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A method of evaluating the productivity of social wasp colonies (Vespinae) and an introduction to the traditional Vespula wasp hunting in Japan

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TITLE:

Evaluation of the Productivity of Social Wasp Colonies (Vespinae) and an Introduction to the Traditional Japanese *Vespula* Wasp Hunting Technique

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KEYWORDS:

colony productivity; reproductive success; social insect; behavioral ecology; wasp hunting; local knowledge; edible insects

SUMMARY:

This methodological paper evaluates the productivity of a social wasp colony by examining the number of meconia per 100 cells of comb, to estimate the total number of adults the wasps produced. The associated video describes how to search for *Vespula* wasp nests, a method developed by amateur wasp chasers.

ABSTRACT:

For vespine wasps, colony productivity is typically estimated by counting the number of larval cells. This paper presents an improved method that enables researchers to estimate more accurately the number of adults produced, counting the number of meconia (the stools left in the cells by wasp larvae when pupating into adults, per 100 cells) in each comb. This method can be applied before or after colony collapse (*i.e.*, in active or inactive nests). The paper also describes how to locate wild *Vespula* wasp colonies by "flagging" wasp baits and chasing the wasp collecting them, using a method traditionally performed by local people in central Japan (as illustrated in the associated video). The *Vespula* chasing method described has several advantages: it is easy to reinitiate the chase from a point where the forager flying back to the nest was lost, and it is easy to pinpoint the nest location as marked wasps often lose their flag at the nest entrance. These methods for estimating colony productivity and collecting nests can be valuable for researchers studying social wasps.

INTRODUCTION:

Every species is thought to develop an optimal strategy for survival and reproduction among a vast array of possible strategies. In natural selection, individuals with traits that maximize an individual's reproductive success will leave more offspring (and genes) to the next generation. Therefore, the number of offspring produced by an individual can be used as an indicator of the individual's relative evolutionary fitness. In a given ecological context, the comparison of the

number of offspring produced relative to alternative behavioral strategies can help researchers predict the best strategy for optimizing fitness[1].

Social Hymenoptera (such as wasps, bees, and ants) have a system of three different castes, which are workers (sterile females), queens (gynes), and males[1]. Only new queens (gynes) and males count toward fitness in social Hymenoptera. Worker production does not directly contribute to fitness since the worker is infertile. On the other hand, a queen that can produce a higher colony productivity (such as a higher number of total cells or a heavier nest) is considered to have a higher fitness in social Hymenoptera, regardless of the number of actually produced new queens and males (see, *e.g.*, Tibbetts and Reeve[2] and Mattila and Seeley[3]). In general, it is difficult to precisely count the number of offspring produced by a colony of social Hymenoptera. In fact, the queens of many social insects live for more than 1 year (*e.g.*, leaf-cutter ant queens can live >20 years[4] and honeybee queens may live for 8 years[5]). In addition, one queen may produce thousands of reproductive offspring over the course of several weeks or months, even in annual species of genera *Vespa* and *Vespula*[6-8]. Furthermore, the lifespan of workers is shorter than that of their mother queen, and workers often die away from their nests. Hence, even if one could accurately count all adults in a nest at any given point in time, such a count would not accurately depict the number of offspring produced. Therefore, the number of offspring produced has been roughly estimated from the size of the nest, the number of workers in the nest, or the weight of the nest at a given point in time[3,9,10]. The number of larval cells could result in an overestimation of the offspring production when some cells are empty. The same method could also result in a potential underestimation of the offspring production because combs of small cells that contain worker brood can produce two or three cohorts of larvae[6,7,11].

The first aim of this work is to provide an improved method for estimating vespine wasp colony productivity in terms of the number of adults produced. Yamane and Yamane suggested that the best way to estimate the number of offspring produced by a colony is to count the meconia in the nest[12]. The meconia is the fecal pellet comprising larval cuticle, gut, and gut contents that a larva leaves in its cell when pupating (**Figure 1A**). The total number of meconia produced per comb is calculated by multiplying the total number of cells present by the average number of meconia per cell. There are often multiple layers of meconia in a cell, and each meconia indicates that an individual successfully pupated in that cell[6,11] (**Figure 1B**). When estimating the mean number of meconia per cell, if the number of cells examined is small (a small sample size), the standard error (SE) increases, and as a result, the error for the total number of meconia per comb becomes higher than if the sample size was larger. The SE of the mean (SEM) is a measure of the dispersion of sample means around the population mean. Therefore, in this study, we focus on the SEM of the number of meconia per cell to estimate the population (the number of adults produced) from the sample mean (the average number of meconia per cell). This study attempts to determine how many samples are required to obtain an SE rate of less than 0.05 per cell. To do this, a numerical simulation is performed with real data on the number of meconia per comb, to determine the minimum sample size (for both worker and queen combs) needed to estimate this value accurately within the defined SE of 0.05.

Vespine wasp colonies live in concealed nests (underground or aerial) composed of multiple horizontal combs, built in series from top to bottom[6,7,11]. The average size of the cells increases from the first (top) to the last (bottom) comb. In the bottom combs, a sudden shift in the average cell size can be seen. These wider cells are built for the development of new queens. Hence, a more accurate estimate of colony productivity (*i.e.*, the number of individuals produced) can be obtained when the total number of meconia in the worker cells (small cells) and queen cells (large cells) are considered. In order to estimate fitness at the colony level, researchers could estimate the number of queens produced and focus on the meconia in the queen cells alone. As for reproductive males, these are reared either in worker or queen cells, depending on the species. Thus, it may be difficult to estimate the male production of a colony, except in species where males have a third, unique cell size[13] (*e.g.*, *Dolichovespula arenaria*).

The second aim of this work is to present a useful technique for locating wild vespine wasp colonies in the field and transplanting them into laboratory nest boxes. Although some researchers obtain wasp nests from pest control calls (*i.e.*, people reporting them as pests[14,15]), this method is not always possible or desirable. Researchers might need to collect nests in wild and inhabited areas where pest controllers do not operate, or to conduct their research by more flexibly obtaining nests at specific times. Interestingly, people living in the mountainous areas of central Japan traditionally collect and rear wasps (*Vespula shidai*, *Vespula flaviceps*, and *Vespula vulgaris*) for food. Therefore, collecting and artificial rearing techniques for these wasps are well developed in those areas[17].

This paper also summarizes the methods employed to rear *Vespula* wasps. The experimental organism for this study was *V. shidai*, a social, ground-nesting wasp inhabiting western Asia and Japan. *V. shidai* possesses the largest colony size among all Japanese vespine wasps, with a total of 8,000 to 12,000 cells per nest, with a maximum of 33,400 cells[14,18]. Workers of *V. shidai* have an average wet weight of 67.62 ± 9.56 mg. Males are usually reared in worker cells; in contrast, new queens are reared in specially constructed, wider queen cells[14].

[Place **Figure 1** here]

PROTOCOL:

1. Evaluation of Colony Productivity

1.1. Estimation of the number of cells per comb

1.1.1. Separate the combs one by one. Sweep away all adult wasps from the comb and pull out all larvae and pupae from the cells with tweezers.

1.1.2. Measure the square measures of 10 randomly chosen cells per a comb, by using imaging software (*e.g.*, Image J version 1.48, see http://imagej.nih.gov/ij/).

1.1.2.1. Take a picture with the scale reference so that all the cells are pictured from right above.

1.1.2.2. Based on the actual length of the scale, convert all measured lengths to pixels.

1.1.2.3. Measure the areas of the 10 cells in pixels and convert them to the actual areas.

1.1.2.4. Calculate the average area of worker and queen cells.

1.1.3. Estimate the number of worker and queen cells by dividing the area of each comb by the average cell area per comb.

1.2. Counting the number of meconia for the evaluation of colony productivity

1.2.1. Count the number of meconia per 100 cells for each comb by carefully breaking the comb and examining meconia.

NOTE: This number of cells was determined here to be sufficient (the SE of the number of meconia per cell is within 0.05, see the representative results section). Meconia may have solidified into two or more layers in the cell (**Figure 1**).

1.2.2. Calculate the average number of meconia per cell for these 100 cells.

1.2.3. Calculate the total number of meconia for each comb (*i.e.*, the number of individuals produced, the colony productivity), extrapolated from the estimated number of cells and the average number of meconia per cell for that comb.

2. Finding *Vespula* Nests

2.1. Baiting

2.1.1. Hang pieces of cuttlefish, freshwater fish, or chicken heart (approximately 10 g in total) on a tree branch at a height that can be easily reached by hand (**Figure 2**).

2.1.2. Place these baits along a transect (*e.g.*, along a road crossing a forest or along a river) at 50 to 100 stations, with at least 5 m between each station.

[Place **Figure 2** here]

2.2. Providing wasps with a "flagged" bait

2.2.1. Flag construction and attachment

2.2.1.1. Cut plastic (polyethylene) bags into strips of 3 - 5 mm in width and 15 cm in length by using a box cutter.

2.2.1.2. Prepare 1.5 mm^3 of chicken heart or cuttlefish on a bamboo skewer or thin branch (the diameter of the meat bait can be 1 - 2 mm, less than 15 mg for a *V. shidai* worker; **Figure 2**).

2.2.1.3. Tie a thread to the flag (plastic strip, less than 10 mg) and then to meat bait, attaching it within 3 mm from the flag (this is called the "flagged" bait). Cut off the loose thread above the knot.

NOTE: Use extremely fine polyester thread normally used with sewing machines.

2.2.2. Presentation of the meat bait to a wasp

NOTE: A nest is found most efficiently by following wasps that return to the bait repeatedly within 4 min of leaving. This is because wasps that take the bait and return quickly have a nest nearby.

2.2.2.1. Paint a unique mark on each thorax to identify the wasps individually when they are biting the baits (preferable with water-based paint pens, see **Table of Materials**).

2.2.2.2. Orient the flag with the thread under the wasp while it bites the flagged bait when presenting the bait to the wasp (place the flag so that it and the thread pass under the wasp's abdomen from below its thorax).

2.2.3. Following a marked wasp

2.2.3.1. Gather the baits from the surrounding area, so that the returning wasp is more likely to return to the same spot, before following a wasp.

NOTE: Following marked wasps is best accomplished with a group of two or more people. At least one person stays on at the transect, providing the foraging wasps with flagged baits, while the other(s) follow the marked wasp. When more than one wasp is attracted to the same bait, mark and follow only wasps that fly away in the same direction.

2.2.3.2. Follow a wasp with a flagged bait.

2.2.3.3. When a followed wasp lands somewhere on the way to its nest, gently lift the wasp with a long stick (branch) or fishing rod and watch it until it resumes flight.

NOTE: Be gentle and do not strike the resting wasp because it will drop the bait and fly away.

2.2.3.4. When the wasp is shaping another meat ball before flying back to its nest again, readjust the flag, if necessary.

NOTE: Wasps will sometimes land and chew through the thread, removing the flag from the meat bait. If this happens frequently, make the flags shorter to increase forager flying ability.

2.2.3.5. When a wasp escapes detection while being followed, wait for the wasp to return to the bait station on the transect before resuming the chase. This time, while the wasp is biting the new bait, carry the bait stick (and wasp) to the point where it had last escaped detection.

NOTE: Foraging wasps do not let go of their baits easily, and do not sting if handled gently. Hence, the wasp with the flagged bait can be moved to the desired location by holding the flag, without the wasp escaping.

3. Transfer of the Nest

3.1. Structure of the carrying box

3.1.1. Construct nest boxes of various sizes, from 10 to 20 cm in length and width and from 10 to 20 cm in height, to accommodate nests of various sizes.

NOTE: Boxes of this size are big enough to accommodate young nests of *V. shidai* (collected in Central Japan between mid-July and mid-August). Make a carrying box according to the nest size of each species, for each growth stage.

3.1.2. Construct the bamboo grid and attach it to the inside of the box, about 2 cm above the bottom of the box, to facilitate the placement of the nest inside the carrying box.

3.1.3. Cover the bottom of the carrying box with newspaper and paste it to a wooden, removable board (**Figure 3**).

NOTE: The newspaper will, later, allow the wasps to chew through it as they build additional combs below the carrying box when this is placed in a nest box (see section 3.2).

3.2. Excavation of the nest

3.2.1. Before the exposure of the whole nest

NOTE: Wear protective clothing to avoid being stung by the wasps defending their nest.

3.2.1.1. Once the wasp nest is found, excavate the nest.

3.2.1.2. Vigorously stamp on the ground around the nest for about 10 to 20 min so that workers leaving and returning to the nest remain inside to protect it, to collect as many workers as possible.

NOTE: If the wasps continue to stay outside the nest, it is better to capture them using an insect net. Although the stamping is useful for *V. flaviceps*, *V. shidai*, and *V. vulgaris*, other species' workers from the nest may attack the individual performing the stamping. In the case, skip this step.

3.2.1.3. Shine a light directly into the nest entrance to determine the direction in which the nest entrance runs. Use a finger to confirm the orientation of the nest hole, while gently excavated soil from around the nest.

3.2.2. After the exposure of the whole nest

3.2.2.1. When the whole nest is exposed, spread a cloth and place the nest on top of it to prevent wasps from escaping into the ground under the nest.

3.2.2.2. Place the excavated nest into a wooden (carrying) box for transportation to the lab (**Figure 3**); then, cover it with branches and newspaper. Leave the top of the nest uncovered while it is in the box.

3.2.2.3. Place the carrying box on a cloth for 5 to 10 min, until the wasps become calm.

3.2.2.4. Collect any wasps in the vicinity with an insect net and transport them to the laboratory with the nest.

NOTE: As an alternative collection procedure, anesthetize the nest occupants by fanning celluloid smoke or diethyl ether into the nest before excavating it.

[Place **Figure 3** here]

4. Rearing *Vespula*

4.1. Structure of the nest box

NOTE: The nest box is made of wood, with dimensions of 50 cm in length and width and 70 cm in height for rearing *V. shidai* (a mature nest is approximately 40 cm in diameter in the wild). Make a nest box according to the nest size of the species to be reared.

4.1.1. Provide the nest box with an entrance hole (usually placed in the upper part of the box) to allow wasps to leave the nest to forage.

4.1.2. Fill about 1/3 of the nest box with soil like that occurring at the location where the nest was collected.

4.1.3. Install a wire mesh (with a mesh size of 1.5 cm^2) at the entrance of the nest box to prevent any intrusion by other wasps (predators, such as *Vespa mandarinia* and *Vespa simillima*).

4.1.4. Place two wooden bars in the nest box that can bear the carrying box (**Figure 4**).

[Place **Figure 4** here]

4.2. Transplantation of the carrying box into the nest box

4.2.1. Keep the nest box in a dry place while rearing wasps in the collected nests (*i.e.*, somewhere not exposed to rain).

4.2.2. Remove the wooden board at the bottom of the carrying box and put it in the nest box for a long-term study (**Figure 4**).

NOTE: Often, wasps will have bitten holes in the newspaper covering the bottom of the carrying box, and so, there is a danger of being stung by wasps escaping through the holes. Therefore, wear protective clothing when transplanting the nest.

4.3. Feeding the wasps

4.3.1. Place various types of meat (squid, freshwater fish, chicken breast, or chicken heart) and a 1:3 solution of honey and water at approximately 3 m from the nest box.

4.3.2. Provide enough food for the feeding requirements of 1 day. Replenish fresh food every day (Vespinae do not forage on old/rotting meat).

REPRESENTATIVE RESULTS:

One goal of this study was to determine how many samples are required to obtain an SEM of the number of meconia per cell which is less than 0.05. In this study, a comb with an average cell size of <20 mm^2 was defined as a worker comb, whereas larger combs were defined as queen combs. We counted the number of cells for queen combs and worker combs (in this study, counts were made of six queen combs and six worker combs from five *V. shidai* colonies). The actual number of cells per comb was estimated from these data *via* extrapolation (**Table 1**).

[Place **Table 1** here]

An analysis of the relationship between the sample size and the SEM of the number of meconia per cell demonstrated that the sample size should be established using a bootstrap approach based on the number of meconia counted (from real data). Using real data, the mean and standard deviation (SD) of the number of meconia per cell were calculated, with the number of samples set at 1,000 for each sample size (the number of cells to be examined were 1 to 500; **Figure 5**). We did not allow for an iterative extraction from the data at sampling. The SEM for the

number of meconia per cell was calculated for each sample size for each set of real data. Then, the sample size at which the SEM was less than 0.05 was examined. All calculations were made using software R.3.2.4.[19] This analysis showed that the SEM was <0.05 when the sample size was 100 cells (for both worker and queen combs) (**Figure 5**). Therefore, the following results are based on examining the number of meconia per 100 cells per comb.

The actual and estimated numbers of cells in six worker combs and six queen combs and the number of meconia per comb are shown in **Table 1**. The estimates of the number of cells in the worker combs, based on comb area measurements, were both higher and lower than the true count. The mean number of meconia in the cells of worker combs, which represents the number of workers produced, ranged from 1.96 times more than the number of estimated larval cells to 0.89 times less than the estimated number of cells (**Table 1**). In the queen combs, the actual number of cells was often less than the estimated number of cells. The number of meconia in the queen combs, which can represent a component of fitness (*i.e.*, a part of the reproductive success of the founding queen), was 0.53 to 1.02 times the estimated number of cells.

All cells and meconia were counted in six randomly selected worker combs and six randomly selected queen combs from the five nests (**Table 1**). The total number of cells counted in the worker combs was 7,165, whereas the number of meconia counted in the worker combs was 10,851. The average number of cells per comb was 1,194.2 ± 720.3 (average ± SD), whereas the average number of meconia in the worker combs was 1,808.5 ± 1,368.2. In the queen combs, the total number of all cells was 6,572, whereas the number of all meconia was 5,244. The average number of cells per comb in the queen combs was 1,095.3 ± 174.8[20], whereas the average number of meconia was 874.0 ± 223.8. Meconium layers in worker cells ranged from zero to three, whereas the queen cells had either one or no meconium layer.

[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Meconium in a larval cell. (**A**) Cross section of a comb of *Vespula shidai*. Meconia is indicated by red arrows. (**B**) Two meconia are layered. Each blue arrow indicates one meconium.

Figure 2: Providing wasps with a flagged meat bait. (**A**) Baiting wasps with meat attached to the tip of a stick. (**B**) The piece of meat is tied with a thread to a plastic flag. (**C**) The wasp holds on the meat which is tied to the flag. Such "flagged" baits will increase the visibility of the flying forager. The photos in panels **B** and **C** were taken by Fumihiro Sato.

Figure 3: Carrying box. (**A**) Box for carrying nests collected in the field. (**B**) A bamboo grid is placed at on the bottom of the box. The two boxes in the image on the right are upside down.

Figure 4: Laboratory setup. (A) Setting a carrying box into a nest box used for a long-term study. Before placing the carrying box in the nest box, the wood board at the bottom of the carrying

box was removed, leaving only the newspaper to cover the bottom of the nest. (**B**) A series of nest boxes with food resources hanging from a wire line.

Figure 5: The relationship between the sample size and the standard error (SE) relative to the number of meconia counts. (**a**) Meconia per cell in worker combs. (**b**) Meconia per cell in queen combs. Each circle depicts an SE relative to the number of meconia per cell obtained *via* simulation with actual data. Color differences represent the data from each sampled nest. Simulating the SE for the number of meconia per cell in comb WWkb02 (worker comb) was accomplished with a sample size of 300 because that comb only had 347 cells.

Table 1: The actual and estimated numbers of cells in six worker combs and six queen combs and the number of meconia per comb. WW = a worker comb from a wild nest, WR = a worker comb from a rearing nest, QW = a queen comb from a wild nest, QR = a queen comb from a rearing nest. Alive = viable wasp larvae in cells, Collapse = no viable larvae in cells.

DISCUSSION:

The colony productivity of bees, ants, and wasps has been estimated previously by the number of workers and cells in nests or by the weight of the nests[3,9,10]. This study shows that the estimate of the number of meconia provides a better estimate of the overall number of individuals produced (*i.e.*, a better indicator of colony productivity). In fact, it was found that, for both worker and queen combs, the number of meconia ranged from 0.53 to 1.96 times the number of larval cells in the comb. These findings quantify how inaccurate the determination of the number of workers and queens produced can be when it is based on the number of cells in a comb. Despite being more labor-intensive, estimating the number of meconia in a nest seems to guarantee a more precise evaluation of colony productivity. On the other hand, in this study, it was not evaluated how accurately the number of meconia represents the number of individuals produced.

This paper shows how many cells of a *V. shidai* nest should be examined to estimate colony productivity, based on the results of a bootstrap simulation approach using sample data on the number of meconia in the nest. Based on these results, it would be appropriate to investigate 100 cells per comb of both worker and queen cells. The method for counting meconia can also be applied to a nest after it has collapsed (*i.e.*, is inactive), which can be advantageous for researchers: the reproductive period of vespine wasp colonies is quite long[8] and studying a nest after it has collapsed means that the total number of adults produced over the entire reproductive period can be estimated. Such colonies are also easier to collect.

To collect nests of *V. shidai*, some researchers have followed either marked (*e.g.*, coated with fluorescent powder) or unmarked wasps[21]. The nest location method presented here (feeding the wasps “flagged” meat) facilitates following wasps to their nests. This approach is also helpful if a tracked wasp is lost because the same wasp will eventually return to the bait along the transect. Provide new flagged bait to this wasp and carry it to the point where it was last lost, thus allowing chasers to resume the chase from that point onward (closer to the nest). Some of the flags brought to the nest are dislodged at the nest entrance, which also facilitates finding ground nests.

However, this method is not suitable for rainy days because markers tend to stick to branches and leaves when they get wet. Although chasing flagged wasps is useful for *V. shidai*, *V. flaviceps*, and *V. vulgaris* in Japan, this method could not be applied to *Vespula rufa* because these wasps do not come to the bait and do not grab flagged bait. The nest location method can probably not be used for some *Vespula* wasps.

More sustainable diets are needed by an ever-increasing global population. In addition, the demand for edible insects increases daily. Many edible insects, which are being consumed locally and traditionally throughout the world, have been identified by the Food and Agriculture Organization of the United Nations[21] as a promising alternative protein source for overcoming food insecurity worldwide. Larvae and pupae of *Vespula* have traditionally been used as food in mountainous areas of Japan[16], and so, they could be used to provide a source of protein elsewhere in the world. The set of protocols developed in this study is likely applicable to locating nests of other yellowjacket species. Therefore, the protocols outlined in this paper will be useful for collecting yellowjackets as an edible resource and studying wasp behavior.

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DISCLOSURES:

The author has nothing to disclose.

REFERENCES:

1. Davies, N.B., Krebs, J.R., West, S.A. *An introduction to Behavioural Ecology*. John Wiley & Sons (2012).
2. Tibbetts, E.A., Reeve, H.K. Benefits of foundress associations in the paper wasp *Polistes dominulus*: increased productivity and survival, but no assurance of fitness returns. *Behavioural Ecology*. **14**, 510-514 (2003).
3. Mattila, H.R., Seeley, T.D. Genetic Diversity in Honey Bee colonies Enhances Productivity and Fitness. *Science.* **317**, 362 (2007).
4. Weber, N.A. *Gardening Ants, the Attines.* American Philosophical Society. Philadelphia, PA (1972).
5. Baer, B., Schmid-Hempel, P. Sperm influences female hibernation success, survival and fitness in the bumble-bee *Bombus terrestris. Proceedings: Biological Science.* **272** (1560), 319-323 (2005).

6. Spradvery, J.P. *Wasps. An Account of the Biology and Natural History of Social and Solitary Wasps, with Particular Reference to Those of the British Isles.* Sidwick & Jackson Ltd. (1973).
7. Matsuura, M., Yamane, S. *Comparative Ethology of the Vespine Wasps.* Hokkaido University Press. Sapporo, Japan (in Japanese) (1984).
8. Greene, A. Production schedules of vespine wasps: an empirical test of the bang-bang optimization model. *Journal of Kansas Entomological Society*. **57** (4), 545-568 (1984).
9. Cole, B.J. Multiple mating and the evolution of social behavior in the Hymenoptera. *Behavior Ecology Sociobiology*. **12**, 191-201 (1983).
10. Goodisman, M.A.D., Kovacs, J.L., Hoffman, E.A. The significance of multiple mating in the social wasps *Vespula maculifrons. Evolution.* **61** (9), 2260-2267 (2007).
11. Greene, A. *Dolichovespula* and *Vespula.* In *The Social Biology of Wasps.* Edited by Ross, K.G., Matthews, R.W., 263-305, Cornell University Press. Ithaca, NY (1991).
12. Yamane, S., Yamane, S. Investigating methods of dead vespine nests (Hymenoptera, Vespidae) (Methods of taxonomic and bio-sociological studies on social wasps. II). *Teaching Materials for Biology.* **12**, 18-39 (in Japanese) (1975).
13. Loope, K. J. Matricide and queen sex allocation in a yellowjacket wasp. *The Science of Nature.* **103** (57), 1-11 (2016).
14. Matsuura, M. *Social Wasps of Japan in Color.* Hokkaido University Press. Sapporo, Japan (in Japanese) (1995).
15. Foster, K.R., Ratnieks, F.L.W., Gyllenstrand, N., Thoren, P.A. Colony kin structure and male production in Dolichovespula wasps. *Molecular Ecology.* **10** (4), 1003-1010 (2001).
16. Loope, K.J., Chien, C., Juhl, M. Colony size is linked to paternity frequency and paternity skew in yellowjacket wasps and hornets. *BMC Evolutionary Biology.* **14** (1), 1-12 (2014).
17. Nonaka, K. Cultural and commercial roles of edible wasps in Japan. *Forest Insects as Food: Humans Bite Back. Proceedings of a workshop on Asia-Pacific resources and their potential for development.* Chiang Mai, Thailand. 123-130 (2010).
18. Yamane, S. The unique ecology of *Vespula shidai amamiana* and the origin of distribution. In *Ecological Society of Japan*. Edited by Funakoshi, K. *Biodiversity of the Nansei Islands, its formation and conservation* (in Japanese) (2015).
19. R: The R Project for Statistical Computing. https://www.R-project.olrg/. (2018).
20. Saga, T., Kanai, M., Shimada, M., Okada, Y. Mutual intra- and interspecific social parasitism between parapatric sister species of Vespula wasps. *Insectes Sociaux.* **64** (1), 95-101 (2017).
21. Van Huis, A. *et al. Edible insects: future prospects for food and feed security*. Food and Agriculture Organization of the United Nations. Rome, Italy (2013).

Figure3

Click here to access/download;Figure;Figure3_Saga_2018_.eps

Figure4

Click here to access/download;Figure;Figure4_Saga_2018_.eps

Figure5

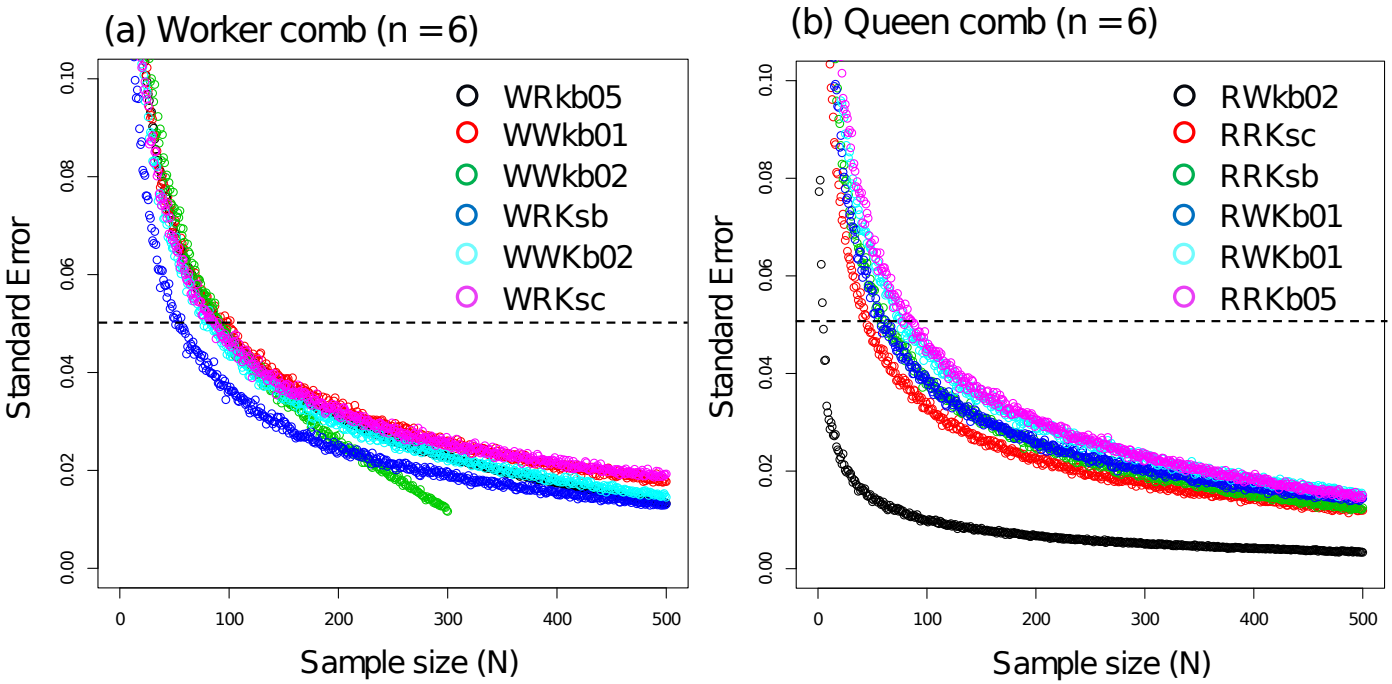

Figure1

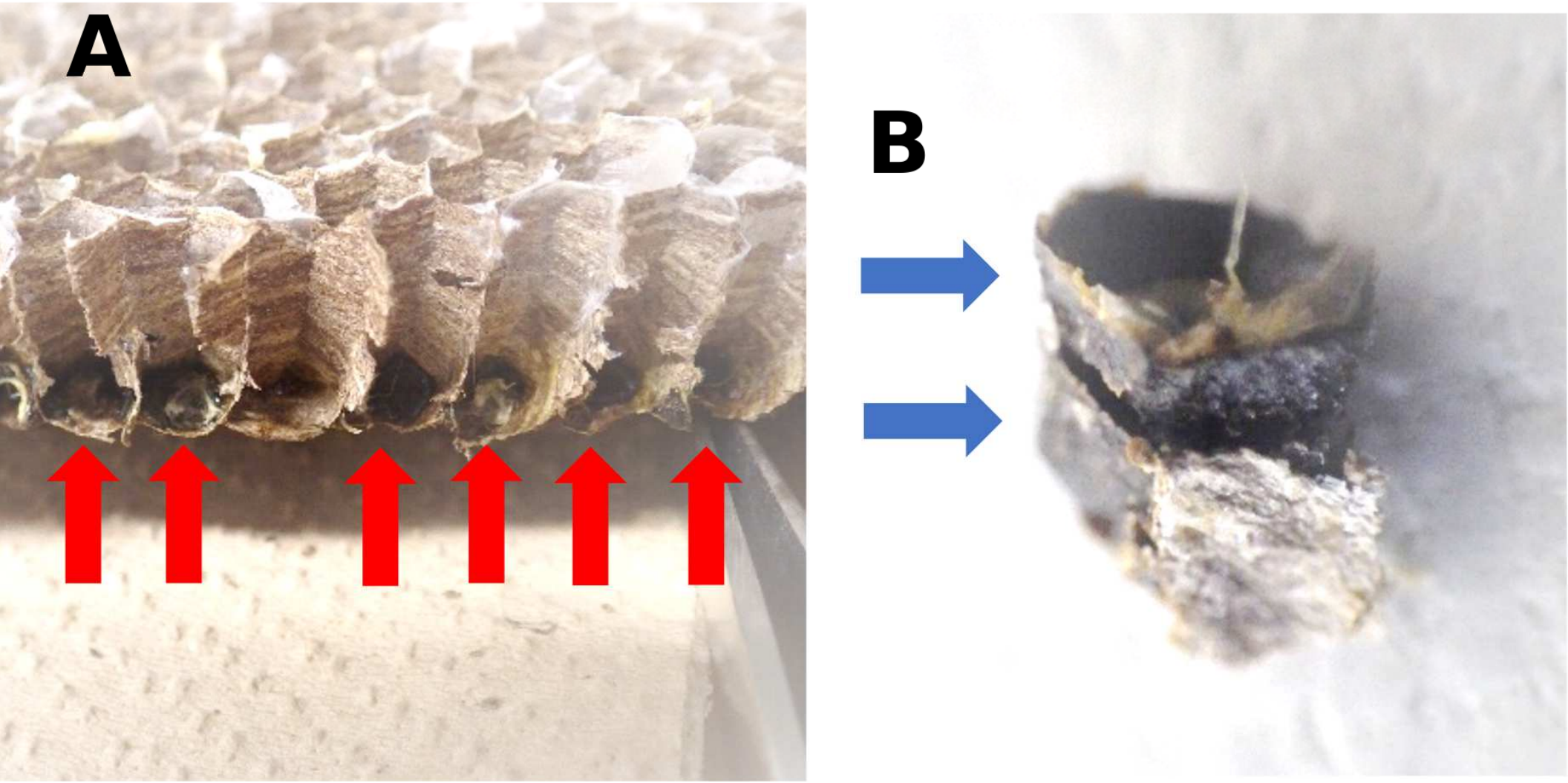

Figure2

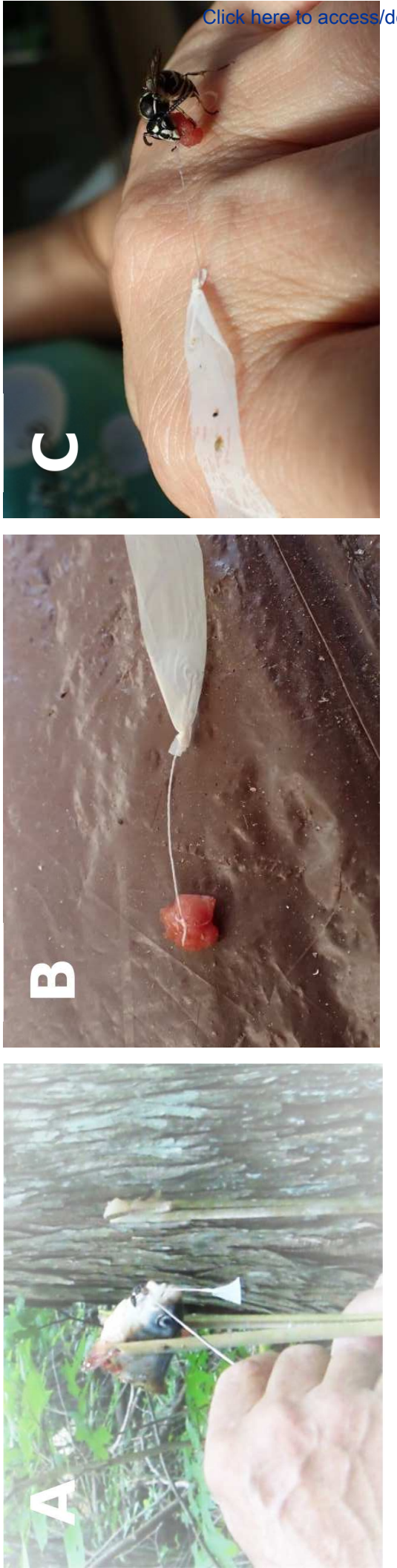

Table 1. **The actual and estimated numbers of cells in six worker combs and six queen combs of meconia per comb.**

ID	State	Collection date	Area (mm^2)	Estimated number of cells (ENC)	Actual number of cells (ANC)	Actual number of meconium (ANM)
WW-Kb01	Alive	18-Oct-16	27756.7	1599.9	1433	2430
WW-Kb02	Alive	18-Oct-16	4098	381.9	347	494
WW-Kb02	Alive	18-Oct-16	22439.3	1118.9	986	1317
WR-Ksb	Collapse	3-Nov-16	19094.9	1098.6	1,181	974
WR-Ksc	Collapse	27-Nov-16	38,933.40	2,198.70	2,455	4,321
WR-Kb05	Collapse	29-Nov-16	10970	860	763	1315
QW-Kb01	Alive	18-Oct-16	29186.2	1094.4	1095	759
QW-Kb01	Alive	18-Oct-16	36920.5	1361.6	1341	1075
QW-Kb02	Alive	18-Oct-16	37295.9	1047.2	1080	1068
QR-Ksb	Collapse	3-Nov-16	24811.2	1011.9	893	701
QR-Ksc	Collapse	27-Nov-16	33352.8	1384.5	1241	1069
QR-Kb05	Collapse	29-Nov-16	25157.6	1071.4	922	572

WW = a worker comb from a wild nest, WR = a worker comb from a rearing nest, QW = a queen
wild nest, QR = a queen comb from a rearing nest. Alive = viable wasp larvae in cells, Collapse = r

and number

ANM /ENC
1.52
1.29
1.18
0.89
1.96
1.53
0.69
0.79
1.02
0.69
0.77
1.97

comb from a
no viable

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
cuttlefish	Any		fresh/ as a bait
dace	Any		fresh/ as a bait
chichken heart	Any		fresh/ as a bait
plastic bag (polyethylene)	Any		as a flag
bamboo skewer	Any		
industrial sewing thread	FUJIX Ltd.	King polyester, No.100	
paint marker pen	Mitsubishi pencil	UNI, POSCA, PC5M	
fishing rod	ANY		
carrying box			made of wood
nest box			made of wood

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Author(s): Tatsuya Saga

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CORRESPONDING AUTHOR

Name:	Tatsuya Saga
Department:	Tajimi Senior High school
Institution:	Tajimi Senior High School
Title:	Evaluating the productivity of social wasp colonies (Vespinae) located, collected and reared following traditional Japanese techniques.
Signature:	Tatsuya Saga
Date:	6 September 2018

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Dear Dr. Alisha DSouza

EMID: 82c35267b56053f8
JoVE59044 “Evaluating the productivity of social wasp colonies (Vespinae) located, collect and reared following traditional Japanese techniques”

I am returning herewith the above-mentioned manuscript revised according to your e-mail of September 28. I appreciate the editor’s and reviewers' careful and precise comments on my manuscript. I considered that the all the comments/questions were appropriate. I carefully revised protocol section according to the instructions of the editor. Furthermore, following the reviewers’ advices, I believe that the method I proposed has been clarified further by this revision.

The one-by-one replies to the editor’s and reviewer’s comments (BLACK) were replied in “Response to Reviewers” in BLUE letters (also see PDF). The changes made in the original text were indicated in RED.

I would like to thank the editor, the associate editor, and the reviewers for their helpful comments and hope that the revised manuscript is now suitable for publication in Journal of Visualized Experiments.

Yours sincerely,

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Thank you very much for inviting me to submit a revised draft. I carefully proofread the manuscript.

2. Please revise the title to be clear and concise.
I changed the title ”Evaluating the productivity of social wasp colonies (Vespinae) located, collect and reared following traditional Japanese techniques ” to “A method of evaluating the productivity of social wasp colonies (Vespinae) and an introduction to the traditional *Vespula* wasp hunting” (Line 2-3). I hope that you agree.

3. Line 101: Please update the reference format to a superscripted number.
I updated the reference format to a superscripted number (Line 112).

4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
I ensured to avoid using of any personal pronouns in protocol.

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
I revised most of the protocol as you indicated (Line 118-259). I have rewritten action items in the imperative tense. I also added the notes in protocol.

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
I have added some sentences for clarifying the protocol steps, especially first step (Evaluating colony productivity) (Line 118-127).

7. 1.1: It is unclear how to obtain and prepare the cells for counting. Please add such details.
I have added more details of protocol steps in 1.1. (Line 120-121).

8. Lines 115-116: Please describe how to calculate the average area of worker and queen cells. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.)
I added sentences that I calculated the area from the ratio of the actual scale length to the number of pixels (Line 122-127).

9. Line 147: What is used to cut?
I use box cutter to cut a plastic bag for making flags (Line 152-153).

10. Line 148: Please specify the type of meat.
I use chicken heart or cuttlefish as flagged bait meat (Line 154-156).

11. Line 161: How many baits are used for a specific area?
I use 50-100 baits which attract wasps for a specific area (Line 145-146).

12. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

I have simplified the protocol so that each step contains only 2-3actions (Line 118-259). I moved the explanation for understanding the result to representative results section (Line 296-298). I also moved the discussion about the protocol to Figure legend (L348).

13. Please include single-line spaces between all paragraphs, headings, steps, etc.
I revised the manuscript as include single-line spaces between all paragraphs, headings, steps, etc.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
I highlighted 2.75 pages for identifying the essential steps of the protocol for the video.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
I ensured that the highlighted part of step includes some actions.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
I highlighted some steps according to the above instructions.

17. Figure 5: Please change the x-axis label “sample size(N)” to “Sample size (N)”.
I have revised the x-axis label.

18. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.
I uploaded each Figure individually as an EPS file as you indivated.

19. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file.
I uploaded Table 1 as an .xlsx file.

20. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.
Yes. I removed the title and Figure legends from the figures.

21. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.
I have added some sentences that showed some limitation of the technique (Line 374-375, 394-397).

Again, thank you for giving me the opportunity to strengthen my manuscript with your valuable comments and queries.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this nice paper, the author in effect describes three useful techniques to improve the study of vespine wasps. The first is the use of meconia counts, rather than cell counts, to estimate colony productivity. While the former has been used before, the latter is much more common, so a paper demonstrating its superiority and effectiveness is welcome. The most valuable contribution I can see is the second method described (the proposed subject of the video): the techniques used to locate wild vespula colonies. I have used similar methods but find this approach attractive, and hope to try it soon. Finding colonies in the wild is perhaps the most limiting step in studying these fascinating but often difficult animals. As these insects cannot be reared artificially, nests must be collected from the wild for study. Thus, this technique addresses an important problem in wasp biology methodology. The fact that it has been developed as the result of a cultural practice in Gifu of collecting these wasps for use by the wider community is interesting as well. Finally, the paper also describes how the authors transplant and keep these wasps in the lab. The details of this will be interesting to anyone who has tried to do this. It is not an easy task, and the methodology presented here is an improvement on my previous attempts. Publications like these are a service to the community, where otherwise such methodological advances are either lost, or spread very slowly and imperfectly via word of mouth.

I appreciate your precise and valuable comments.

Major Concerns:
I have none.

Minor Concerns:
I find the paper generally well written and clear. I suggest light copyediting by

the journal editors to ensure proper grammar, though mistakes are few. Another minor suggestion would be to group the sections 2.4 (The structure of the carrying box) and 2.5 (excavating the nest) with the third section on rearing Vespula, rather than the end of the second. To me, this would be a more logical arrangement, since these methods pertain directly to transplanting the nests. Some studies will not require transplanting, and instead study colonies where they are found. Thus, I think these fit better with the third section than the second.

I agree with your suggestion that some studies will not require the transplanting (the section 2.4. and 2.5.). Therefore, I moved the section 2.4. and 2.5. from section 2 “Finding *Vespula* nest”. On the other hand, I think that transplanting and rearing a colony are different topics. Therefore, I made new section “Transferring the nest” which included “The structure of the carrying box” and “excavating the nest” as sub-steps. I believe that this revision is more appropriate.

Reviewer #2:

Manuscript Summary:

General comments:

The ideas of counting meconia and flag-tracking adults are not noevl to wasp researchers. However, illustrating these methods visually in a well-done videa could be a valuable contribution.

Thank you for giving me the opportunity to strengthen my manuscript with your valuable comments and queries.

Major Concerns:

None

Minor Concerns:

Lines 40-46: Careful when talking about fitness. Workers in social insect colonies do not directly count toward fitness- they are more like investment in body growth. Only the gynes (new queens) and males count toward fitness.

I have incorporated this comment in Line 51-57.

Line 57 correct: number of larval cells will could overestimate

I corrected it as you indicated (Line 66).

Line 122: Sufficient by what criterion? Explain…

That criterion is that standard error of the number of meconial per cell is within 0.05. I have added it (Line 145-146).

Line 143: This requires marking the wasps…

I agree with your comment. I added a sentence about marking wasps to identify individuals before an action required marking the wasps (Line 179-180).

In Figure 5, add a horizontal dashed line to indicate the target SE of 0.05.

I have incorporated this comment and added the horizontal dashed line in Figure 5.

Reviewer #3:

This is an interesting method that could be useful for a number of different wasp species, including many species of Polistes and Vespula. My main concern with the study is how do we know that this method is accurate. The author suggests that is a useful and accurate method, but doesn't provide data to assure us of this statement. I am, however, not sure how you'd test the method. You'd need to estimate the total number of workers produced over the lifespan of a nest and correlate that with the meconia count estimates. However, I don't think that is a problem for the publication of the method, but perhaps this issue should be acknowledged.

I understood the concern about accuracy of estimation the number of adults by counting meconia by reviewer3. Therefore, I have added a sentence to inform readers about it (Line376-377). I think this change now better.

Overall, I think this is an interesting method worthy of publication. The manuscript could use a degree of revision as outlined below. A key point might be to try and generalise the manuscript: at the moment it is focussed on the Japanese Vespula species. However, methods used for this species will not work for all Vespula.

Thank you for your suggestion. I have incorporated your comments in discussion (Line 396-399). I mentioned that this tracking method cannot be used for all vespula.

Key points:

-- On page 4 there is a single square bracket "[" that should be removed. There are also no units on the average wet weight of the Vespula worker. I'm not actually sure why the worker weight is needed?

I removed “[” as you indicated. I described the worker weight to readers to understand the relationship between the weight of an adult wasp and the flag.

-- In section 2.1 on Baiting, I don't think that the exact species of fish are

needed here. Japanese dace aren't frequently available in countries like New Zealand. I think just a description of the fish and approximate weight of fish is needed here. The suggested flag size must also be dictated by the size of the wasp and what it is able to carry.
I cancelled to describe the exact species of fish. Approximate weight of the pieces of fish is about 10mg.

-- On lines 195- 197 the author suggests stamping on the ground to have workers stay in the nest. For many species including those that I work with, stamping your foot on the ground would result in a flood of wasps coming out of the nest to attack.
Although the stamping on the ground is useful for collecting *V. shidai*, *V. flaviceps* and *V. vulgaris* nests in Japan. I understood that the stamping on the ground makes it hard to get nests in other species. Therefore, I have added a note that the stamping method may be not suitable for collecting nests in other species (Line 236-238).

-- Section 3 talks about "Rearing Vespula". The methods suggested are brief and may need more details. I'd also note that the nest box size and dimensions are very small for many Vespula. Too small for species like V. germanica or V. vulgaris.
I added a step (Line 275). I also added a note that the size of the nest box should be made according to each species (Line 264-265).

-- Lines 257-258. Why not look at the coefficient of variation here rather than the SE?
The number of individuals produced is calculated by multiplying the sample mean by total number of cells. Therefore, I believe that the standard error which represents the magnitude of error of the sample mean would be more appropriate than the coefficient of variation because easy to know the error of the estimation.

-- On lines 307- 309 you state that "our study shows that the estimate of the number of meconial provides a better estimate of the overall number of individuals produced and of the number of queen wasps produced (i.e., a better indicator of colony fitness". As noted above, how do you know that? What data do you have to make that comparative statement? The number of queens produced is really the key estimator of fitness, but there should only ever be one cohort of queens, so that there should only ever be one meconial deposit left in each queen cell. Also, the author switches between "I" and "we" and "our" in the paper. I suggest standardizing.

I have reflected this comment to the discussion (Line 369-371). I understood that I can not discuss about estimation of fitness from my data by your indication. I canceled to claim that the number of new queens produced (a part of the fitness) can be known by the number of meconia in queen combs as you indicated. I hope that you agree.

Sincerely,

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