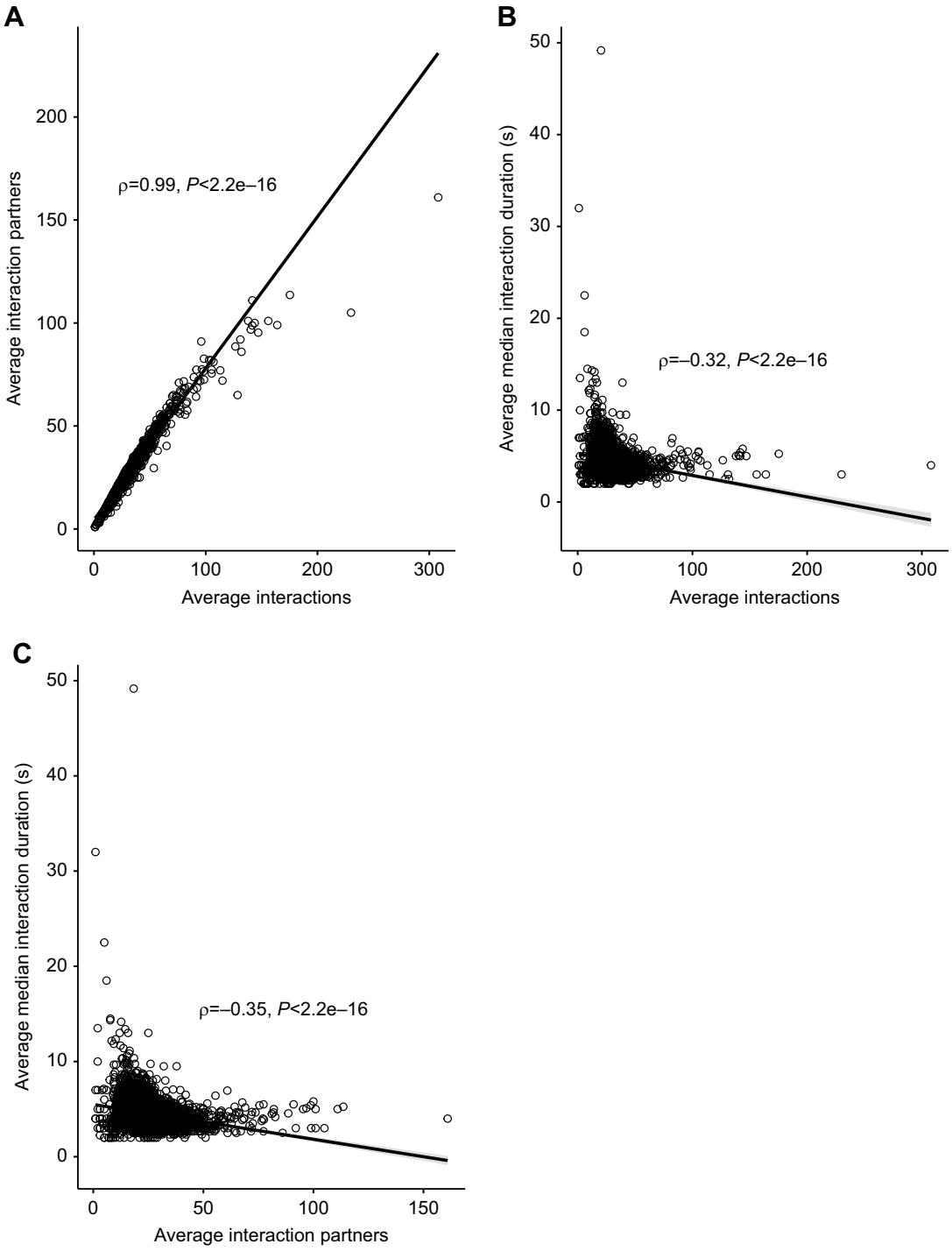

Fig. 2. Metrics of honey bee sociability are highly correlated with each other. (A) A strong positive correlation exists between the number of interactions and the number of interaction partners. (B) Moderate negative correlations were found between interaction duration and the number of interactions and (C) interaction partners. Spearman's correlation, P<2.2e-16 for all comparisons. Analyses were performed with data averaged across all days of the experiment (n=5779 bees observed in eight observation hives). Shaded areas around the regression lines represent 95% confidence intervals.

Although foragers and nurses both engage in affiliative behaviors while performing their respective tasks, there were significant differences in median interaction duration between these two groups (Fig. 4C), even though both groups exhibited comparable numbers of interactions and interaction partners. Perhaps this is related to the fact that the purpose of trophallaxis is distinct in each case. Foragers generally perform trophallaxis to acquire resources for extended periods of flight or when depositing resources after returning to the colony from a successful foraging trip, implying that comparatively large volumes are being transferred. Nurses, in contrast, do not commonly leave the hive, and therefore may not require as intensive food-exchange sessions.

The results of the laboratory behavioral assays demonstrate that individual differences in sociability are strongly associated with division of labor. These results also indicated that number of interactions or number of interaction partners could be used

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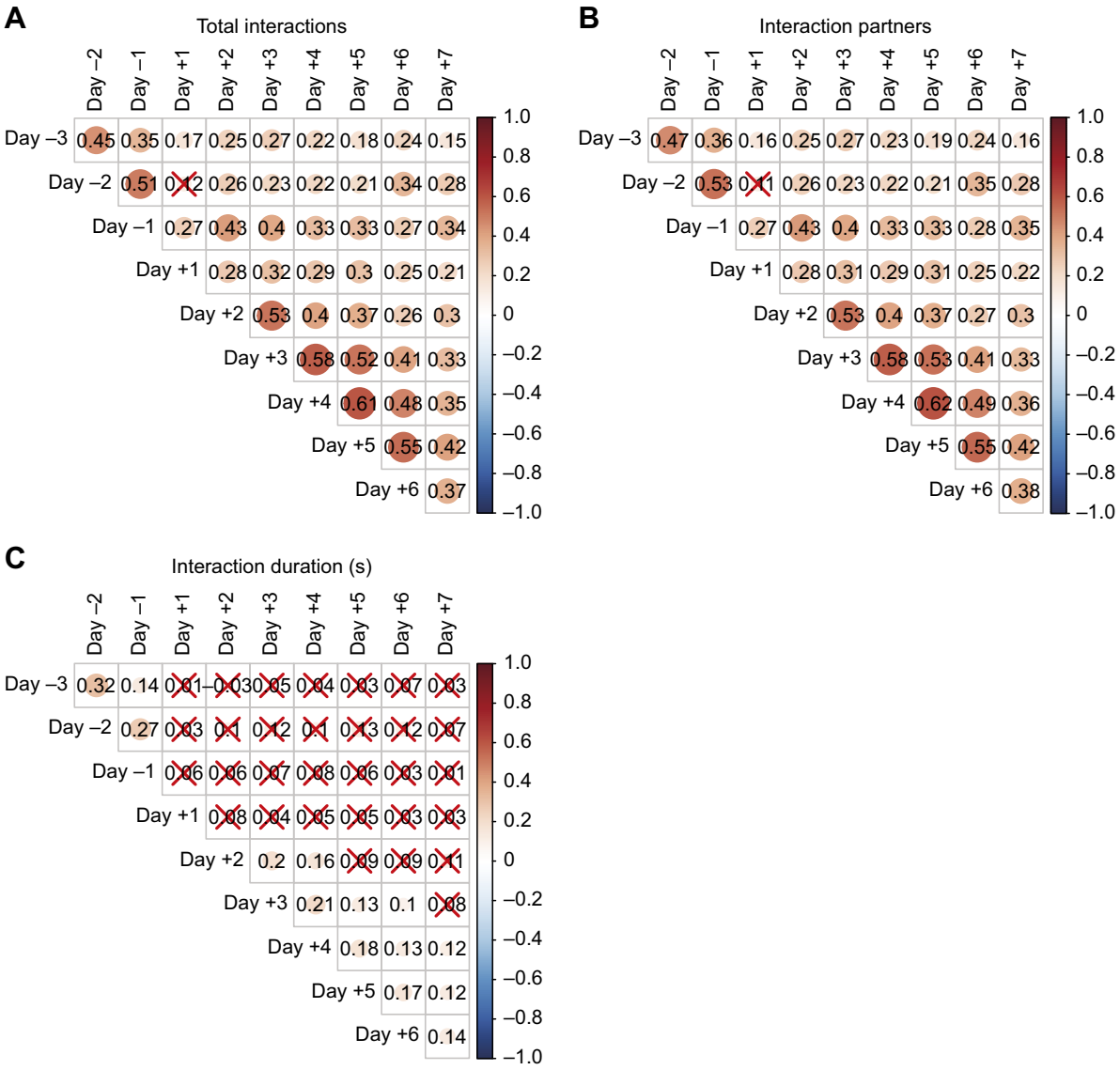

Fig. 3. The total numbers of interactions and interaction partners were stable over time, but not the median interaction duration for individual honey bees. Correlograms depict Spearman's correlation between days of observation before (day –) and after (day +) colony-wide perturbation (see Materials and Methods). The correlation coefficient of each comparison is listed for all comparisons; underlying circles, shown only for significant comparisons, are colored according to the correlation coefficient as depicted by the color bar on the right, and circle diameter represents the absolute value of the coefficient. Red Xs denote correlations that were below a *P*-value of 1e-10 (*n*=5779 bees observed in eight observation hives).

interchangeably for assessing sociability as related to division of labor; for simplicity, we used the former as a proxy for sociability for the rest of the analyses.

We next explored the robustness of the relationship between sociability and division of labor. Both JHA and cold treatment significantly altered colony-level sociability ($P<0.0001$; Fig. S2) in ways that could not be easily disentangled, so we combined both perturbations in our analysis; their role in this study was limited to providing a point of comparison for the effects of colony disturbance in experiment II. The perturbations in experiment I caused prolonged, significant increases in sociability relative to baseline in generalists, foragers and nurses, lasting as long as 7 days post-perturbation ($P<0.05$; Fig. 5A–C). By contrast, neither guards nor non-responders showed any consistent changes in sociability relative to the perturbation (Fig. 5D,E), again demonstrating differences between guards and the other task-defined groups of bees. The perturbations of experiment I did not impact the stability of individual-level sociability metrics over time (Fig. S3). As expected (Hamilton et al., 2019; Robinson and Ratnieks, 1987), JHA treatment caused precocious foraging (Cox proportional hazards, $z=5.47$, $P<5e\text{-}10$), while there was no effect of acetone treatment ($z=0.31$, $P>0.7$).

Experiment II: Changes in sociability following threat experience are dependent upon task-related behavioral state

We performed a colony manipulation specifically designed to target guards. We placed 30–35 aggressive foreign bees (collected from an unrelated colony and marked for identification) inside a glass-walled observation hive. This provoked a rapid defensive response from a subset of resident bees; we called them guards because, like guards at the hive entrance, they responded to the threat. Moreover, the presence of bees that specialize in nest defense but are located inside the hive is well known (Breed et al., 1990; Moore et al., 1987). We used

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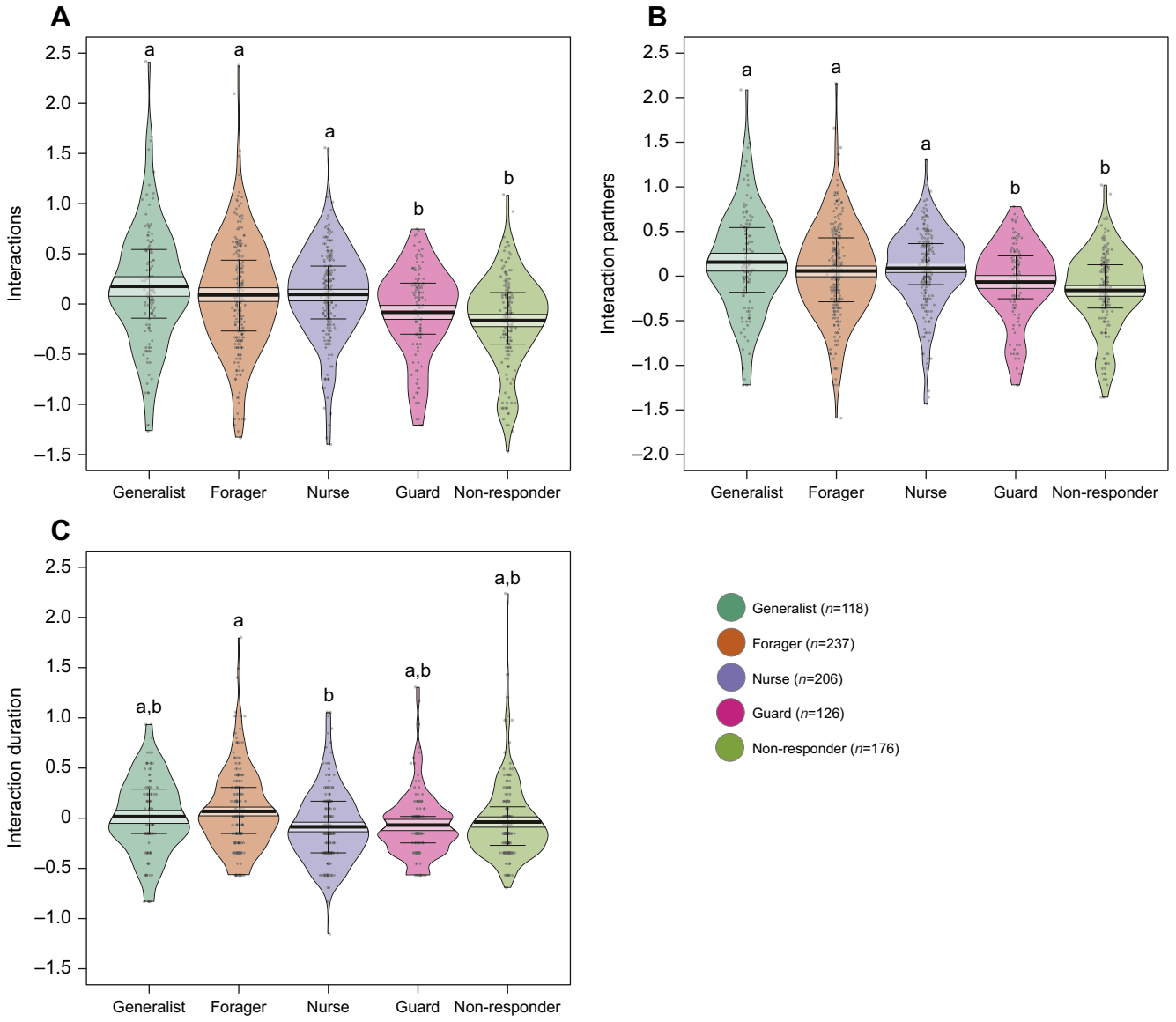

Fig. 4. Task-related and individual differences in sociability in trophallaxis social network. (A–C) Violin plots depict the mean number of interactions (A), interaction partners (B) or median interaction duration (C) over the last 2 days of the experiment, log-scaled and normalized to the mean value of the relevant metric in baseline bees (n=1526 bees across six observation hives). Letters designate significantly different groups after Tukey correction (P<0.005). Violin plots are constructed as follows: raw data are shown as points, solid black line represents mean with 95% confidence interval as pale white above and below, whiskers show lower and upper quartiles (25% and 75%, respectively), and overall plot shape represents a smoothed density curve outlining the complete data distribution.

automated monitoring to detect trophallaxis interactions continuously on days before and after this disturbance. For each colony, we focused on an average of ∼25 guards and ∼20 bees that formed the queen's retinue (affiliative individuals that licked, groomed, fed the queen and ignored the disturbance) across five replicates each performed on a different colony. We compared these two behavioral groups with the other bees in the colony selected randomly with respect to task-related behavior, which we considered to be baseline.

There were significant effects of experimental day on the number of social interactions per bee ($\chi^2_{33,878}$=259.82, P<2e-16). Guard bees had relatively few social interactions prior to the disturbance, consistent with the results of experiment I, but the disturbance caused a large increase in their sociability. Specifically, we found increased sociability in guards compared with baseline starting just after the administration of the disturbance (day 0, P<0.0001), and this effect persisted for the remainder of the experiment (day +1, P<0.0005; day +2, P<0.05; day +3, P<0.001). There were no differences between guards and baseline bees beforehand (day −2, P>0.10; day −1, P>0.10). There also were no differences between retinue and baseline bees in trophallaxis sociability on any day before, during or after the colony disturbance (Fig. 6).

This increase in guard sociability appears to be a specific response to colony threat, as we did not observe any increase in guard sociability following the perturbation in experiment I, which selectively affected non-guard bees. Because the presence of intruders can increase the number of bees defending the hive entrance (Alaux and Robinson, 2007), we considered whether the observed increase in guard trophallaxis sociability could have been caused by decreased time on the honeycomb frame, either owing to increased time at the entrance or disturbance-related injuries or death. This was not the case, as the time on honeycomb frame for our focal guard bees was very similar to that of baseline bees following the

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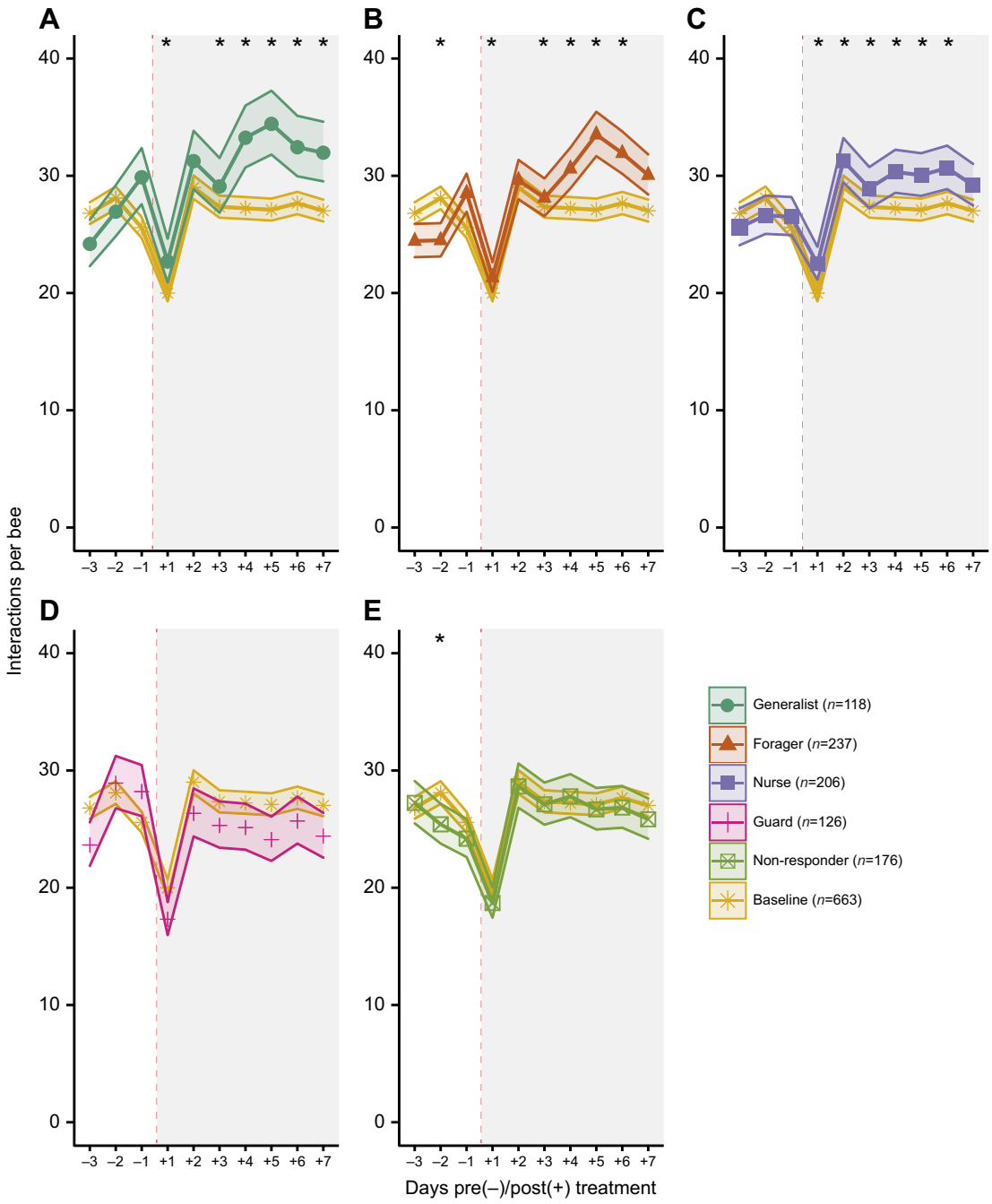

Fig. 5. Colony perturbation causes long-term changes in sociability in all task-related behavioral groups except guards. Contrasts between baseline bees and either behavioral groups or non-responders for each day of the experiment reveal sustained differences in sociability for (A) generalists, (B) foragers or (C) nurses, but not (D) guards or (E) non-responders (GLMM with Šidák-corrected contrasts, *P<0.05) following perturbation (n=1526 bees across six observation hives). Shaded areas around line graphs represent the 95% confidence interval; gray shaded rectangle represents time post-perturbation.

disturbance (Fig. S4); there also was no differential mortality, with rates of 3–5% for each behavioral group by the end of the experiment.

Experiment III: Threat experience and behavioral state are associated with changes in future behavior and brain gene expression

Using the same laboratory assays as in experiment I, we measured the impact of a threatening stimulus on the opportunity to provide affiliative caregiving. We quantified behavioral variation among guards and nurses, defined as above.

Threat experience significantly reduced levels of subsequent larval care displayed by nurses ($\chi^2_{1,74}$=4.32, P<0.05; Fig. 7A), even though these individuals did not display aggression in response to threat. Taking a subset of individuals used for these behavioral analyses, we measured MB expression levels of four genes previously shown to be associated with the response to a queen larva: *hr38*, *egr1*, *obp14* and *CREB* (Shpigler et al., 2018). qPCR revealed significant differences in threat-experienced and/or threat-naïve (no experience of threat) nurses for each tested gene relative to baseline controls (Table 1). In addition, *post hoc* analyses showed that threat-experienced nurses tended to have the

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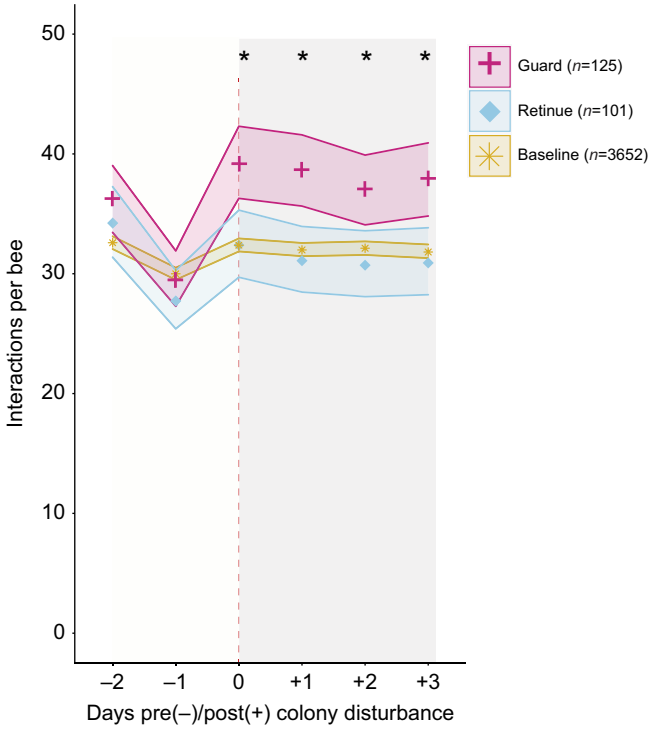

Fig. 6. Automated behavioral monitoring reveals changes in sociability following colony disturbance. Bees that responded aggressively to colony disturbance ('guards', pink) showed an increase in the frequency of social interactions relative to a negative behavioral control ('baseline', yellow) shortly after the disturbance (day 0, morning), and this effect persisted for the remainder of the experiment (days +1, +2 and +3). Significance (P<0.05, black asterisk) is based on total daily social interactions in guards compared with baseline. We found no differences between bees that tended to the queen ('retinue', blue) and baseline for any day pre- or post-disturbance. *Y*-axis represents average interactions per bee per day. n=3878 bees across five observation hives. All *P*-values resulting from *post hoc* comparisons are corrected for multiple testing via the Šidák method. 'Day' is denoted relative to the disturbance (i.e. day 0 is the day of the disturbance, day –2 is 2 days prior to the disturbance, day +2 is 2 days after the disturbance, etc.). Shaded areas around line graphs represent the 95% confidence interval; gray shaded rectangle represents time post-disturbance.

highest levels of *hr38*, *egr1* and *CREB*, but not *obp14* (Fig. 7B, Table 1).

DISCUSSION

Understanding biological embedding requires knowledge of how the processing of environmental experience is shaped by intrinsic and extrinsic factors (Boyce et al., 2012; Ellis and Boyce, 2008; Hertzman, 1999; Traniello and Robinson, 2021). For social animals, it remains challenging to predict how biological embedding at the level of the individual scales to affect emergent, group-level properties such as social structure. We showed that there are strong and consistent individual differences in sociability related to division of labor. Moreover, specific division-of-labor-related changes in sociability and caregiving occur following environmental threat, and we identified the changes in caregiving to be associated with changes in MB gene expression. These findings suggest that division-of-labor-related differences in behavioral state influence how an individual will integrate internal physiology and environmental cues to modify future behavior.

Individual variation in sociability was shown to be related to division of labor, not just after colony disturbance, but also on an intrinsic basis. Workers that specialized in tasks that require affiliative interactions (i.e. foraging and nursing) had substantially higher levels of trophallaxis sociability than non-responders or bees specializing in tasks that did not, such as guarding the nest. The metrics of affiliative behavior quantified in this study were highly stable over time, suggesting that sociability in the honey bee is defined by a constellation of factors that may be maintained throughout adult life. It is therefore possible that differences in sociability not only reflect task specialization but also influence an individual's proclivity for performing particular tasks. Similar differences in honey bee 'personality' have been observed for other tasks as well (Liang et al., 2012; Walton and Toth, 2016; Wray et al., 2011).

Experience with threat caused a decrease in affiliative caregiving, consistent with reports from a variety of taxa, including crustaceans (Arundell et al., 2014), birds (Ghalambor and Martin, 2002; Ghalambor et al., 2013), teleost fish (Gallagher et al., 2016) and humans (Doulougeri et al., 2013; Dozier et al., 2012; Elfgen et al., 2017). The cross-context effects of adverse experience have been characterized as dynamic 'trade-offs' between agonistic behaviors and affiliative caregiving, as animals must use environmental cues to optimize behavioral investments (Ros et al., 2004). What was striking about our results is that experience with threat was sufficient to decrease affiliative caregiving in individuals that merely perceived the threat but did not engage it. One explanation for this is that threat perception activates similar physiological pathways in caregiving individuals to pathways activated in guards during or after an aggressive encounter. Evidence for a similar mechanism exists in diet-restricted fruit flies (*Drosophila melanogaster*), in which food odor alone was shown to modulate feeding-related longevity (Libert et al., 2007).

Experience with threat also caused a change in the expression of several genes previously shown in the honey bee MB to be associated with affiliative caregiving (Shpigler et al., 2018; Traniello et al., 2019), further substantiating the behavioral results. For example, expression of olfactory binding protein (OBP) gene *obp14*, previously found to be among the most upregulated genes in the MB following a display of caregiving but not after response to a threat (Shpigler et al., 2018), was suppressed in threat-experienced nurses compared with naïve nurses that lacked experience with social adversity. The function of OBPs outside of olfactory receptor neurons is unknown, but OBPs have been identified outside of antennal tissue and the nervous system altogether in insects (Dippel et al., 2014), including honey bees (Forêt and Maleszka, 2006). Although expression of *obp14* was reduced with threat experience, the expression of immediate early genes *hr38* and *egr1* as well as *CREB* – three genes previously shown to increase in expression following both adverse and affiliative social encounters (Shpigler et al., 2018; Traniello et al., 2019) – was increased following caregiving in threat-experienced nurses compared with threat-naïve nurses.

Although we measured only a few genes, and the functional significance of the changes in gene expression is not known, our results indicate a clear embedding-type response, such that threat experience potentiates the expression of some genes while reducing the expression of others in parallel to the reduction of an important affiliative behavior. One explanation for these findings is that social experience shapes brain gene expression via modification of the brain's epigenetic landscape, a hypothesis supported by previous studies linking changes in DNA methylation to threat experience in bees (Herb et al., 2018; Shpigler et al., 2017a). In mammals, DNA methylation plays a significant role in translating early-life stress

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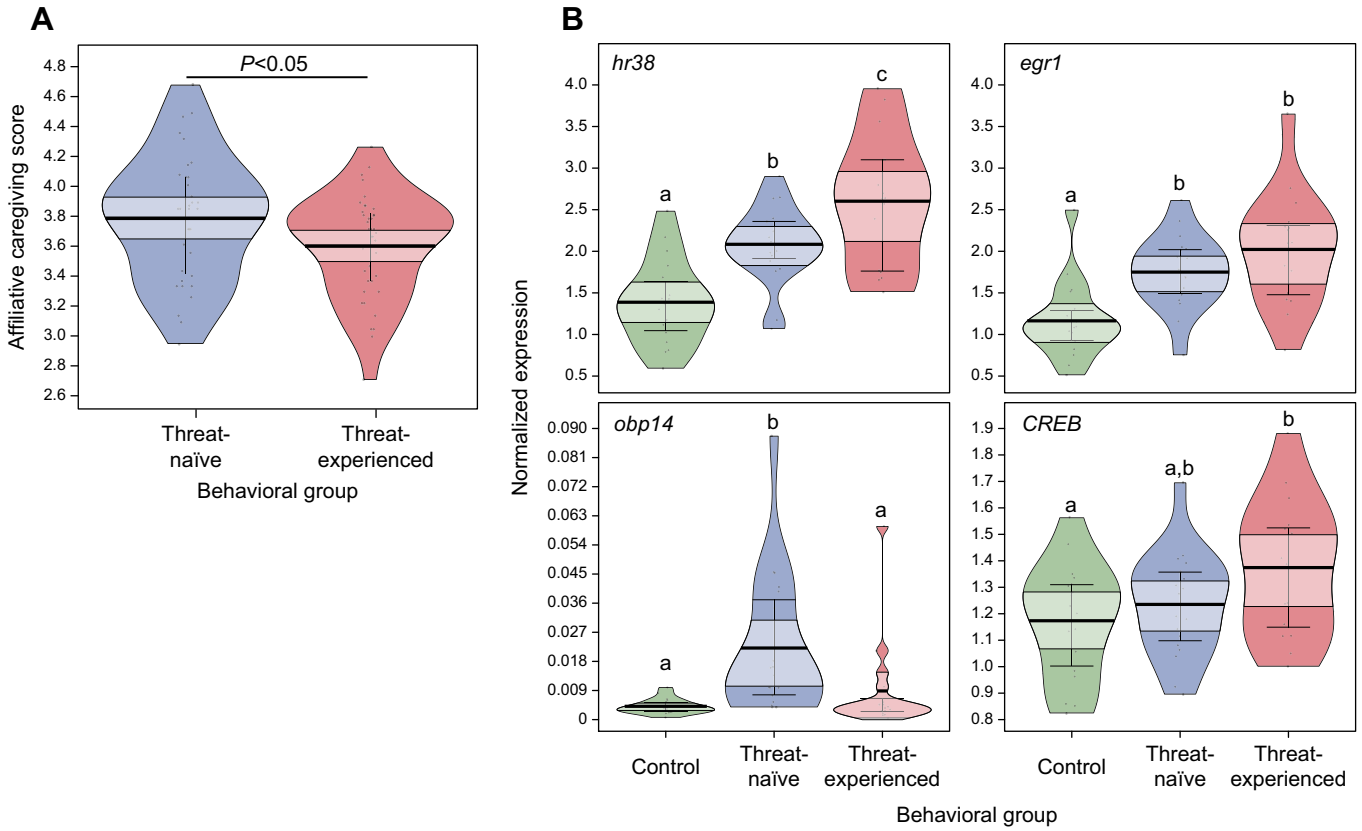

Fig. 7. Social adversity affects affiliative caregiving and brain gene expression. (A) Threat-experienced nurses displayed significantly less caregiving than threat-naïve nurses (that received an inanimate 'control' object); to avoid the possibility that physical harm reduced caregiving performance, we only considered nurses that did not engage the intruder. Linear mixed model, $P<0.05$, $n=35$ threat-experienced and 39 threat-naïve nurse bees. Affiliative caregiving scores were log-transformed (see Materials and Methods). (B) Mushroom body qPCR results showed consistent differences in gene expression between threat-experienced and -naïve groups. Letters denote $P<0.05$, Tukey's HSD *post hoc* tests following ANOVA; $n=16$–18 individual bees per group. Sample sizes are pooled across two colonies, assayed separately. Statistical details are shown in Table 1. Violin plots were constructed as described in Fig. 3.

to long-term changes in adult gene expression and behavior (Aristizabal et al., 2019; Demetriou et al., 2015; Sasaki et al., 2013), and specific connections between methylation, neuronal IEG induction and adaptability to stress have been made (Saunderson et al., 2016). Future work will be necessary to explore the evolutionary conservation of these mechanisms, especially in affiliative contexts, across vertebrates and invertebrates.

Guard sociability was specifically affected by colony disturbance but unaffected by perturbation, suggesting that an individual's societal role influences the extent to which certain environmental phenomena can be biologically embedded. Why do guards, which had fewer social interactions when compared with individuals that carry out affiliative behaviors such as caregiving and foraging, rapidly increase sociability to levels above other groups only after responding to a brief intrusion? This is unlikely to be related to defensive recruitment, as honey bees rely primarily on the release of alarm pheromone to alert, recruit and orient nestmates to a disturbance (Nouvian et al., 2016; Wager and Breed, 2000). In addition, the observed increase in sociability far outlasted previous reports of the duration of heightened vigilance post-disturbance (Alaux and Robinson, 2007; Rittschof, 2017; Shpigler et al., 2017a). However, we note that these previous observations were made either at the hive entrance or in dish-based assays, and the behavior of guards within the hive following a disturbance is less well understood. It may be the case that the prolonged increase in trophallaxis is related to an increase in energetic demands, owing to both the acute aggressive response to intrusion as well as a lasting increase in vigilance. Guards may also more frequently receive food from nestmates as a means of 'freeing up' time to surveil the hive and be better prepared to rapidly respond to a future threat. Alternatively, the increase in interaction rate may itself be a means of performing social surveillance, with aggressive bees more

Table 1. Mushroom body gene expression analyses

				Post hoc group		
Gene	Description	*F*	*P*	Control	Threat-naïve	Threat-experienced
hr38	Hormone receptor 38	18.04	**<1e-05**	a	b	c
egr1	Early growth response protein 1	11.75	**<1e-04**	a	b	b
obp14	Odorant binding protein 14	12.47	**<1e-04**	a	b	a
CREB	Cyclic-AMP response element binding protein	4.38	**<0.05**	a	ab	b

The same data were used to generate Fig. 7B and compare control, threat-naïve and threat-experienced groups. Significant results (in bold) via ANOVA were followed by Tukey *post hoc* analysis. $n=16$–18 individuals per group.

regularly monitoring for the presence of intruders, as has been suggested for colonies of the dampwood termite *Zootermopsis angusticollis* (Thompson et al., 2020). This is plausible because a threat perceived deep in the hive on a honeycomb itself could signify a breach of the defensive mechanisms in place at the hive entrance (Breed et al., 1990, 2004; Winston, 1991).

The framework of biological embedding contains an ecologically relevant component, in that all salient social experiences, both agonistic and affiliative, can drive context-dependent shifts in behavioral inclinations, thus allowing an animal to better anticipate a changing environment (Traniello and Robinson, 2021). In this regard, all animals must have responsive neurogenomic mechanisms in place for behavioral flexibility. We note that the behavioral and molecular impact of adverse social experience presented here was on the order of hours to days, far shorter than the projected consequences of, for example, PTSD, which can persist for months to years in vulnerable human populations (Yehuda and LeDoux, 2007). However, honey bees, like most social insects, are short-lived, meaning that the observed behavioral changes following adverse social experience occupy a much larger proportion of their lifespan than would be the case for similar behavioral changes in humans. Days of life for a honey bee may reflect developmental physiology analogous to a much longer time period in humans (Münch et al., 2008).

Modification of social networks in response to a threatening environment has also been observed in humans (Taylor, 2006; von Dawans et al., 2012) and non-mammalian vertebrates (Bruintjes et al., 2016; Krams et al., 2010). In these cases, it has been hypothesized that such modifications serve to increase intragroup cohesion and mitigate harm by synchronizing behavioral activity. Food sharing is a common affiliative, altruistic behavior in animal societies that strengthens social bonds (Wilson, 2017), so the observed changes in the trophallaxis social network we report are consistent with this hypothesis. Other hypotheses have suggested that a strengthening of intragroup bonds post-conflict facilitates the formation of a support network that can offer protection from future environmental threats (Taylor, 2006; von Dawans et al., 2012). In weaver birds (*Philetairus socius*), aggressive experience increased cooperative nest-building (Leighton and Meiden, 2016), and intragroup aggressive conflict has been shown to facilitate cooperative work in mammals as well (Reeve, 1992). These studies, together with ours, hint at widely conserved evolutionary mechanisms of plasticity in sociability to respond to the changes in group life.

Acknowledgements
We thank Alison Sankey for expert beekeeping assistance, Nathan Beach, Laima Licitis, and Safa Khan for barcoding bees and assembling colonies, and members of the Robinson Lab for comments that improved the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: I.M.T., A.R.H., T.G., G.E.R.; Methodology: I.M.T., A.R.H., T.G.; Software: T.G.; Validation: I.M.T., A.R.H., G.P.H.; Formal analysis: I.M.T., A.R.H., A.C.C.; Investigation: I.M.T., A.R.H., A.C.C., G.P.H., A.M.R., A.G., J.T.; Resources: T.G., G.E.R.; Data curation: I.M.T., A.R.H., A.C.C., T.G.; Writing - original draft: I.M.T., A.R.H., G.E.R.; Writing - review & editing: I.M.T., A.R.H., T.G., A.C.C., G.P.H., A.M.R., G.E.R.; Visualization: I.M.T., A.R.H.; Supervision: N.G., G.E.R.; Project administration: N.G., G.E.R.; Funding acquisition: N.G., G.E.R.

Funding
This research was supported by grant R01GM117467 from the National Institute of General Medical Sciences (G.E.R. and N.G., PIs). Deposited in PMC for release after 12 months.

Data availability
Computer code for analysis of automated tracking data is publicly available at https://github.com/gernat/btools. All datasets and relevant code for analysis are available from Figshare: doi:10.6084/m9.figshare.16556505.

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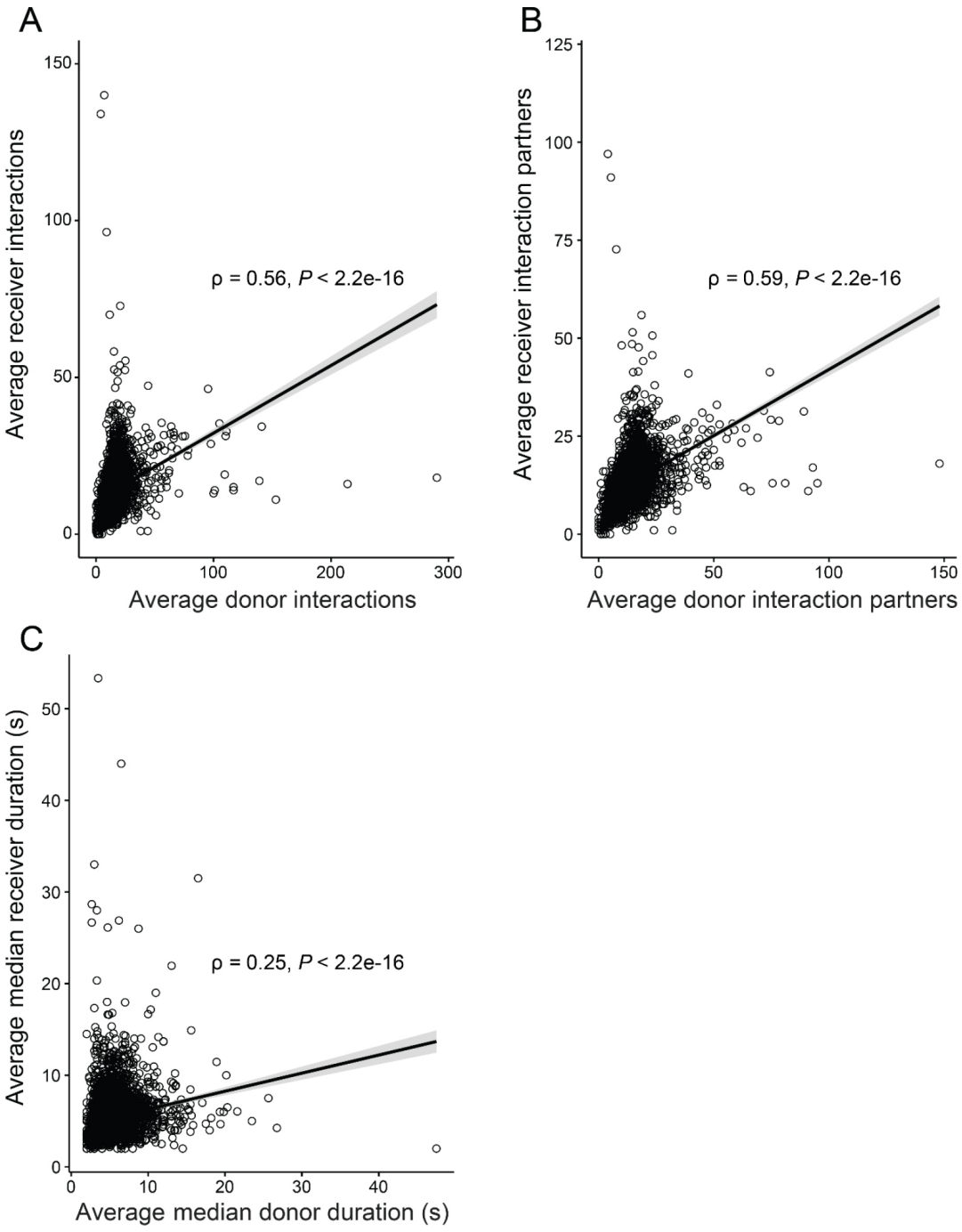

Fig. S1. Trophallaxis donor and receiver behaviors are highly correlated with each other for different metrics of sociability. Strong positive correlations exist between (A) the number of donor and receiver interactions and (B) interaction partners. (C) A moderate positive correlation was found for the mean donor and receiver interaction duration. Spearman's correlation, $P < 2.2e\text{-}16$ for all comparisons. Analyses performed with data averaged across all days of the experiment (n = 5,779 bees observed in eight colonies). Shaded areas around the regression lines represent 95% confidence intervals.

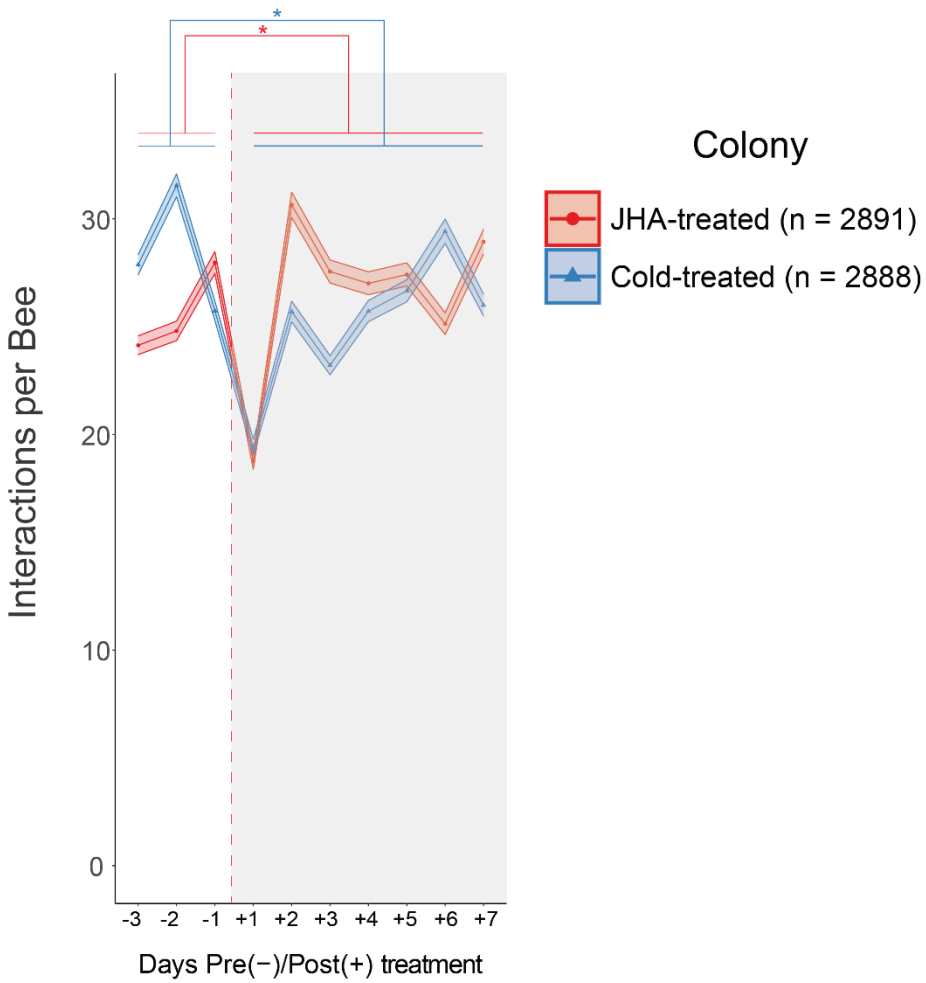

Fig. S2. Colony sociability pre- and post- treatment. Both juvenile hormone analog (JHA) and cold anesthesia perturbations caused significant changes in colony-level trophallaxis sociability (*P < 0.0001, four colonies per treatment). Note that JHA-treated colonies are composed of individuals treated with JHA, acetone, and cold anesthesia, as described in Materials and Methods. Statistical analyses, X-axis annotation, and figure convention follow descriptions in Figs. 5-6.

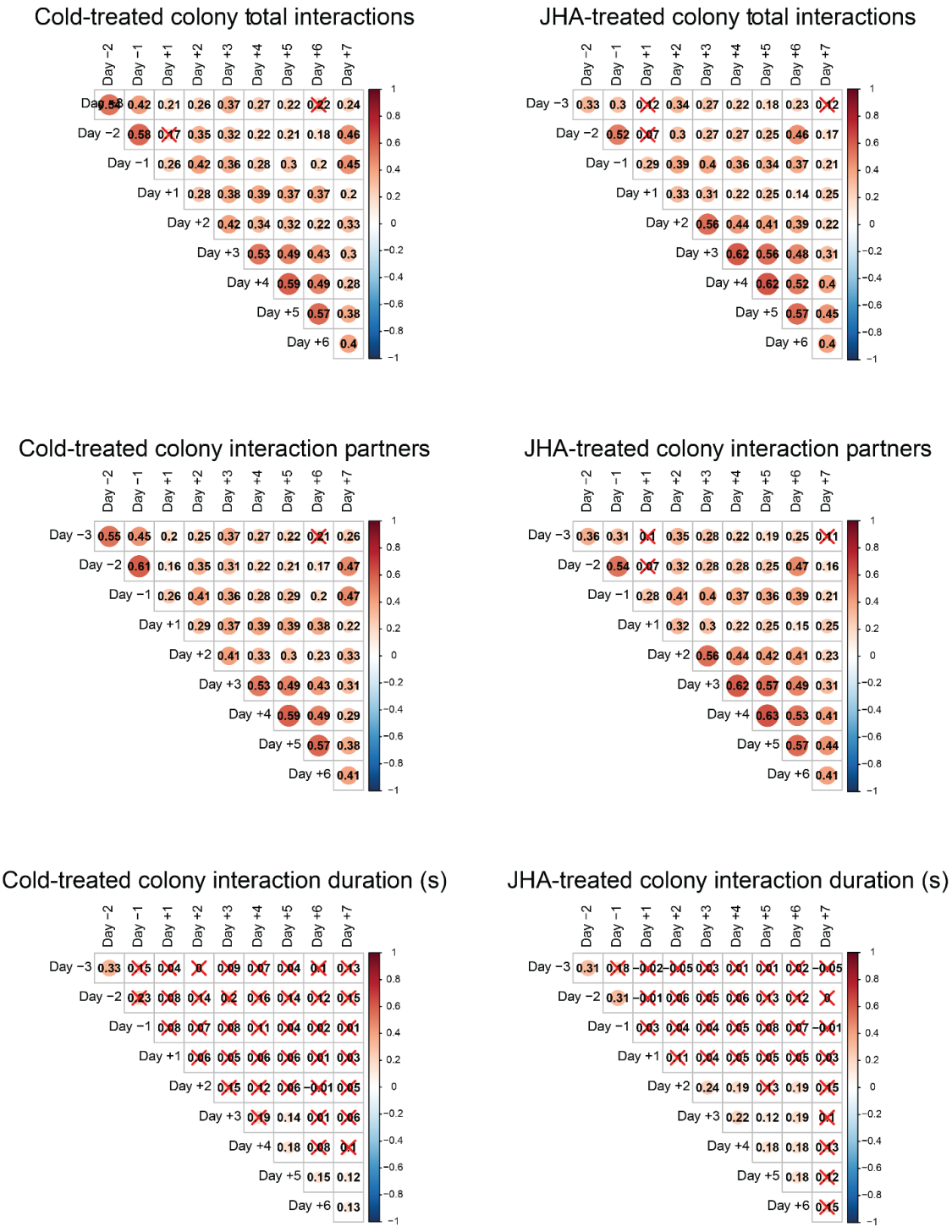

Fig. S3. The stability of individual-level sociability metrics over time is not affected by colony perturbation. Correlograms depict Spearman's correlations between days of observation before (Day -) and after (Day +) colony-wide perturbation. The correlation coefficient of each comparison is listed for all comparisons; underlying circles, shown only for significant comparisons, are colored according to the correlation coefficient as depicted by the color bar on the right, and circle diameter represents the absolute value of the coefficient. Red X's denote correlations that were below P = 1e-10. n = 5,799 bees observed in eight colonies. JHA, juvenile hormone analog.

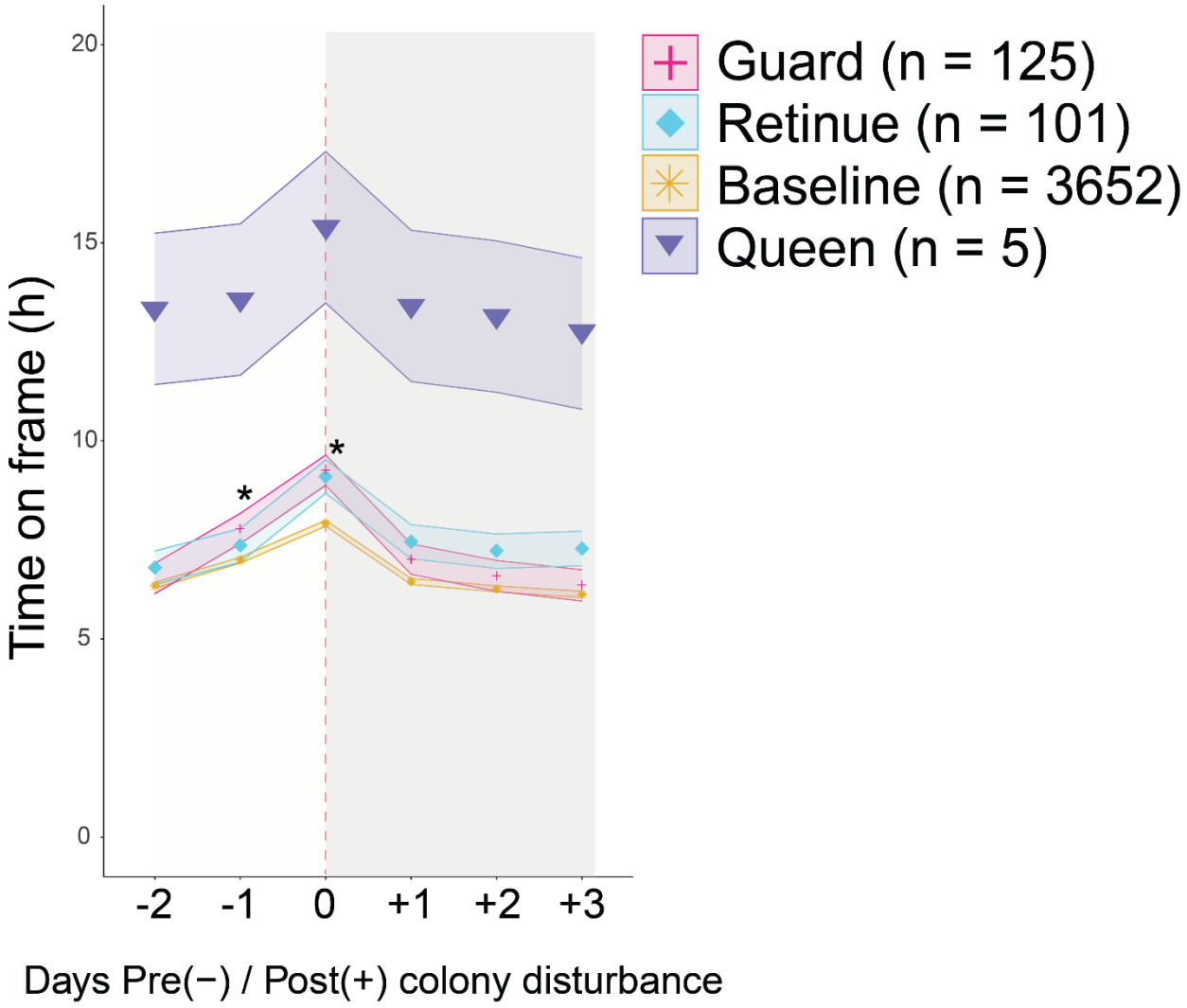

Fig. S4. Automated behavioral monitoring reveals that colony disturbance only minimally influences time on frame (TOF) for each behavioral group. We found that colony disturbance did not affect how much time guards spend on the frame relative to baseline, and significant variation (*$P < 0.01$) could only be detected on Days -1 and Day 0, the latter being the day of the disturbance. We include queen TOF here as a positive control: queens rarely leave the hive and therefore spend significantly more time on the frame than any other group ($P < 0.0001$), but this value is well below 24 h, as egg-laying completely obscures the tracking barcode and therefore reduces TOF estimates. Statistical analyses, X-axis annotation, and figure convention follow descriptions in Figs. 5-6.

Table S1. Primer sets used for target genes. We designed primers to target genes associated with the mushroom body response to aggression or affiliation, following transcriptomic results from lab- and field-based studies (Rittschof, 2017; Rittschof and Robinson, 2013; Shpigler et al., 2017a; Shpigler et al., 2018; Traniello et al., 2019).

Gene	HAv3.1 ID	Strand	Primer Sequence
hr38	551232	Sense	5'-GAGACTTACACGGCTCAACG-3'
		Antisense	3'-CCCTCGTCCATTTTGATGCC-5'
egr1	726302	Sense	5'-GCAAACGGTGCAGCTCAGT-3'
		Antisense	3'-CCGCATACGATCGAATTCG-5'
obp14	677673	Sense	5'-ACCACAAGGAATCAAAGCAGT-3'
		Antisense	3'-ATGGTTGAACAATCGGAGACT-5'
CREB	409401	Sense	5'-CTGTTGACCCATTGTCTG-3'
		Antisense	3'-GAGTTTGCTGCTGTGTTC-5'