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MASTER'S THESIS

Idiosyncratic social behavior within bumble bee (*Bombus impatiens*) colonies impacts reproductive potential of workers

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Declaration of Authorship

I, Claire GUERIN, declare that this thesis titled, "Idiosyncratic social behavior within bumble bee (*Bombus impatiens*) colonies impacts reproductive potential of workers" and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree in this Master's program.
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- Where I have consulted the published work of others, this is always clearly attributed.
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- I have acknowledged all main sources of help.
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Date: August 14, 2017

University of Groningen

Abstract

Faculty of Science and Engineering
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Erasmus Mundus Master Programme in Evolutionary Biology

Idiosyncratic social behavior within bumble bee (*Bombus impatiens*) colonies impacts reproductive potential of workers

by Claire GUERIN

Primitively eusocial bumble bee colonies enter a competition phase towards the end of the colony cycle, during which a few workers lay eggs at the expense of the foundress queen. Consistent variability in behaviors among workers, called behavioral idiosyncrasies, have been recently shown to occur in bumble bee colonies between closely related sisters. However, it is still poorly understood whether and to which extent social behavior plays a role in worker ovarian development. Using automated tracking, I assessed individual social interactions in three whole colonies of *Bombus impatiens*, and dissected workers in order to measure their ovarian development. I found that social interaction level negatively correlates with ovary development of an individual. I conclude that social environment is an important factor to the onset of the competition phase in primitively eusocial bumble bees, and thus can help determine reproductive workers who will compete against the queen for males production.

Contents

Chapter 1

Introduction

Social insects provide a very compelling framework for studying the emergence of distinct behaviors within groups of individuals that share both a genetically and environmentally similar background. Eusocial insects in particular are well-studied social groups, or colonies, within which reproduction is divided between a single (sometimes several) founding queen on one hand and sterile workers — who in most cases are the queen's daughters — on the other hand. Honey bees (genus *Apis*) are often referred to as the paramount, most complex eusocial species. In honey bee hives, individuality arises among workers as the worker force is subject to division of labor, also called task allocation. Individuals devote to specific tasks within the colony, typically nest defense, brood care, foraging or undertaking (in other words, the removal of dead bodies from the nest).

Bumble bees (genus *Bombus*) are a phylogenetically close taxon of honey bees, eusocial too. However, unlike its sister group, *Bombus* retained ancestral features of eusociality (Michener, 1974; Kapheim et al., 2015). Indeed, bumble bees cooperatively maintain and defend their nest, but are reproductive and behavioral generalists. Although bumble bee queens undertake most of the reproductive endeavor, workers retain the ability to reproduce (Sladen, 1989). A bumble bee colony life follows an annual cycle that can be broken down into several phases (Amsalem, Orlova, and Grozinger, 2015). Once a young queen has mated, she enters diapause. This process allows her to overcome winter, but is also considered to hold a role in activating the queen's reproductive function. At the end of winter, diapause terminates and the queen enters the solitary phase, during which she starts a nest on her own. She produces diploid, female workers, marking the transition to a cooperative phase. Once enough workers support nest maintenance, the queen starts to conceive gynes and drones (female and haploid male reproductives, respectively). Up to this stage, workers typically do not reproduce. Van Oystaeyen et al. (2014) found a conserved class of queen pheromone that represses ovary activation in insect workers, and was later argued to diffuse an honest

signal for fertility rather than chemically sterilize workers (Oi et al., 2015). However, when targeted on *B. impatiens*, this class of pheromones shows contradicting results across studies (Holman, 2014; Amsalem, Orlova, and Grozinger, 2015). It has been further suggested that worker reproduction was determined behaviorally by the simple perception of, or through physical intimidation from the queen (Alaux, Jaisson, and Hefetz, 2004).

In spite of the queen's presence, towards the end of a colony life cycle, some workers develop ovaries and engage into reproduction. A study on *B. terrestris* revealed that 63.8% of workers develop ovaries and 38.4% actually lay unfertilized eggs (Alaux et al., 2004). Even though *B. impatiens* show patterns of worker ovarian development similar to those of other bumble bees such as *B. terrestris*, the proportion of reproductive workers in *B. impatiens* colonies is smaller. Cnaani, Schmid-Hempel, and Schmidt (2002) evaluated it at 9% . The onset of worker reproduction marks the competition phase of the colony, where workers overtly compete against their queen for males production. While the queen is suspected to dump eggs sired by the so-called anarchist workers (O'Connor, Park, and Goulson, 2013), the latter reply with egg cannibalism and can even go as far as killing the queen herself (Trivers and Hare, 1976; Bourke, 1994). The causes and mechanisms for the onset of worker ovary development in bumble bee colonies remain unclear so far. Theory predicts that competition between queen and workers arises from competition at the genetic level, as closely related workers value nephews more than brothers (Hamilton, 1964; Loope, 2015). Moreover, there may be a predisposition for reproduction due to the ability to produce the juvenile hormone (JH) at the larval stage. Similarly, JH and ecdysteroid levels in the adult influence worker reproduction potential, with an interacting effect of social context (Cnaani et al., 1997; Bloch et al., 2000; Geva, Hartfelder, and Bloch, 2005). Several clues seem to suggest that different environmental factors are responsible for ovarian development in workers, with a higher effect than the genetic component. Mechanisms go from a decrease in the production of repressing pheromone by the queen as she ages, or a change in nest wax composition synchronized with the increased population density within the nest. This may be used by workers as a cue to indicate that there is enough workforce to support additional brood (Rottler-Hoermann, Schulz, and Ayasse, 2016).

Bumble bee queens are mostly singly mated (Estoup et al., 1995), resulting in colonies composed of highly related sisters. Unlike highly eusocial hymenopterans such as honey bees, task allocation among workers is plastic, and not clearly predicted by neither morphology nor age (Harrison, Hammond, and Mallon, 2015). However, weak task allocation arises in bumble bee colonies (Jandt and Dornhaus, 2014). Jandt and Dornhaus (2009) and

Crall et al. (2017) describe behavioral idiosyncrasy in *B. impatiens* colonies, with individuals showing consistent differences in behavioral characteristics such as preeminent commitment to certain tasks, speed and distance covered during nest maintenance, spatial distribution and centrality to the nest, etc. Variation among workers within the same colony arises from the joint action of worker interactions with both their environment and each other (Gordon, 2015). Social behavior in eusocial insects in general (Kühbandner, Modlmeier, and Foitzik, 2014) and in bumble bees in particular is linked with ovarian development on many levels. It is hypothesized that competition occurs not only between queen and workers, but also within the worker caste itself. In ants for instance, worker competition should discriminate reproductive workers from the others (Bourke, 1988b; Heinze, 2008). It is conveyed through social interactions, and notably aggression. Reproductive hierarchy among bumble bee workers is highly correlated with both chemical and behavioral patterns. Aggressive behavior (Amsalem et al., 2010; Amsalem and Hefetz, 2011) increases with ovarian development, although this link is not as clear in *B. impatiens* (Sibbald and Plowright, 2013). Non reproductive workers display chemical sterility signals to weave aggressive pressure from dominant workers (Amsalem et al., 2013; Amsalem et al., 2014; Shpigler et al., 2014).

The present work aims to explore the social behavior of the common eastern bumble bee *Bombus impatiens*, and link behavioral idiosyncrasies to differential ovarian development in workers of queenright colonies. I hypothesize that the global social environment experienced by an individual worker in the colony should be related to reproductive potential. I aim to understand whether an individual worker's level of social activity, as measured by both positive and negative interactions with peers, rather than aggressive behavior only, may have a role in enabling the onset of reproductive workers in a colony under the foundress queen's regency. I recorded activity in bumble bee colonies kept in the laboratory, and automatically estimated the level of social interactions for each tracked individuals. I show that the global level of social interaction experienced by bumble bee individuals negatively correlates with worker ovarian development.

Chapter 2

Material and Methods

2.1 Tracking individuals in the hive

In order to test the link between social environment and ovarian development in bumble bee workers, I record life within three colonies of *Bombus impatiens* in the lab. I designate the three colonies by the names A, B and C in this report. Each bee within the colonies is tagged with a unique number that allows automatic tracking of individual activity. Once behavior is recorded, bees are dissected to assess ovarian development.

2.1.1 Tracking arena design

We designed experimental arenas and other acrylic-based tools used in the lab (anesthesia box, CO_2 pad, etc.) with Autodesk Inventor CAD or Illustrator softwares, and laser-cut them at the University's workshop. For simplified drawings of the experimental arenas, see fig. 2.1 and 2.6, and see Supplementary Materials A to access design files. The experimental tracking arena is entirely made of laser-cut acrylic of $^1/_4$" (6.35 mm) thickness. The whole arena surface is of white color, except for the cover and one side-wall of the arena that are kept transparent for light to go through as well as for the experimenter to be able to see the hive when handling the arena. Four ventilation holes covered with mesh are included on the higher parts of the side walls to prevent condensation. The arena is constituted of two separated chambers: the nest chamber (NC) and the foraging chamber (FC). The structure on which the colony's nest is built is inserted and held up in the nest chamber from below. The foraging chamber simulates the outside environment for foraging workers. It provides access to both a carbohydrate source —- nectar -— and a protein source for the build-up of the colony —- pollen. Nutrient sources are accessible to the bumble bees through holes covered with mesh in the side wall and the arena floor respectively. A pollen feeder is attached onto the side wall, while a nectar feeder is placed below the arena. The nectar feeder

holds a cotton swab impregnated with nectar, accessible to bees through the mesh. Nectar is taken from the Biogluc bottle supplied by the crop management company, and pollen (Y.S. Eco Bee Farm©, kept in refrigerator) is regularly ground fresh. Workers can move freely between the nest and the foraging chamber by means of a plastic tunnel on the side of the arena. The tunnel width excludes the queen from the foraging chamber.

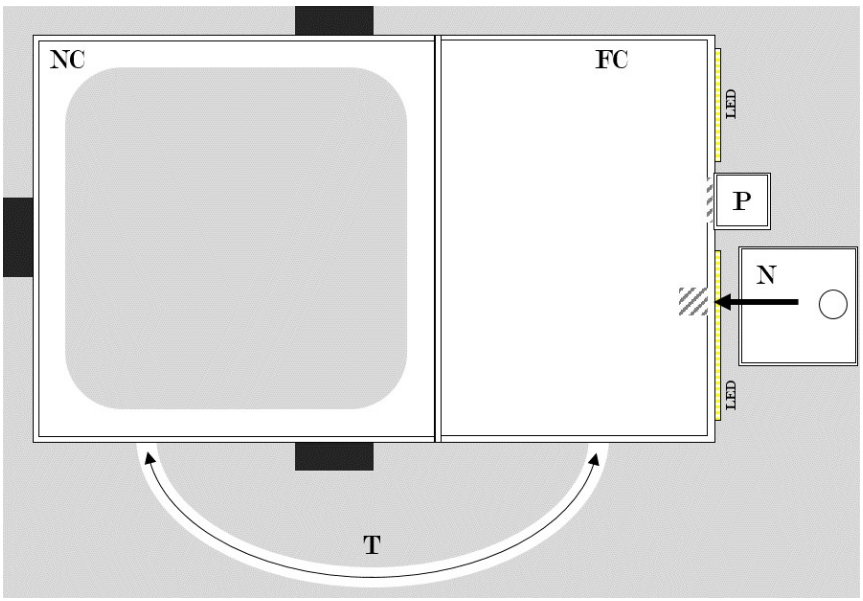

FIGURE 2.1: **Schematic of the experimental arena used for the reproductive potential experiment.** The arena is elevated and held in place in an enclosed rig (see section 2.1.4) with stoppers (in black). **NC** is the **N**est **C**hamber, with an open floor that allows us to insert from below the structure on which the hive built its nest (grey area). **FC** is the **F**oraging **C**hamber, to which a **P**ollen feeder (annotated P on the figure) is abutted. Pollen simply falls into the FC through the mesh. The **N**ectar feeder (designated as **N** on the figure) slides underneath the FC. Nectar goes by capillarity through a cotton swab up to the mesh on the FC floor. Bumble bee workers can commute between the two chambers through a **T**unnel (**T**), but due to her greater body size, the queen cannot pass the tunnel and is thus restricted to the NC. LED strips are attached to the transparent side of the FC.

2.1.2 Hive transfer into the arena

Three colonies of *B. impatiens* were grown and shipped from Canada by the crop management company Biobest. Hives are transported and stored in boxes equipped with a ventilation system, a 1.5L bottle of ready-to-use sugar solution Biogluc, and see-through Plexiglas on top. Upon arrival, the Biobest box that contains the hive is placed into a transparent

acrylic air-tight container, which is filled with carbon dioxide (20 LPM). The disturbance generated to the colony by moving the box and flowing CO_2 causes bees in the box to aggressively fly up and hiss as a defense signal (Kirchner and Röschard, 1999). CO_2 narcosis is considered sufficient when no more individual is showing such aggressive behavior. This usually lasts for 5 to 10 minutes, depending on how sensitive to CO_2 bees in the hive are. Once the bees are calmed down, they are taken out of their box with an insect vacuum. The aspirator's nose holds a removable collecting chamber that can host up to about 15 bees at once. It has an aluminum mesh at one end and a flap valve at the other, so that bees enter the chamber but cannot escape, once the end cap closes it. When a collecting tube is filled with bees, it is removed and placed in the refrigerator. Cold reduces stress experienced by the insects during the transfer process. Indeed, it slows down the metabolism of captured bumble bees, meanwhile the rest of the hive is being removed from the Biobest box. The whole colony (workers and queen) is then transferred into the experimental arena with the nest kept intact. The colony is left undisturbed for two to four days in order to allow bees to accommodate to their new environment and resume foraging activity.

2.1.3 BEETag

Once the colony is familiarized with the experimental arena, each individual bee in the hive is attributed a unique identification number, corresponding to a QR-like tag taken from Crall et al. (2015b) (fig. 2.2A). CO_2 narcosis is used as described in section 2.1.2 to collect the bees from the experimental arena. Individuals are then placed on a pad, in an enclosed transparent box specifically designed for tagging (find link to designs in Supplementary Materials A). The pad diffuses CO_2 at about 5 LPM. 0.24×0.24 cm (0.11×0.11") tags are printed on waterproof paper, and painted over with transparent nail polish. Bumble bee individuals are tagged one-by-one under CO_2 narcosis: tags are attached on the dorsal side of the thorax center, with gel-like super glue (fig. 2.2B). Bees who are not yet tagged or who have already been tagged are kept in the refrigerator. After tagging, bumble bees are transferred —- along with their already existing nest —- into the experimental arena. The colony rests for one day so that individuals recover from the procedure.

2.1.4 Image setup and data collection

The transportable arenas are loaded into an enclosed rig of black acrylic with frosted surface on the inside to reduce light-bouncing. The whole arena is lit up from above with infra-red light-emitting diodes (IR LEDs) attached to the ceiling of the rig. The foraging chamber only

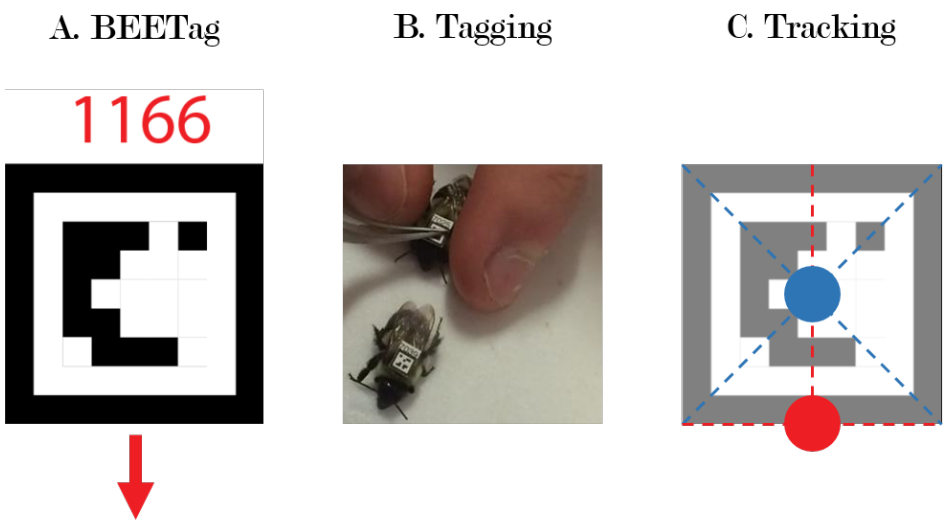

FIGURE 2.2: **Tracking with the BEETags. A.** Example of the QR-like tags used to identify unique identity numbers (here, 1166). Each tag is composed of a unique combination of black and white 5×5 pixels. The black border, when contrasted with white, allows MATLAB's BEETag software to locate potential tags before identification. Tags are oriented, with the red arrow indicating the front part. *Adapted from Crall et al. (2015b).* **B.** Photo taken during the tagging process. Bumble bees are placed on a pad that diffuses CO_2 as anesthetic, and tags are glued on the dorsal part of their thorax, between the wings. **C.** The BEEtag software allows identifies the tag. The coordinates (in pixels) of the centroid and the front part of the tag (spotted in blue and red respectively) can then be extracted.

is punctuated by a daily-like cycle of white light which simulates the outdoor environment (light turns on automatically from 6am to 6pm). White light comes from an LED strip fixed to the bottom part of the chamber wall (fig. 2.1). We assume that under poor lightning conditions, bees tend to crawl instead of fly, which prevents individuals to be out of focus. Like most insects, bumble bees have a poor vision for long wavelengths, starting with red light. Thus in the nest chamber, bees are shrouded in darkness, while only the lower part of the foraging chamber is lightened. Two Point Grey BlackFly high resolution cameras hang right above the nest chamber and foraging chambers. When recording, I open a lid on top of each chamber, so that cameras fit exactly in. This is to avoid light bouncing and image distortion that could occur while recording through Plexiglas. The cameras are of resolutions 2448×2048 and 1288×964 pixels respectively, in order to ensure readability of the tags. Filters for the appropriate wavelength are added to the two cameras. Movement in the tunnel between the foraging and the nest chambers is not recorded.

Both cameras are connected to the same computer, and videos are recorded for each colony over 3 consecutive days, for 1 hour between 3pm and 6pm. The order of the time slot attributed to the colonies each day is changed everyday, so that each colony is recorded once at each time (3pm-4pm; 4pm-5pm; 5pm-6pm) to account for variation in bee activity on a daily cycle. On the second day, one video did not record and thus was recorded again for the corresponding colony between 6pm and 7pm. Videos are acquired and processed with computer vision under MATLAB, with a frame rate of 5 Hz.

2.1.5 Automated individual tracking

Image data is processed by the software BEETag (Crall et al., 2015a) run under MATLAB. This software detects the position of bee tags by identifying their unique pattern. It is used on every recorded video, for each colony, to identify tags taken from the list of all tags used on the colony. It produces a structure array which contains the coordinates of each identified bee over every frame. Coordinates actually correspond to the centroid and the front of the tag, as shown in fig. 2.2C.

Tracking of the BEETag allows for accurate inference of the position, orientation, and social interactions of each individual through time.

Note that on every video recorded for the 3 colonies, a technical issue internal to the PointGrey recording system caused video frames to be altered. Corrupted videos are corrected as detailed in the Supplementary Material A.

2.1.6 Estimate social interactions

Social interactions between individuals are automatically inferred through algorithms written under MATLAB software R2016b. Fig. 2.3 is a comprehensive flowchart that summarizes the step-wise use of the scripts that process acquired videos and lead to an estimation of social interactions.

Once images are acquired at rate 5 Hz, the output video file (format `.avi`) is processed in MATLAB software R2016b. BEETag software detects tags faster and more efficiently when using a Bradley local image thresholding algorithm. For a brief description of the Bradley method, see Supplementary Materials A. The first step in analyzing the video thus consists in finding the optimal Bradley parameters for tag discovery. The Bradley adaptive thresholding algorithm relies on two main parameters: pixel brightness threshold and locally adaptive filter size. Once I obtain the values for these two parameters that enable to BEETag to efficiently detect a maximum of tags on one frame of the video, I can track individuals on every frame to extract individual bee's coordinates. The coordinates calculated for corrupted frames are corrected. Due to the orientation of the tag or movement of the bee, the track of a tag can be lost over a few frames, but shortly retrieved a few frames later. For short gaps in tracking data (< 2 seconds), coordinates of the tag position can be interpolated using the estimated trajectory of the tag between the last frame where the tag was identified before being lost, and the first frame where it is tracked again. I then set the scale for each video, by measuring the edge of a random tag on one frame of the video. The actual length for the tags' edge is fixed and is of 0.25 cm (0.11"). This is assuming that for a given video, focus and zoom remain stable throughout the recording time, and that the measured size of a randomly selected tag on the image is representative of the whole population. In reality, due to differences in heights between individuals, the measured tag size on an image varies slightly from one individual to the next. Body length and width of every tracked bee in each colony is measured on the computer and transformed to inches. Using body size and position data, I estimate social interactions between individuals in the hive.

Two methods for estimating interactions are designed and detailed hereafter as the "Ellipses" and the "Probabilistic Body Density" methods, EM and PBD. Body width and length of each individual in the three colonies is measured post-hoc on the videos, and converted into real size, using the known length of the edge of the tags (0.11", *i.e.* 0.25 cm).

Based on the tag coordinates (center and front), one can deduce a bee's orientation on every frame where it has been identified. Combined with the size of the bee measured as previously, this is used to calculate an ellipse that estimates the body of the bee (pseudo-code

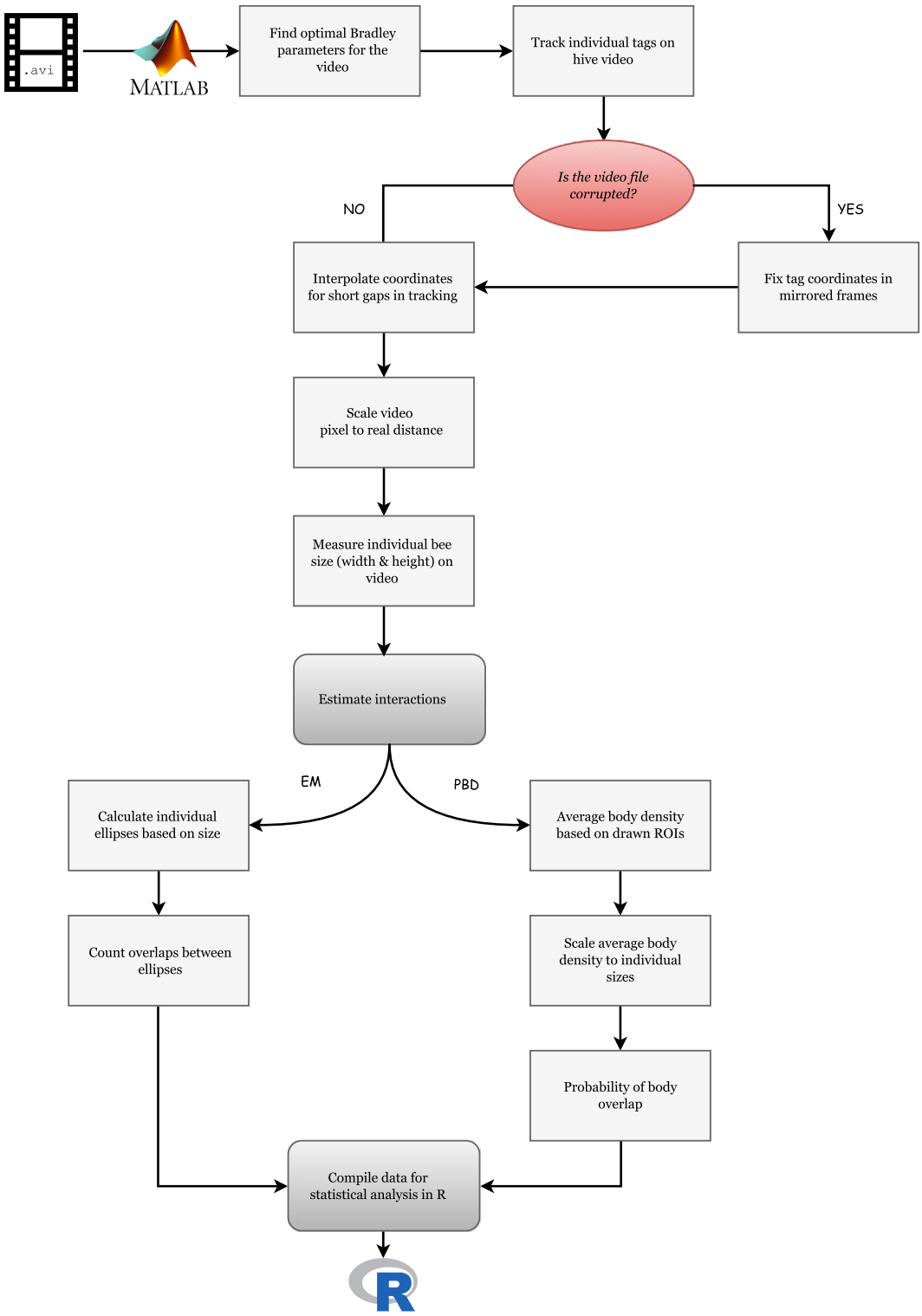

FIGURE 2.3: Flowchart for image processing and social interactions estimation.

1).

Algorithm 1 Estimate interactions between individuals with the ellipses method EM.

function ELLIPSES($tagCoordinates, bodySizes, expansionFactor$)
    $xCenter, yCenter, xFront, yFront \leftarrow$ ordered elements of the vector $tagCoordinates$ of length 4
    $bodyWidth, bodyLength \leftarrow$ first and second element of the vector $bodySizes$ of length 2
    $semiMinorAxis \leftarrow bodyWidth \times expansionFactor/2$
    $semiMajorAxis \leftarrow bodyLength \times expansionFactor/2$

    $orientationAngle \leftarrow \text{atan}_2(yFront - yCenter, xFront - xCenter)$
    $x0, y0 \leftarrow xCenter$(resp. $yCenter$) $- 1/2 \times a \times \cos(orientationAngle)$
    $t \leftarrow$ linearly spaced vector of n values from 0 to 2π
    $X, Y \leftarrow a$(resp. b) $\times \cos(t)$
    $x \leftarrow x0 + X \times \cos(orientationAngle) - Y \times \sin(orientationAngle)$
    $y \leftarrow y0 + X \times \sin(orientationAngle) + Y \times \cos(orientationAngle)$

    $regionMaskBees \leftarrow$ convert polygon (x, y) to region mask

    for $beePair \leftarrow 1$: all combinations of bees **do**
        $overlap \leftarrow$ does $regionMaskBees$ overlap between bees in $beePair$?

        if $overlap =$ TRUE **then**
            $interaction(beePair) \leftarrow 1$
        end if

    end for
return interaction

end function

Require: tagCoordinates, bodySizes

$expansionFactor \leftarrow 1.2$

for $frame \leftarrow 1$: number of frames **do**
ELLIPSES(tagCoordinates, bodySizes, expansionFactor)
end for

I increase the ellipse size by 20% from the measured width and length of the bee's body, in order to account for potential interaction between bees that do not physically touch each other but are extremely close to one another. One interaction is accounted for between two bees whenever on a frame, the ellipses calculated for each bee in the pair overlap. The number of interactions per bee is averaged over the total number of frames in each video (which amounts to one hour of video acquisition). This gives an estimate of the mean time each individual spends interacting with others.

After identifying limitations of the EM (see Chapter 4), we decided to re-analyze the data with a more complete method using density distribution (PBD). The rationale of this method is as follows: I calculate the mean bee size based on the body size data from the three colonies. The average bee body length and width are of 14.96 mm ($\pm$3.47) and 6.63 mm ($\pm$1.74) respectively. I then select five bees out of the three colonies, whose size is closest to the mean width and length, and use them as reference for estimating body density. Looping through every video in the experiment, I extract every frame on which these five bees appear (330 in total). Resizing, centering and cropping each of these frames around the reference individuals, I then pivot the image so that the body is oriented on a north-south axis, front facing north (fig. 2.4 A, B, C and D). This allows me to draw Regions Of Interest (ROIs) around the bodies, and calculate a mean bee body density. The mean body is centered and oriented as shown on fig. 2.4 E.

The method using body density to estimate social interactions is probabilistic. It can thus be mapped onto any individual bee, knowing its position and its orientation. Each pixel of the mean body density image is a value between 0 and 1. 0 corresponds to a pixel where the body never appears, and 1, where the body is always present. Thus, the further the pixel from the centroid of the body, the less likely it is to belong to the body of the bee.

I also use collected behavioral data to ensure behavioral consistency. Indeed, I want to confirm that individual social behavior as estimated with the ellipse and density methods is steady enough over time, so as to assess whether individuals display consistent, intrinsic behavioral idiosyncrasy rather than irregular behavioral variation.

2.2 Estimate worker reproductive potential

Reproductive value of worker bumble bees can be estimated by the state of development of their ovaries. Every female bumble bee individual, regardless of its reproductive status, possesses a pair of ovaries, stretching from the anterior to the posterior part of the abdomen, and with a more or less advanced development stage. Each ovary is constituted of four ovarioles, which amounts to eight ovaries per individual, in both queen and workers. Completely sterile individuals have thin, transparent string-like ovarioles, which can be seen as empty tubular structures. On the other end of the ovarian development spectrum, a fully developed ovariole bears oocytes, that will progressively mature up to the stage at which the egg is laid in a nest's wax cell. The most mature oocytes are thus located on the posterior end of the ovarioles, closest to the ovipositor. For a good illustration of developed bumble bee ovaries (species *B. terrestris*), see Figure 2 in Amsalem et al. (2015).

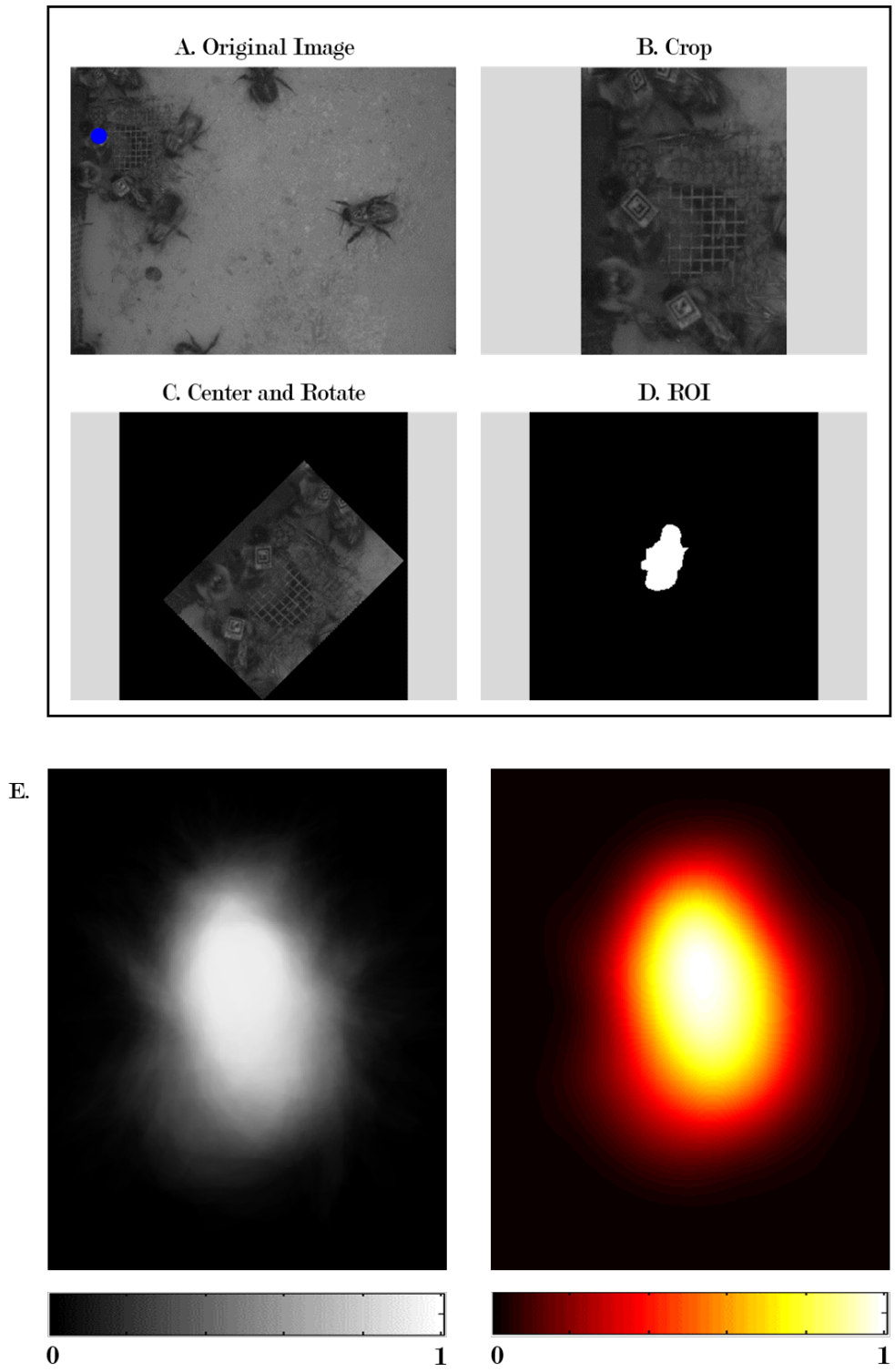

FIGURE 2.4: **Calculate mean body density**

Algorithm 2 Estimate interactions between individuals with body density method PBD.

function PROBABILITY($tagCoordinates, bodySize$)
    $xCenter, yCenter, xFront, yFront \leftarrow$ ordered elements of the vector $tagCoordinates$ of length 4
    $bodyWidth, bodyLength \leftarrow$ first and second element of the vector $bodySize$ of length 2
    $orientationAngle = \text{atan}_2(yFront - yCenter, xFront - xCenter)$
    $beesDistribution \leftarrow matrix$(frame height, frame width, number of bees)

    for $bee \leftarrow 1$: number of bees **do**
        $beeDensityProbability \leftarrow$ resize $scaledDensity$ according to $bodySize$ of bee
        $orientBeeDensity \leftarrow$ rotate $beeDensityProbability$ according to $orientationAngle$ of bee
        $beesDistribution(:, :, bee) \leftarrow$ place $orientBeeDensity$ on frame according to $xCenter(bee), yCenter(bee)$
    end for

    for $beePair \leftarrow 1$: all combinations of bees **do**
        $P(beePair) \leftarrow max(beesDistribution(:, :, beePair[1]) \times beesDistribution(:, :, beePair[2]))$
    end for
return P

end function

Require: meanDensity

for $frame \leftarrow 1$: number of frames **do**
PROBABILITY(tagCoordinates, bodySize)
end for

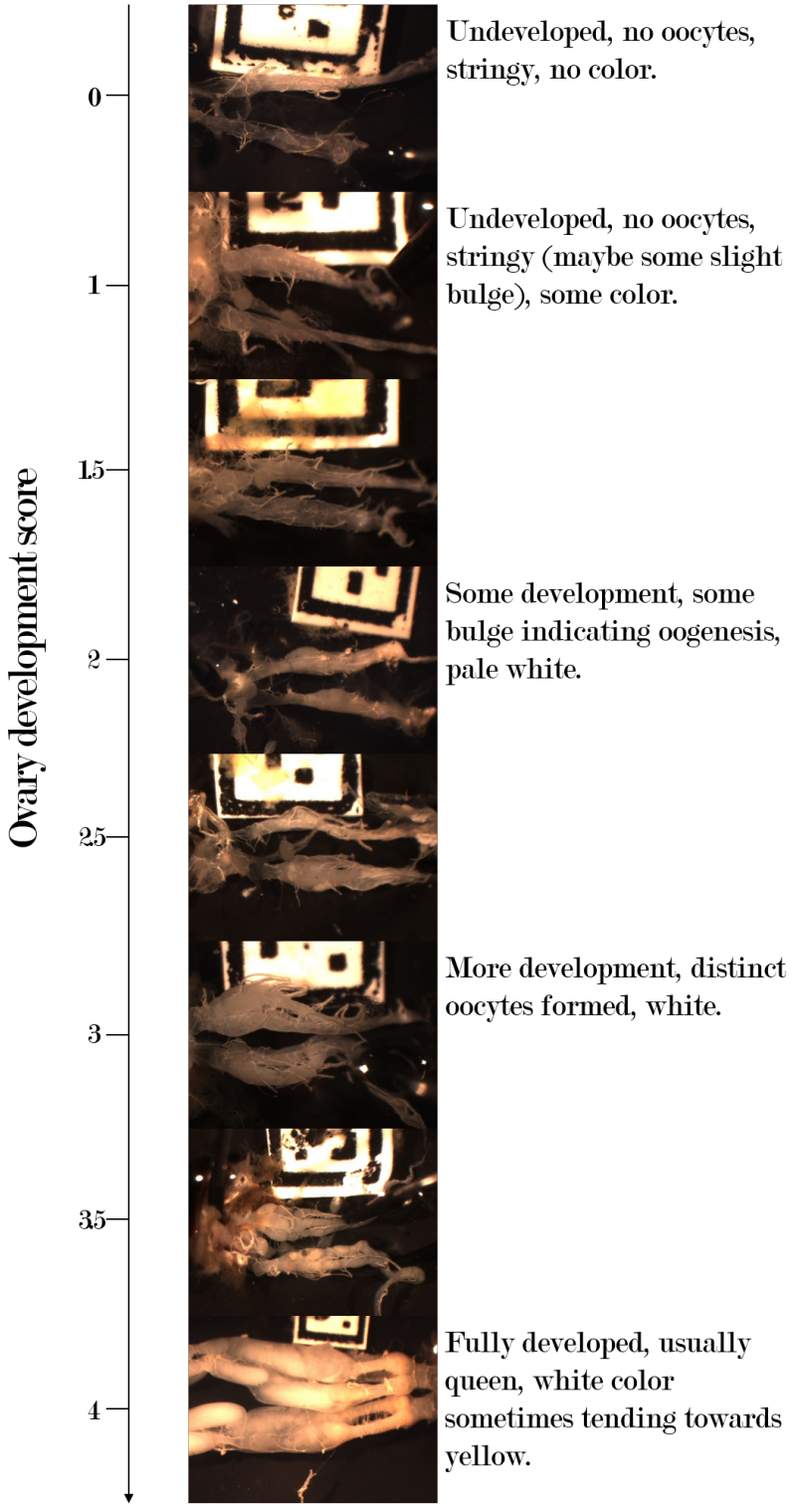

FIGURE 2.5: **Ovarian development score.** I score ovaries from 0 to 4 (top to bottom), according to the degree of ovarian development. When I was undecided between two scores, I attributed intermediate values (e.g. 3.5). Note that the score of 4 is specific to queen ovaries, which are very distinct from the worker's. Some workers may display similar characteristics in ovarian development in queenless (QL) colonies. The criteria for the scoring system were provided by Robert Oppenheimer.

Once activity within a colony was recorded, I anesthetized the whole hive one last time by CO_2 narcosis. I then froze and stored all individuals of the same colony in a plastic box, at $-70\,^{\circ}\mathrm{C}$. I weighed every individual in the colonies with a precision balance (10^{-4}g precision). I then randomly selected individuals whose tag was still attached on their back, out of the box for dissection. Dissection was performed under the microscope, in a small petri dish. I cut the bumble bee laterally with micro-dissecting scissors at the connection between thorax and abdomen. I put the abdomen in the petri dish and fully submerge it with deionized (DI) water. I then delicately remove both dorsal and ventral tergites one and two (sometimes three) with thin tweezers. This step, as every single next step, must be performed with high care, at the risk of tearing apart the fragile ovaries. From this point onward, the ovaries should become detectable on both sides of the gut. It is often required to carefully move the swollen air chambers on both sides of the abdomen in order for the ovaries to appear. Once I detect the ovaries, I remove every remaining cuticle segments and organs (mainly the gut, air chambers and nervous ganglia), so that the ovaries are cleanly separated and clearly visible for a picture to be taken with a camera attached on the microscope and connected to a computer. I assign an ovarian development score to the individual, and report her identification number (which can be read on the tag).

2.3 High-throughput multiple colonies' individuals tracking

Bearing in mind the time required to manually load, acquire data and unload single hives into the experimental rig as described in section 2.1.4, we aimed to increase the replicates size, *i.e.* the number of colonies in which we assess behavior. We therefore decided to automatize data acquisition and built a robot inspired from the "Santa Fe" robot, constructed earlier in the de Bivort lab (unpublished work). This FlySorter fly dispenser was built in collaboration by Benjamin de Bivort and Dave Zucker for handling large amounts of fruit flies automatically. Adapted to recording behavior in bumble bee colonies, it consists of a large rig that can host up to 12 arenas (12 colonies) at once (fig. 2.6 A). The rig is kept in darkness with an opaque cloak. The two cameras, along with IR-LEDs from the previous setting are attached to a platform. The platform can hover over each arena. Platform movement and camera and IR-light activation are automatically controlled from a computer.

The arena design was slightly adapted to fit the robot rig (fig. 2.6 B). The arena is now made up of thinner acrylic ($^1/_8$" thickness). The tunnel is removed and bumble bees can commute between the nest chamber and the foraging chamber through a hole in the wall between the two chambers. The floor of the foraging chamber is perforated and covered in

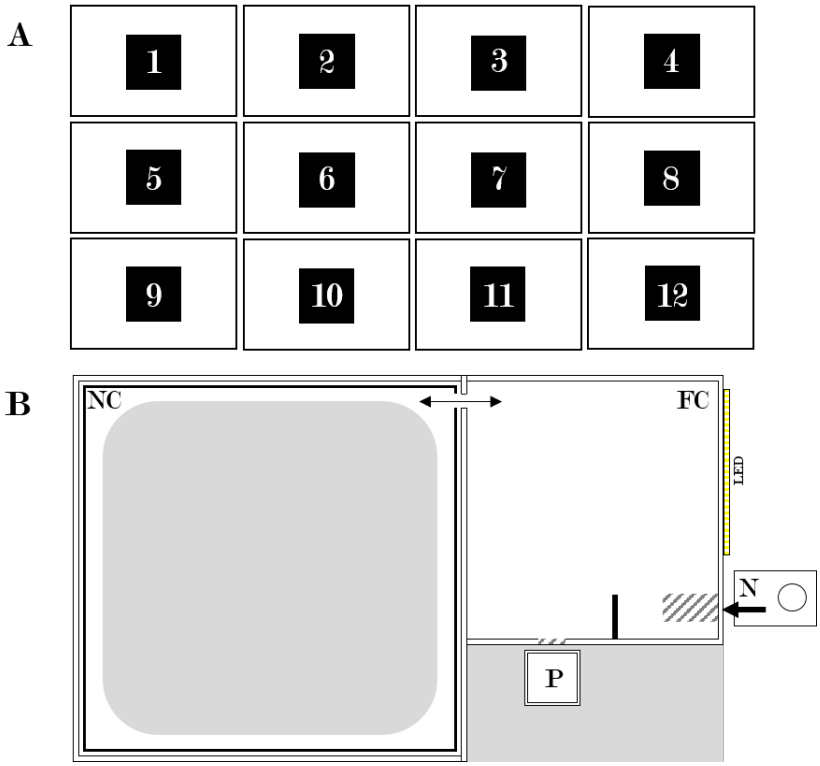

FIGURE 2.6: **High-throughput multiple colonies tracking. A.** Top-view map of the robot rig, with arena positions $1 \rightarrow 12$. The platform holding the two cameras and IR-LEDs hovers over desired positions for recording. **B.** Schematic of the experimental arena. The arena can be inserted into slots on the robot rig floor. **NC** is the **N**est **C**hamber, hosting the nest structure as described in fig. 2.1. Thin black acrylic sheets slide down inside the NC to ensure dim lightning conditions. **FC** is the **F**oraging **C**hamber, to which a **P**ollen feeder (annotated **P** on the figure) is abutted. The **N**ectar feeder (**N**) slides into a compartment below the arena. Bumble bee workers can commute between the two chambers through a hole in the wall separating the two chambers. The queen is precluded from the FC due to her significantly bigger size. An LED strip is attached to the transparent side of the FC.

mesh, so as to ease cleaning-up of the arena between housing of two consecutive colonies. We suspect ventilation may have been of poor quality in the previous design. Indeed, we noticed condensation within the arena, which may have induced developmental issues for some individuals (undeveloped wings). We therefore replace the 4 ventilation holes from the previous design by multiple horizontal lines of slots running the width of every wall. To compensate for the extra amount of light coming into the nest chamber and bouncing-off white walls, we added supplementary sheets of acrylic ($^1/_{16}$" thickness) inside the chamber, which are IR-transparent (but opaque to white light).

I endeavor to explore the link between behavioral idiosyncrasy and success at the colony level. For this experiment, I use eight colonies. Hive transfer, tagging and behavior recording is executed in parallel for pairs of colonies. The experimental design consists of recording individual behavior within the colony for 3 consecutive days, using the BEETag tracking system (section 2.1.3). Social interactions are estimated with the ellipses method as described in section 2.1.6. Individuals within each colony are ranked according to their overall level of social interaction. Each colony population is split into two groups: the most socially active on one hand, and the least socially active on the other. I then proceed to a "swapping experiment". I create artificial colonies by grouping together socially active individuals from each pair of one colony, and gathering bumble bees with a lower rank (from the same pair of colonies), in another group. Colonies are all of similar size. Before performing this experiment, I conducted a preliminary swapping experiment between individuals randomly selected in two colonies of *B. impatiens*, and observed them over several consecutive days. No aggressive behavior from the host colony was displayed towards migrated individuals, in neither of the two colonies. Due to technical issues discussed in chapter 3, only four out of eight colonies survived, meaning that in fact, I only swapped individuals between two pairs of colonies.

Chapter 3

Results

3.1 Tracking individuals and quantifying their behavior

We tracked activity of 3 bumble bee colonies (species *Bombus impatiens*) in a confined environment. The colonies — A, B and C — were composed of 84, 84 and 95 individuals respectively. The queen of the hive lost her tag in both colony B and C. Only the queen in colony A was successfully tracked throughout the experiment. The output data of estimations of social interactions with the PBD method were corrupted. The cause for data corruption needs to be identified, and the tracking data must be re-analyzed. The analysis pipeline for a single video file is correct, as accurate data could be extracted from one video. Data correctness for both methods is checked visually by plotting the calculated ellipses or body densities of individuals, over the video.

Using social interaction estimations from the EM method, I can calculate a mean level of interaction per day of recording, for each tracked individual. In order to check the stability of individual behavior over time, I performed pairwise Spearman correlation tests on mean interaction level of every tracked individual, between days. The results (correlation coefficient and p-value) are shown in table 3.1.

		Correlation coefficient r			
	Day	1	2	3	6
p-value	1	1.00	0.44	0.25	0.42
	2	<0.001	1.00	0.21	0.24
	3	0.060	0.111	1.00	0.32
	6	0.001	0.075	0.016	1.00

TABLE 3.1: **Spearman correlation statistics.** The correlation coefficient r of individual behaviors between days is indicated in the upper panel of the table, and the p-value statistic, in the lower-panel.

3.2 Ovarian development

Upon dissection of the first colony in the experiment (colony A), 47.8% of the randomly chosen bumble bee specimens were in a degradation state that did not allow the assessment of ovarian development. Their abdominal organs were either moldered, or completely dried and shrunk, or organs were not present in the abdomen. This is probably due to the fact that those bees died naturally during the experiment, shortly before flash-freezing. As a consequence, the decomposition process of the internal organs had already begun. Individuals that died a few days before freezing could be singled-out and discarded before dissection, as their body was more dry, hard and brittle. However, individuals who died shortly before the freezing process were hardly distinguishable from specimens suitable for dissection. As this could not be determined externally and impacted the efficiency of the dissection, I listed individuals that were moving on frames of the very last recording of the colony. I only selected these specimen for dissection, in order to reduce the probability that the individual died before it was frozen. This significantly reduced the proportion of rotten bees discovered during the dissection process (20% and 30.3% in colonies B and C respectively). Twenty individuals were successfully dissected and assigned a score in each colony, for a total of 60 ovary scores obtained. Most workers had a score of 0 or 1. The maximum score of ovarian development, 4, was only attributed to queens.

The proportion of individuals from the scored population decreases as the ovarian development scores increase in both colony A and C (fig. 3.1). However, ovary scores are more evenly distributed in the dissected subset of colony B. Excluding queens, who are clear outliers in terms of weight and ovary score, I test the correlation between individual weight and score with a linear regression. There is a significant correlation between ovarian development score and weight of the individual (p-value < 0.01). Although significant, the linear model does not fit the data perfectly, with a low R^2 value of 15%.

3.3 Effect of social interactions on ovarian development

I want to test the link between the intensity of social interactions experienced by a bumble bee, and the individual's reproductive potential. To do so, I fit a regression model to the social interactions estimated with the EM. The count data obtained with the EM method do not follow a normal distribution. I use the methods of generalized linear models to test the hypothesis that social interactions impact reproductive potential. Some of the data of ovarian development scores was transformed before statistical analysis. In some occurrences during

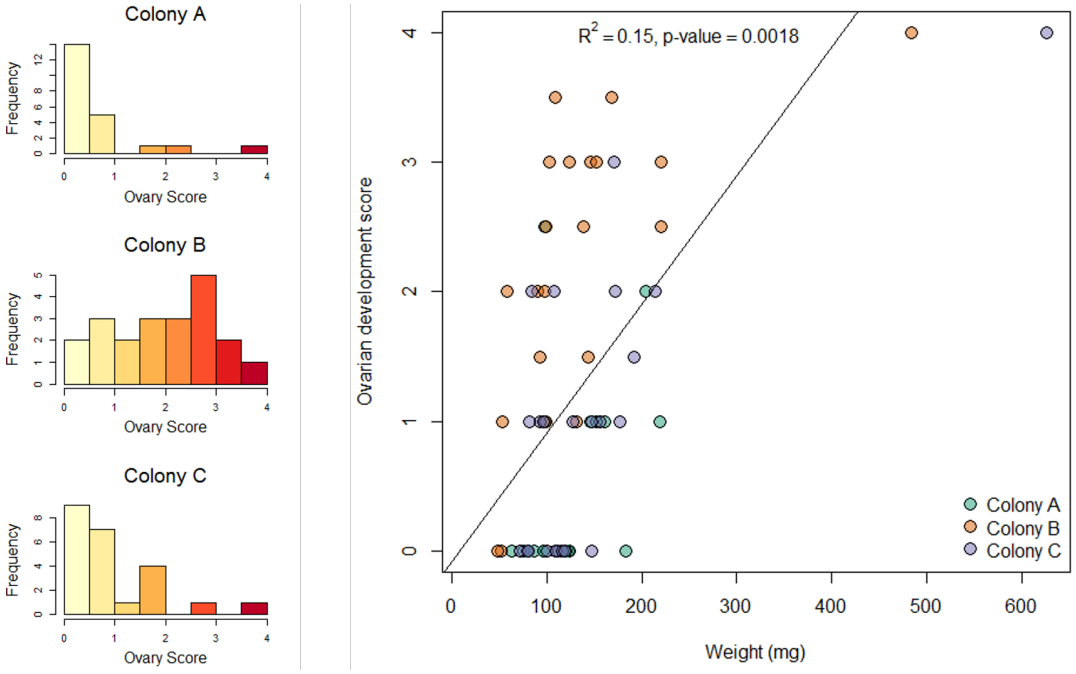

FIGURE 3.1: **Ovarian development distribution in the three colonies A, B and C.** There is a significant correlation between ovarian development and weight.

dissection, worker ovaries seemed developed to a stage close to a high scoring value of 2 or 3 (even 4 in one instance), but the score was not as clearly differentiable from the previous lower score as for other specimen of comparable ovarian development. Since I was not confident enough in scoring ovarian development, I chose to attribute an intermediate score (1.5, 2.5, 3.5). Such intermediate scores are sparse in the data (5%), which diminishes both the statistical significance and biological meaningfulness of the evaluated scores. Posterior to the dissections, I rounded-off intermediate scores by flooring them to the closest round value. The only exception is the case of the worker to which I initially attributed a round score of 4. After comparing the picture of this individual's ovaries, to the ones of the queen and another worker scored lower, I changed the score of 4 to 3 instead (Supplementary Material A). Over-dispersion of the interaction level data was too high for a Poisson model to fit the data (residual deviance of 2870847 on 478 degrees of freedom), and values of zeros for the estimation of interactions were highly overrepresented (74%), as can be observed on fig. 3.1. I thus use a hurdle model to test the hypothesis of a link between level of sociality and reproductive potential. This type of model corrects for excess of zeros as well as over-dispersion of the data. Hurdle models treat zero and non-zero response data separately, by fitting a zero-inflated model to data with a positive y-value, and treating zero values in

the response variable with a binomial model. I tested the fit of three closely related count distributions on non-zero values: Poisson, Negative-binomial and Geometric, respectively referred to as P, NB and G hereafter. All models were significant ($p < 0.001$). Ovarian score affects the level of social interaction. The higher the ovarian score, the lower the counts of social interaction is. I compared the goodness of fit of each model by means of the Akaike Information Criterion (AIC) and tested the significance of the models with the Likelihood-ratio test (LRT). Both tests confirm that the non-zero count data follows a NB distribution. The variance of every model was better than that of a simple Poisson model, but it remained relatively high, meaning that although the hurdle models better represent the variation of the data, a lot of variance in estimated individual social interaction remains unexplained by the model.

3.4 Colony swap

At the time of the swapping experiment, there was a blizzard event in the region of New England. Transportation in the region was affected. The arrival of ordered material necessary to build arenas suitable to recording multiple colonies with the robot was therefore delayed. As a consequence, the tracking setup was ready for data acquisition later than originally intended. The eight colonies dedicated to the swapping experiment were kept in the lab for several weeks before I could start recording their behavior. The rate of mortality was extremely high ($> 50\%$), after the bees in each eight colonies were anesthetized and tagged.

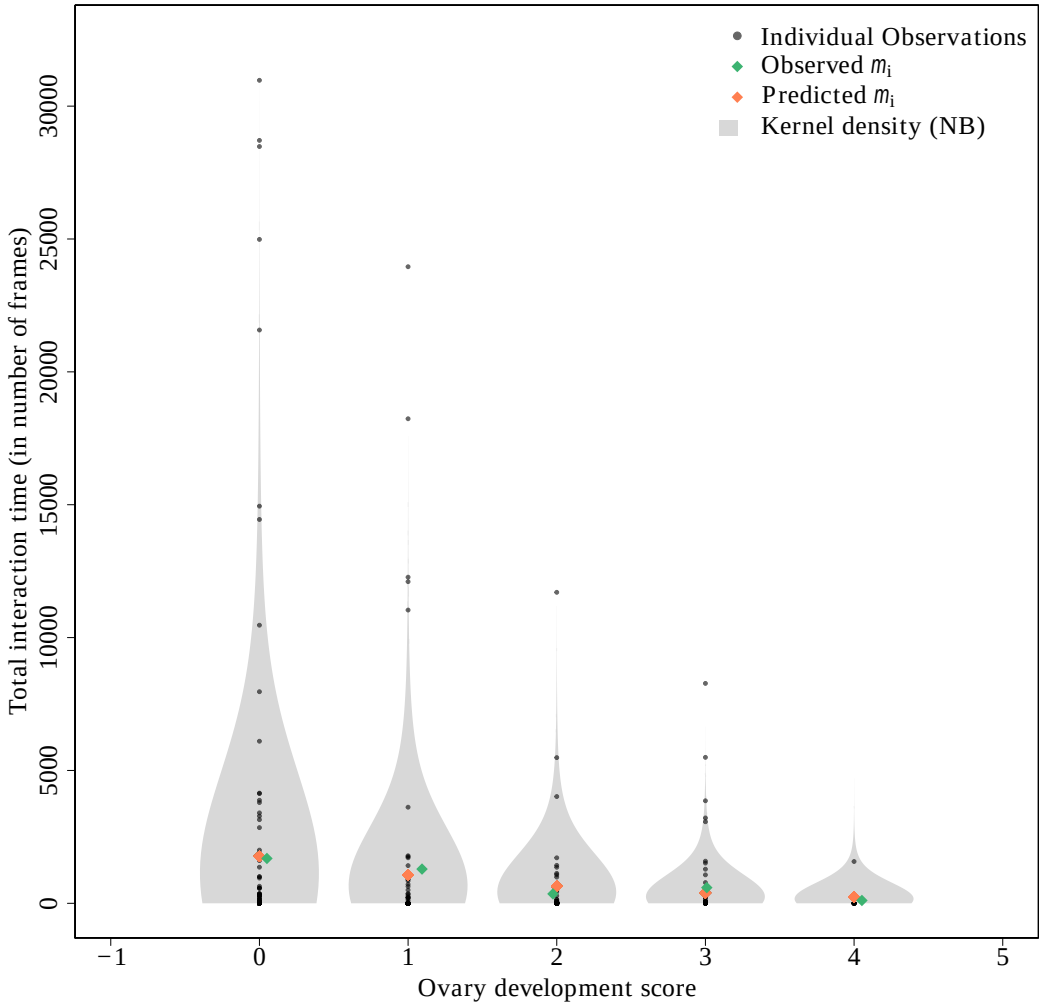

FIGURE 3.2: **Ovarian development and level of social interactions.** Level of social interaction as a function of reproductive potential. Interaction levels follow an over-dispersed, zero-inflated negative binomial distribution. Variance in social interaction counts decreases as ovarian development score increases. A hurdle model predicts the mean interaction level with ovarian development score ($p < 0.001$). Density probability of the response variable is estimated with non-parametric kernel density (Parzen-Rosenblatt) method.

Chapter 4

Discussion

4.1 Anesthesia

We anesthetize bees at several stages of the experiment: transfer to the experimental arena and tagging. There are three main ways to anesthetize insects, which are narcosis by cold, nitrogen N_2, or carbon dioxide CO_2. We opted for the latter as a main way of anesthesia for reasons of practicality and efficiency when handling bees. We also used cold as a way to keep bees' metabolism down when individuals in the colony needed to be handled in batches. Individuals that were not being handled were kept in the fridge. After several anesthetizations, bees habituated to CO_2. Time necessary for narcosis to take effect, so that all individuals in the hive were calm enough to be handled, doubled after a few usages of CO_2 on the colony. Literature abounds in examples of negative impacts of both narcosis methods — cold, N_2 and CO_2 — on insects in general. A detailed review by Nicolas and Sillans (1989) highlights the wide range of impacts on insects' behavior, biology, physiology, and metabolism. Effects are both long and short term. One main drawback of experimentally induced high rates of CO_2 is the resulting high mortality, as detected in various insect taxa. Indeed, in my first trials of narcosis by CO_2 for tagging the bees, I noticed a high rate of mortality the day after anesthesia was performed. I therefore reduced the time of exposure to CO_2 for the experiment. However, a few dead individuals were always found in the colonies after handling. It is difficult to disentangle the possible roles of the use of CO_2 narcosis on one hand, and the process of gluing tags on the bumble bees' back on the other, in observed mortality rates within the colonies. The age of the colony appears to be also critical for the impact range of CO_2 exposure. The second part of my experiment, the colony swap, intended to uncover the effect of personality composition on the colony's fitness. Due to technical complications, the arrival of material necessary to begin the swapping experiment was delayed. As a consequence, the eight colonies of the experiment had been kept in the lab for several weeks

longer than originally intended, before the experiment could be resumed. More than half of the workers in the hives died after anesthetization and tagging. I suspect that there is an interacting effect of advanced age of the colony and exposure to carbon dioxide. CO_2 narcosis has also been shown to impact behavior. For example in *Apis mellifera*, a single exposure can cause workers to shift tasks within the nest, change individual preferences, etc (see for instance Ribbands (1950) and Mardan and Rinderer (1980)). In the European bumble bee species *Bombus terrestris*, both cold and CO_2 narcosis affected activity, foraging, brood care, nest mate aggression and productivity (Poissonnier, Jackson, and Tanner, 2015). One can therefore suspect that social behavior in *B. impatiens* may have been affected too during the experiments. Although we allowed a few days to the colonies to recover from anesthesia and the process of tagging, the effects of CO_2 on *B. terrestris* behavior are shown to be long lasting. Moreover, in the American bumble bee species *B. occidentalis*, workers exposed to CO_2 have been observed to display larval ejection behavior, which translates into the disposal of larvae out of the nest. Such behavior was also observed in our experiments. The day after tagging, larvae of some colonies were found to have been removed from their cell, and displaced in the foraging chamber. Any form of narcosis is a necessary constraint for tracking individuals with BEETags. The possible effects on behavior are to be kept in mind when interpreting the results presented in this report.

4.2 Ovarian development

In our experiment, ovarian development is assessed in twenty individuals randomly selected in each of the three colonies. Scores attributed to rate reproductive potential is based on the external aspect of the ovaries. It relies on qualitative characteristics such as shape or color, which are judged by the experimenter. Attributed scores are therefore partly subjective and may vary from one experimenter to the other. This second opinion could be done *a posteriori*, based on the ovary pictures. We expected most of the workers to have lower levels of ovarian development, and thus to obtain a majority of scores around 0 or 1 during dissection. This is the case for colonies A and C. However, in colony B, scores are less skewed toward low scores, with a higher proportion of workers displaying advanced ovarian development. To ensure that this unexpected distribution was not due to an overestimation of ovarian development during the dissection of this particular colony, I re-assessed ovarian development of the 20 specimens in colony B a few months after dissection, based on the pictures of the ovaries. This only confirmed that among the randomly selected specimens of colony B, there is a higher proportion of reproductive workers than was expected. A

possible explanation for this result is that colony B was at a more advanced stage of colony life. If this were the case, competition for males production was then already at its peak, with more reproductively competent workers than in the other two colonies, which may have been at an early stage of their life cycles (Amsalem et al., 2015). This scenario seems unlikely, however. Although we did not have information on the age of the colonies that were delivered by Biobest company, these were ordered at the same time, and I am confident that they were of similar age. This certitude is not enough to discard completely the possibility for colony B to have already entered competition phase before the other colonies. The mechanisms that trigger competition phase in bumble bee colonies are not very well understood. Rottler-Hoermann, Schulz, and Ayasse (2016) suggested that changes in nest wax scent would be used by workers as a cue for entering a reproductive phase. Indeed, nest wax scent results from the combination of the cuticular compounds left by all individuals of the colony on the nest, either through footprints or shed scales. Rottler-Hoermann, Schulz, and Ayasse (2016) put forward the hypothesis that nest wax scent evolves as colony size increases, and more individuals participate to the composition of wax scent. It could therefore be used by workers as an indicator of colony size, and thus, the social composition of the hive. Cues indicating that the colony has grown to a size sufficient enough to sustain supplementary reproduction from workers (Amsalem and Hefetz, 2011) could be found in wax composition, and would be used for the onset of worker reproduction in *B. terrestris*. When our colonies arrived in the laboratory, they were all of similar size (about 60 individuals per colony), and grew at similar rates (about 80 individuals per colony at the time they were tagged). Therefore, it seems unlikely that colony B entered the competition phase before the other two colonies. Exposure to carbon dioxide also impacts bee reproduction. In honey bees, queens start oviposition earlier when exposed (Mackensen, 1947), while high levels of carbon dioxide significantly reduce ovary activation in workers of queen-less colonies (Koywiwattrakul et al., 2005). The time and frequency of CO_2 exposure necessary to anesthetize bees differed from one colony to the other, as some colonies were more resistant to carbon dioxide than others. This may have played a role in the observed differences in ovarian activation and development between colonies. Yet no trace was kept of the duration of exposure to CO_2 to reinforce this speculation, nor to measure to which extent CO_2 narcosis may have affected worker ovarian development. Workers' ovarian development score is significantly affected by weight. Size differences between workers are already determined during the larval development stage (Cnaani, Schmid-Hempel, and Schmidt, 2002). They become clear in contrasting molting weights of larvae as early as in the second instar (second development

stage of arthropod larva). However, our results show that weight only predicts 15% of the variability of ovarian development between workers. Although more workers in colony B with a high ovary score lead to a significantly higher mean score than in other colonies, the mean worker weight is not significantly higher in colony B (see Supplementary Material A detailed statistical analysis).

4.3 Social behavior

I used two methods to estimate levels of social interaction based on tracking data. The ellipses method EM simplifies the body of individual bees by fitting an ellipsoid shape, based on measured width and length of the bee body. The second method was designed later on in the project in order to correct some of the issues raised when estimating social interactions with the EM method. Indeed, this simplifying view of the bees does not account for the degree of proximity of two interacting bees. EM estimates interactions in a binary manner: either interaction occurs, or it doesn't. Moreover, with this preliminary method, overlapping ellipses are counted indifferently, regardless of the orientation of the bee. In the probabilistic body density method, PBD, we account for the degree of interaction based on proximity between the individuals. Moreover, we keep track of the bee's orientation during the interaction. However, this method generated mainly null probabilities of interaction or NAs. This may be due to the way probability of interaction is calculated in the PBD method. In this method, we map body density distributions of individuals tracked on a frame. Each pixel value corresponds to a probability for the pixel to belong to the body of each bee. Say there are two tracked individuals on the frame. We then calculate the probability of overlap between two body distributions, by multiplying the pixel values element-wise. This gives the probability of a pixel to belong to the body distribution of both individuals. The last step consists in estimating the total probability that the two bumble bee individuals interact. As the probability for each pixel to belong to both bodies is not independent from the probability of the neighboring pixels to also belong to the same bodies, summing every pixel's probability would overestimate the probability of social interaction between the two individuals. We therefore take the maximum pixel value as an estimate of social interaction probability. Unfortunately, this method seems to largely underestimate probability of interaction between individuals. This issue needs to be fixed before data calculated with the PBD method can be used reliably to estimate social behavior in bumble bee colonies. The results presented in this thesis therefore use estimations of social behavior of individuals in bumble bee colonies from the EM method only.

Task allocation is the most extensively studied type of behavior in eusocial Hymenoptera. Honey bees and ants are known to show clear task organization and specializations into working castes (non exhaustively examples in the literature: Wilson (1984), Gordon (2003), Gardner, Foster, and O'Donnell (2007), Duarte et al. (2011), Jandt et al. (2014), Pamminger et al. (2014), Gordon (2015), Jandt and Gordon (2016), and Walton and Toth (2016)). Ant (Seid and Traniello, 2006) and honey bee (Huang and Robinson, 1996) castes are determined by the age of the individual (temporal polyethism). Although task allocation is not as clearly predefined and determined in bumble bees, consistent individual differences can arise within the colony for different types of behaviors (Jandt and Dornhaus, 2009; Duong and Dornhaus, 2012).

I first tested the consistency of quantified behavior over days, in order to assess how coherent individuals are in their behavior. Individuals' behavior recorded within the hive does not significantly correlate between days 3 and 1, 3 and 2, and 2 and 6. However, individual social behavior is significantly correlated between all other days. Social behavior recorded for individual on the first day is correlated to the behavior of the same individuals, six days later. This gives us some confidence that social behavior displayed by individuals and measured with the EM is fairly consistent across days, and characteristic to each individual.

Results from the hurdle model show that there is a significant relationship between ovarian development score and level of social interaction. Ovarian development is used as a proxy for individual fitness, as it estimates bumble bees' reproductive potential. The outcome of the experiment therefore suggests that workers who interact more with other individuals in the colony have a lower individual fitness. As means of confirmation of the consistency of our results, I use the tools of graph theory in order compare weighted social networks. I build networks for each colony A, B and C, weighted with the EM method for social interaction estimation. I can compare them to the same networks, where the edges are weighted by the inverse of mean distance between tracked individuals (fig. 4.1).

This network comparison is performed on the data collected on the first day of experiment, in the nest chamber. Importance of each node in the network can be measured by centrality. I used both degree and closeness centrality. Degree centrality corresponds to the sum of the edge weights connecting to each node. Closeness centrality calculates the inverse sum of the distance from one node to all other nodes in the graph. Networks with edges weighted by the mean distance between individuals connects individuals that are actually far from each other in the nest, as long as they are tracked. EM estimates social interactions as physical interactions, or interactions at proximity ($+20\%$ of body size). Therefore, less

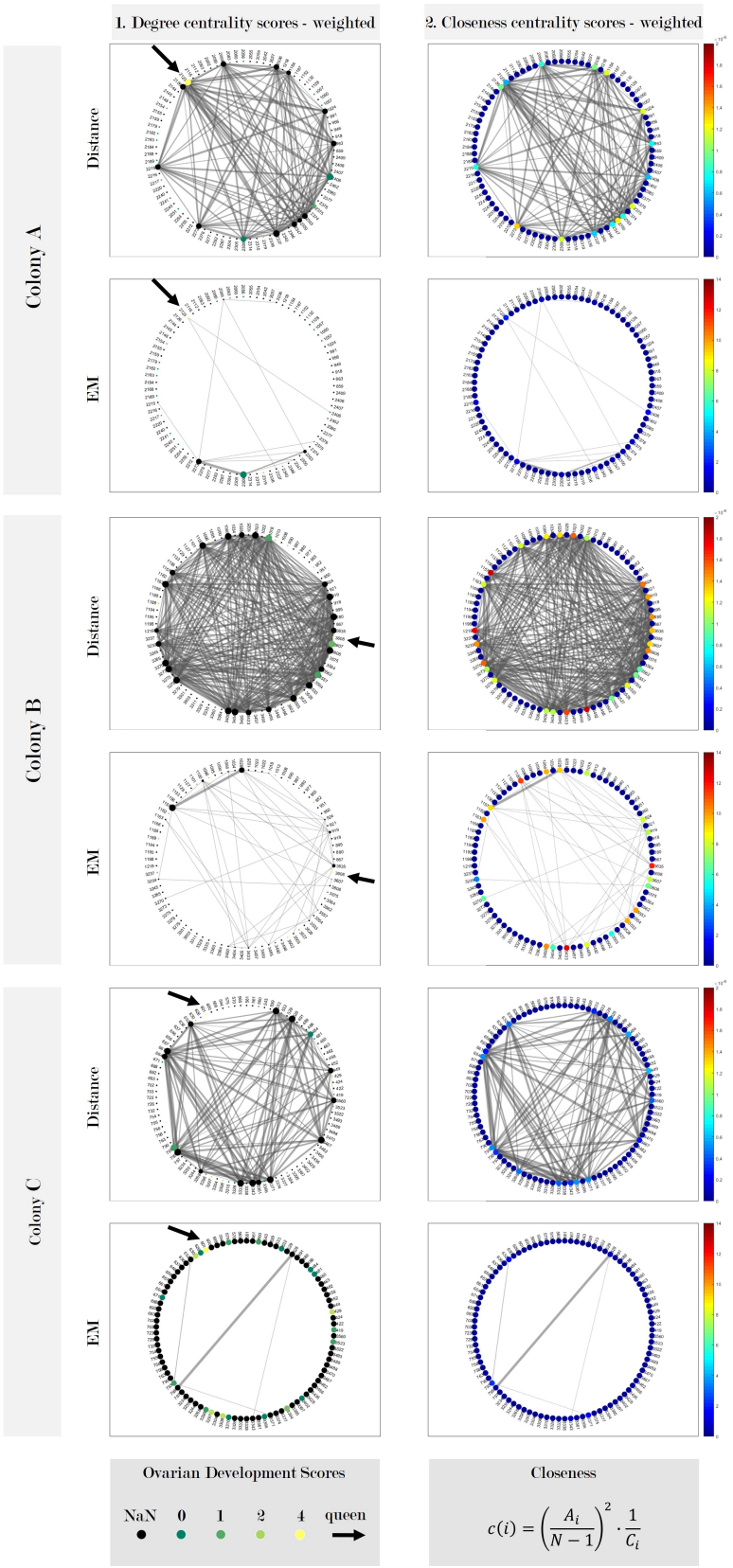

FIGURE 4.1: Nodes are labeled by tag ID, edges are weighted by strength of relation. The upper panels for each colony estimate interaction strength based on the inverse of the mean distance. Unconnected nodes are untracked individuals. The lower panels estimate interaction strength with EM. Unconnected nodes are either untracked or non-interacting individuals. Centrality of each node in the network is calculated. **A.** Nodes are colored by ovarian score and sized proportionally to centrality degree. **B.** Nodes are colored by level of closeness. Individual positions in the network are the same on every graph.

individuals are interconnected in networks weighted by estimated level of social interaction. Despite the fact that networks based on distance overestimate the social importance of nodes, the centrality of nodes is relatively consistent for nodes that are connected with both types of edge weighing. As the units of node weights are different between EM and distance estimations, the range of closeness centrality values differ greatly between the two. However, here we are interested in the relative importance of each node compared to each other. The queen, signaled by an arrow on the figure, could only be tracked in colony *A*. Surprisingly, the queen is shown to be very central in the network, when based on distances between individuals, but far less central in terms of actual social interactions. This seems to indicate that although the queen keeps relatively close to most workers in the nest, it does not actually interact with them. Unfortunately, most of the individuals tracked in this data sample were not assigned an ovarian development score. It is difficult to confirm the negative relationship between level of social interaction and reproductive success in *B. impatiens* colonies based on the networks. However, considering the few tracked individuals that were score on their reproductive potential, there seems to be an emerging trend indicating that the more central nodes are individuals with lower scores, and vice-versa.

Several hypotheses can be put forward to explain a negative relationship between social behavior and fitness. One possible explanation is that sterile workers support the queen and participate to the success of the colony as a whole. These workers, more active in the colony, communicate more with other individuals in the hive as they take part to the daily activities such as brood care or foraging. On the other hand, selfish workers would favor their own interest and focus on their own reproduction. They take less part to the daily activities of the colonies, and as such, do not interact as much with other individuals. One can wonder however, if this is the underlying process defining differences in fitness, why the colony population does not tend towards an equilibrium in which every worker observe an optimal trade-off between nest maintenance and other colony activities on one hand, and reproduction on the other. Another hypothesis, complementary of the former, is drawn from studies lead in queen-less colonies. Queen-less nests provide a setting for the most socially active workers to establish their reproductive status over others through aggressive behavior (Bourke, 1988a; Premnath, Sinha, and Gadagkar, 1995). It is conceivable that in queen-right colonies, individuals who interact with more counterparts, also experience more aggressive encounters than do less social individuals. Therefore, level of sociality and the outcome of previous encounters (e.g. accumulation of aggressive reactions from other workers) are major determinants of ovarian development in an individual worker, as suggested in Amsalem

et al. (2013). This may therefore result in a positive feedback loop, as individuals with a lower reproductive potential interact more with others, increasing the probability of being targets of aggressive behaviors from dominant workers, which in turn will maintain their low reproductive potential or even decrease it further.

One question however still remains, which is why such diversity in social behavior is maintained within eusocial colonies of bumble bees *B. impatiens*?

4.4 Behavioral diversity and group fitness

When studying fitness of eusocial organisms, inclusive fitness is often also taken into account in addition to individual fitness (Hamilton, 1964). Genetic diversity has often be pointed out to increase group fitness in eusocial groups (Hughes and Boomsma, 2004; Tarpy and Seeley, 2006; Mattila and Seeley, 2007; Gove et al., 2009). In several eusocial systems, behavioral variability has also been shown to negatively or positively impact fitness at the level of the colony (Jones et al., 2004; Wray, Mattila, and Seeley, 2011; Modlmeier, Liebmann, and Foitzik, 2012; Jeanson and Weidenmüller, 2014; Leboeuf and Grozinger, 2014; Hasegawa et al., 2016). In *B. impatiens*, diversity in two behaviors — nest thermoregulation and undertaking — appears to not impact colony fitness (Jandt and Dornhaus, 2014). I intended to test whether diversity of social behaviors between individuals within the hive, may affect colony fitness as a whole. The idea was to artificially create colonies of greater or lower diversity in terms of social behavior. After recording and estimating social behavior in several colonies, workers would have been separated into two groups based on their level of social interaction. More social individuals from pairs of colonies would have been gathered into one colony, while the less social individuals would have been gathered into another artificial colony. Technical complications delayed the experiment. The advanced age of the colonies by the time the experiment started, combined with the stress and physiological damage due to anesthetization and tagging of the bees, resulted in a high rate of mortality. Over $^3/_4$ of the colonies were affected, which compromised the experiment. Only a dozen of individuals in total survived long enough to be swapped. Furthermore, these remaining individuals were not very active in general, and showed signs of imminent death. No results can be shown for this experiment.

4.5 Suggestions for later improvements

To conclude, I summarize here the main improvements and work that remain to be done in light of the results presented in this report. First, regarding anesthetization of the bees, it would be interesting to control for the effect of CO_2 narcosis on *B. impatiens* social behavior, in order to better understand the interfering mechanisms that may be in action. One simple way to do so is to consistently keep track of the time and frequency of anesthesia performed on every colony.

As a way to decrease the experimenter's personal bias in assessing ovarian development, I suggest in the future to obtain secondary scoring assessment from another experimenter, without knowledge of the score estimated by the first experimenter. One must also keep in mind that ovarian score is only a proxy for actual reproductive success, as we have no information on whether workers with more developed ovaries actually lay eggs, and at which frequency. Egg laying of bumble bees is a very elusive behavior (a few seconds are enough for laying an egg), which is difficult to record and identify on video. One way to go around this obstacle, would be to use genotyping as a mean to assess the effective reproductive success of every individual in the colony. Bumble bees were tagged all at once indistinctly, so we have no information on age structure of the population and the impact of age on worker behavior or reproductive potential. However, age does not seem as important as in honey bees in determining task allocation and behavior in general (Duong and Dornhaus, 2012).

Last but not least, I intend to complete our methods to automatically estimate social interactions based on tracking data of recorded life within bumble bee colonies. Completing the network analysis by applying it to every recorded video on each day of the experiment would help extracting more information to confirm the negative relationship between social behavior and reproductive potential. I also consider exploring the link between spatial repartition of individuals in the nest, and their social behavior. Indeed, in *B. impatiens*, spatial distribution can be a good predictor of individual behavior (Jandt and Dornhaus, 2009). However, the priority is to resolve the computational issue raised when estimating social interactions with the PBD method. Inferring interaction probability at the individual level, from the probability of body overlap at the pixel level, is the next step to look forward to and the natural continuation of this project.

Acknowledgements

I would like to thank: James for his scientific guidance, his enthusiasm as well as for giving me the opportunity to learn so much on many different topics; Ben for warmly welcoming me in his lab; Adam, Joel and Ben from the workshop, for instilling the basic principles of "do it yourself" in me, and tolerating my endless list of technical questions; Robert Oppenheimer for kindly providing his protocol of bee dissection and Nate for his skillful and patient demonstration of the art of dissecting ovaries; Matt, Kyobi and Kyle for their help with bee emergencies; Zach for his advice on computational matters; Eli for her wise advising, company, and mental support (and the snacks); Kai for our comforting tea breaks; and all those who have participated in many little but significant ways to the progress of this work.

Appendix A

Supplementary Material

A.1 Corrupted frames

The first experiment, linking social behaviour to reproductive potential, is described in section 2. For this experiment, we acquired one hour long videos of activity within bumble bee colonies. Videos were acquired with Blackfly cameras of the company FLIR (formerly PointGrey). Image acquisition was performed under MATLAB, via the software FlyCapture. This software was specifically developed by FLIR to process data from their cameras. Every video recorded during the experiment showed unexpected behavior after about half an hour recording. Frames were split in two vertically and each side was switched as shown on figure A.1.

As a consequence, coordinates calculated of the tags calculated with the BEETag software were erroneous, as it relies on a form of "computer vision" that is based on the analysis of the frame's image itself. We correct the coordinates of tags on corrupted frames as described in algorithm 3. As the image is split vertically, only x coordinates of the tags' center and front need be corrected.

Algorithm 3 Correction of coordinates for corrupted frames. By James Crall.

for $file \leftarrow$ number of files **do**
    $coord \leftarrow$ tracking data of $file$
    $badFrame \leftarrow$ frame at which corruption starts
    $linePos \leftarrow$ x position where frame splits

    $xCoord \leftarrow coord(:, :, 1 \text{ or } 3)$ # x coordinate of tag centers or fronts of every individual, on every frame
    $xCoordCorrected \leftarrow$ modulo$(xCoord((badFrame) : end, :) +$ frame width $- linePos,$ frame width$)$
    $coord(:, :, 1 \text{ or } 3) \leftarrow xCoordCorrected$
end for

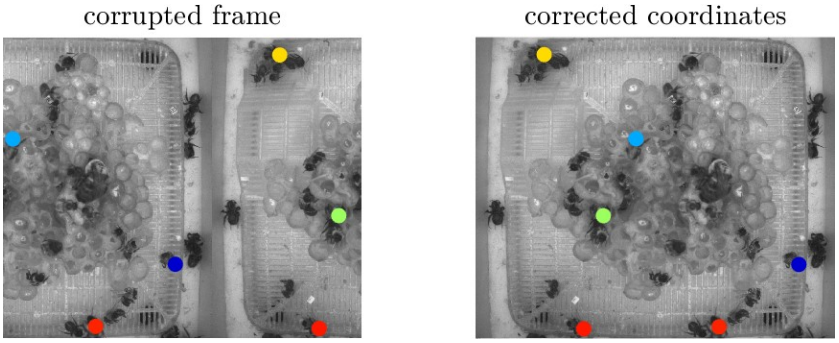

FIGURE A.1: **Example for data correction on a corrupted frame.** Corrupted frames were split in two parts that were switched, with no alteration of the image quality. Tracking the tags was therefore possible, and correction of the data could be applied.

A.2 Brief description of the Bradley method

The BEETag software recognizes QR-like tags on any given image by turning it into a black and white image. First, to recognize the unique numbers associated with the tags on a frame, potential regions for tags have to be singled-out. Recognition of potential tags relies on the identification of highly contrasting square-like white regions with defined black border. The program then analyzes the patterns on this image to try and identify specific bar-code patterns. By default, BEETag uses a global luminescence threshold to turn a frame into a binary black and white image. This process uses the Otsu global thresholding method, which applies a single white $\leftrightarrow$ black threshold level (fig. A.2).

The Bradley-Roth method (often referred to as Bradley method) is a technique for locally adaptive image thresholding developed by Bradley and Roth (2007) (see paper for more detailed explanation of the method). Adaptive thresholding is often used for reading bar-codes for example, and is therefore suitable to ease identification of tags with the BEETag software. QR-like tags will be located and identified more accurately using Bradley thresholding, as opposed to Otsu's global thresholding. The underlying algorithm is also processing images faster than other adaptive thresholding algorithms such as Sauvola's. Two parameters are

necessary for turning the image to black and white: a thresholding value T and size window S. For a specific pixel P on the image with brightness B_P, the Bradley-Roth algorithm will calculate the mean brightness of every pixel around P that belong to the window set by S. Let X be the vector of pixel brightness values within this window. If $B_P < T \times E(X)$, then the pixel P is set to black, and white otherwise. Once a black-and-white image of the frame is generated with this method, using the right parameters T and S, tag regions are recognized faster.

A.3 Individual behavior consistency

Activity within the 3 colonies A, B and C was recorded for one hour per colony, over three consecutive days. It was recorded later on, six days after the beginning of the experiment, in order to confirm consistency of behavior over a longer period of time. To test the dependency of estimated level of social interaction between days, one needs to use non-parametric Spearman correlation test. Indeed, the variables are dependent, since behavior is analyzed on the same individuals day after day. Fig. A.3 shows the results of Spearman correlation tests, along with a visualization of individual behavior consistency between days.

A.4 Ovarian development

Some of the ovarian scores attributed to dissected specimen were transformed before statistical analysis. In some occurrences during dissection, worker ovaries seemed developed to a stage close to a high scoring value of 2 or 3 (even 4 in one instance), but the score was not as clearly differentiable from the next lowest score as for other specimen of comparable ovarian development. Since I was not confident enough in scoring ovarian development, I chose to attribute an intermediate score (1.5, 2.5, 3.5). Such intermediate scores are sparse in the data (5%), which diminishes both the statistical significance and biological meaningfulness of the evaluated scores. Posterior to the dissections, I rounded-off intermediate scores by ceiling them to the lower round value. The only exception is the case of a worker in colony B (individual 3608) to which I initially attributed a round score of 4. After comparing the picture of this individual's ovaries (fig. A.4), to the ones of the queen (score 4) and another worker (original score 3.5 before data transformation), I changed the score of 4 to 3 instead.

I use linear models to disentangle the effect of individual weight on ovarian development, with the confounding differences in ovarian scores between colonies. To do so, I make the simplification that the ovarian score attributed to bumble bee specimens upon dissection

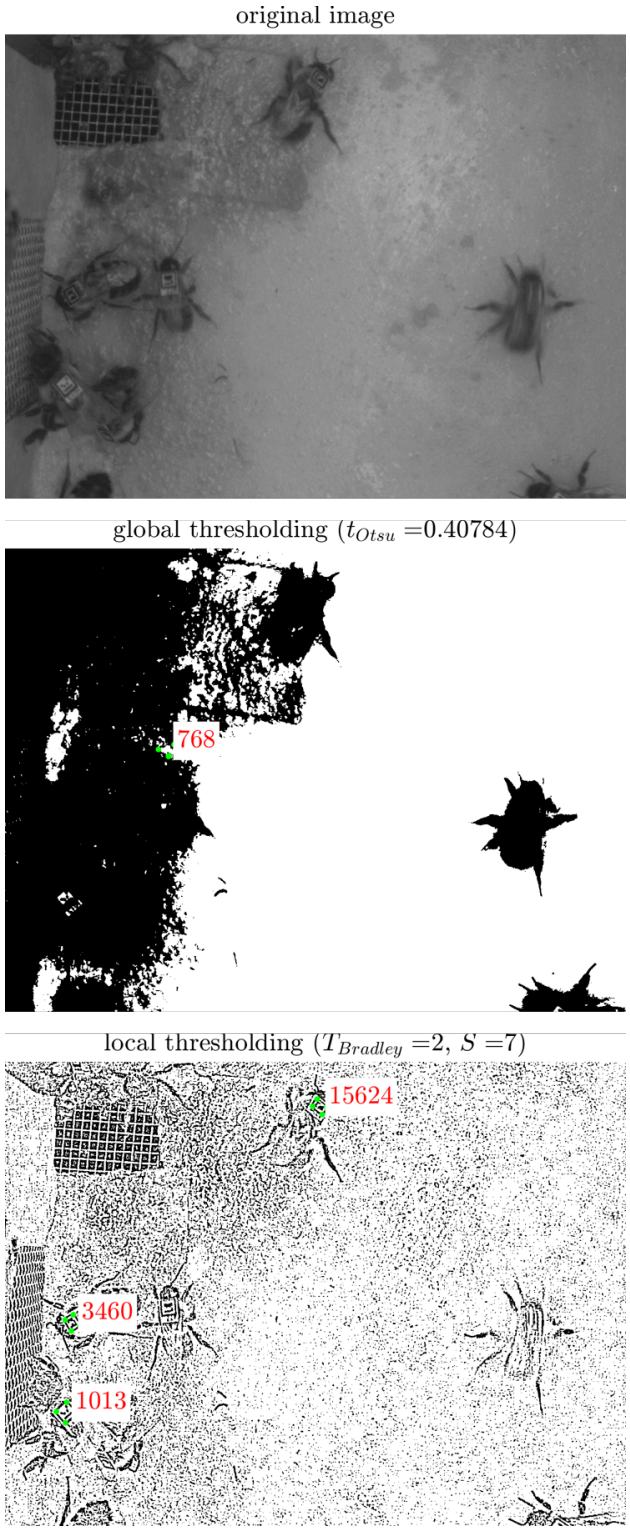

FIGURE A.2: **Example of tag identification using global versus local thresholding.**

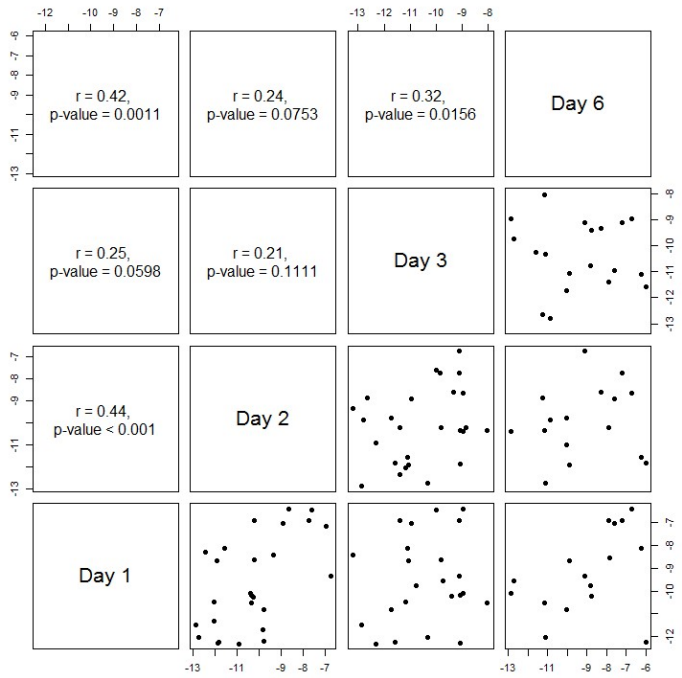

FIGURE A.3: **Spearman correlation for individual behavioral consistency.** The lower-panel shows the log of interaction level of individuals, as estimated with the EM. Each plot compares the behavior of individuals during one day of recording, to their own behavior, on another day of acquisition. Coefficients from the corresponding Spearman correlation test are shown in the upper panel.

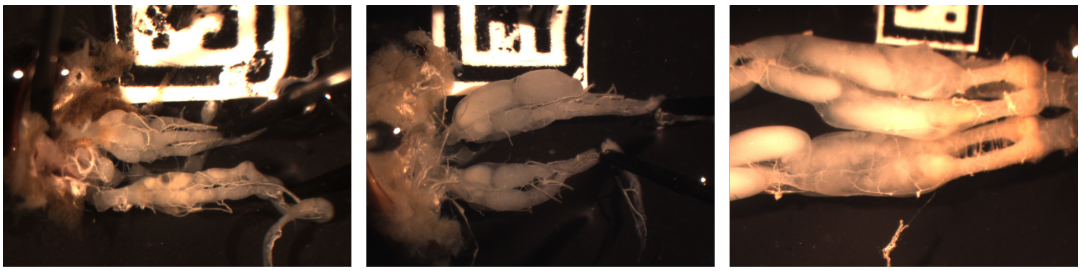

FIGURE A.4: **Comparison between ovary pictures for score assessment.** The picture in the middle shows the ovaries of individual 3608, to whom I originally attributed a development score of 4. When compared to the queen's ovaries (right hand picture) and to the ovaries of another worker, whom I also scored highly (3.5), it appears clear that the ovarian development of individual 3608 cannot be scored as high as the queen's. For reference, tags placed near the ovaries on the pictures are all of the same dimensions.

is an interval variable, although it is in fact ordinal. I excluded queens from the analyses, as they are clear outliers and would defeat the aim of this analysis. I first tested separately the effect of colony differences on individual ovarian score and weight respectively, by means of one-way Analyses of Variance. I performed post-hoc Tukey tests on each results, in order to compare colony effect pairwise. There is a significant effect of colony on both ovary score ($F = 17.44$, $p < 0.001$) and weight ($F = 4.75$, $p < 0.01$). Ovary scores in colonies A and C do not differ significantly, but the mean score in colony B is significantly higher than in colonies A and C ($p < 0.001$). Although there is no significant difference in individuals' weight in colonies A and B, the mean weight of workers in colony C is significantly higher ($p < 0.05$).

I then performed an Analysis of Co-Variance, testing the effect of weight, colony, and the interaction of both on ovarian score. In this model, only weight appears to significantly affect ovary score ($p < 0.001$). After removal of the two non-significant factors, weight explains about 14% of the variability in ovarian score between individuals.

A.5 Note on network closeness centrality calculation

Closeness centrality calculates the inverse sum of the distance from one node to all other nodes in the graph. As in our networks, not every node is reachable, closeness is calculated with:

$$c_i = \left(\frac{A_i}{N-1}\right)^2 \cdot \frac{1}{C_i} \tag{A.1}$$

where i is the node, A_i is the number of nodes that are reachable from i, N is the population size, and C_i is the sum of distances from node i to all the other reachable nodes.

A.6 Access to scripts and arena designs

All MATLAB and R scripts that I wrote and/or used during the project to acquire and statistically analyze data respectively, are stored in a GitHub version control repository. It is accessible via this link. MATLAB scripts written by James Crall or that I adapted from his scripts contain headers with his name. R scripts and MATLAB scripts are kept in different folders of the repository. An additional folder named "realtime" contains MATLAB scripts under construction, to develop a method of real-time tracking with BEETag. Finally, arena designs and other CAD designs for various acrylic material that we created and laser-cut

during the project are stored in the folder "arena_designs". Design files with the extension .dwg were made by James Crall under Illustrator, and .dxf files were designed by myself.

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