# Studies on a novel control system of *Dermanyssus gallinae*

# ワクモの制御方法に関する研究

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# Chapter 1

# General introduction of *Dermanyssus gallinae*

### 1.1. Taxonomy and common name

*Dermanyssus gallinae* was first identified by De Geer in 1778. *D. gallinae* is a blood-sucking ectoparacite belongs to the parasitiformes order Mesostigmata (Gamasida), in the suborder Monogynaspida, cohort Gamasina, superfamily Dermanyssoidea, family Dermanyssidae (Lindquist et al., 2009). The common name is poultry red mite or chicken mite.

### 1.2. Life cycle

*Dermanyssus gallinae* has five development stages: egg, larva, protonymph, deutonymph, and adult (Maurer and Baumgärtner, 1992; Axtell, 1999). Eggs of *D. gallinae* are laid in cracks, crevices, or litter in the poultry house and hatch into six-legged larvae in 2 to 3 days. The larvae molt into protonymphs within 1 to 2 days without feeding. From protonymph stage, this mite has eight legs (Gray and Barry, 2009). The protonymphs feed and molt into deutonymphs within 1 to 2 days. Then the deutonymphs take another blood meal in order to molt into adults within 2 to 3 days. Adults mate soon after molting. After mating, females lay 1-9 eggs every 12 to 24 h following a blood meal. Females will lay about 30 eggs in their lifetime (Sparagano et al., 2014). It takes 7 days to complete development stages (egg-to-egg) under optimal conditions (Chauve, 1998).

### 1.3. Morphology

The egg of *D. gallinae* is small (0.4mm x 0.3mm), oval, smooth and pearly white (Chauve, 1998). The larva is white and same size with egg. From protonymph stages, the mite varies in length and in color from gray to brown/red depending on feeding status (Sparagano et al., 2014). Adult is about 1.0 - 1.5 mm in length (Sikes and Chamberlain, 1954; Sparagano et al., 2014). Adult has a prominent dorsal shield that is oval and tapers toward the posterior part (Di Palma et al., 2012; Murano, 2006). The posterior margin of the dorsal shield is linear or sharply curved. The mite's sternal shield has two pairs of setae. This mite has a roundish sub-triangular anal plate at posterior ventral surface. The anus is open on the posterior part of this plate. Adult female has long stylet-like chelicerae, and adult male has scissor-like chelicerae (Murano, 2006).

**1.4. Ecology**

*Dermanyssus gallinae* usually feeds for 30 to 90 minutes at night every 2 to 4 days (Maurer et al., 1988). This mite parasitizes on its host only during blood-feeding (Chauve, 1998). After blood-feeding, it spends away from host and hides in the cracks, crevices and litter in the poultry farm. Temperatures below -20°C and above 45°C are lethal for *D. gallinae* (Mul et al., 2009). The optimum conditions for growth of *D. gallinae* are at 25°C to 30°C with a humidity of 70 to 85% (Tucci et al., 2008). This mite can survive for 9 months without feeding (Nordenfors et al., 1999).

### 1.5. Hosts

*Dermanyssus gallinae* feeds blood of more than 30 species of domestic and wild birds (Roy and Chauve, 2007) and attacks laying hens in particular (Chauve, 1998; Sparagano et al., 2014). It occasionally bites mammals such as dogs (De Clerq and Nachtegaele, 1993; Ramsay et al., 1975), cats (Grant, 1989), horses (Mignon and Losson, 2008), rodents (Lucky et al., 2001) and even humans (Abdigoudarzi et al., 2014; Rosen et al., 2002; Sparagano et al., 2014) in the absence of birds.

### 1.6. Prevalence

*Dermanyssus gallinae* is distributed worldwide (Sparagano et al., 2014). The massive infestation causes serious problems for poultry production in Europe such as Demark, France, The Netherlands, Germany, Belgium (Mul, 2013), Norway (Øines and Brännström, 2011), Sweden (Höglund et al., 1995), Poland (Tomasz, 2003), Italy (Marangi et al., 2014), UK (Fiddes et al., 2005; Guy et al., 2004), and in United States (Gray and Barry, 2009; Ruff, 1999) and in Africa such as Kenya (Mungube et al., 2008) and Tunisia (Gharbi et al., 2013). Also in Asia, prevalence rate of *D. gallinae* in commercial egg layer farms is 64% and 85% in China (Wang et al., 2010) and Japan (Sparagano et al., 2009), respectively.

### 1.7. Consequences of infestation

*Dermanyssus gallinae* affect animal and public health in particular relating to humans closely associated with poultry as well as the productivity of the egg industry (Chauve, 1998; George et al., 2015; Sparagano et al., 2014).

### 1.7.1. Clinical sign in hens

In hens, *Dermanyssus gallinae* causes higher levels of preening, head-scratching, feather-pecking, cannibalistic behavior, decreasing of growth rates and feed conversion, anaemia and even death (Chauve, 1998; Flochlay et al., 2017; Mul et al., 2009). This mite can lead to decrease in egg quality due to blood spots on the shells and in egg production (Chauve, 1998; Cosoroabă, 2001). In addition, infested chickens fall into immunosuppression, such as the suppression of antibody production (Kaoud and El-Dahsham, 2010; Kowalski and Sokół, 2009) and downregulation of Th1 and pro-inflammatory cytokines/chemokines (Harington et al., 2010).

### 1.7.2. Clinical sign in humans

*Dermanyssus gallinae* can cause irritation, eczema, and allergic skin reactions (Cafiero et al., 2011; Potenza et al., 2008; Sahibi et al., 2008). Therefore, *D. gallinae* is being regarded as an occupational hazard for poultry farm workers in Europe (Cafiero et al., 2011). In Japan, infestation of *D. gallinae* have resulted in turnover of poultry farm employees (Murano, 2007; Murano et al., 2015).

### 1.7.3. Transmission diseases as a vector

*Dermanyssus gallinae* may serve as a disease vector for numerous pathogens including *Salmonella gallinarum* and *Salmonella enterica*, *Erysipelothrix rhusiopathiae*, *Listeria monocytogenes*, *Coxiella burnetii*, *Nocardia brasiliensis*, *Mycoplasma synoviae*, Newcastle disease, Fowlpox virus, Saint-Louis encephalitis, the Eastern, Western, and Venezuelan equine encephalomyelitis viruses and tick-borne encephalitis virus (Chu et al., 2014; George et al., 2015; Valiente Moro et al., 2005, 2009). Its vectorial role in maintaining these pathogens on poultry farms is enhanced by its strong viability enduring to long starvation (Sylejmani et al., 2016).

When *D. gallinae* attacks humans, there is a possiblility of transmission of spirochetes including *Borrelia burgdorferi*, *rickettsiae*, *Salmonellae*, *Bartonellae*, *Pasteurellae*, Sporozoa, hemogregarines, flagellates filariae and avian influenza A virus (Litwin, 1961; Sommer et al., 2016).

### 1.7.4. Economic impact

The annual economic cost for control of this mite and productivity losses caused by *D. gallinae* have been estimated to be approximately €130 million and €67 million in the European Union and Japan, respectively (Sparagano et al., 2009; van Emous, 2005). However, recently this estimated cost in Europe were revised upward to € 231 million (van Emous, 2017), due to an increase of laying

hen population and infestation rate of *D. gallinae.*

### 1.8. Control management

#### 1.8.1. Conventional methods

Cleaning of poultry farms in particular during the unoccupied period and maintaining good hygiene practices help to reduce mite populations of *D. gallinae*, while only these methods are not sufficient for control the mite in most cases. The control of *D. gallinae* has relied on the application of synthetic acaricides, over 35 compounds (e.g., organochlorines, organophosphates, pyrethrin, pyrethroids, carbamates, amitraz, and endectocides) (Chauve, 1998; Marangi et al., 2012; Thind and Ford, 2007). However, many countries now restrict acaricides which can be used against *D. gallinae* due to regulations on active ingredients. In addition, resistance of *D. gallinae* to acaricides has been reported in many countries, including Japan (Beugnet et al., 1997; Fiddes et al., 2005; Marangi et al., 2009; Murano et al., 2015; Nordenfors et al., 2001; Thind and Ford, 2007). For example, more than 60% of poultry farms had experienced infestations of acaricide-resistant mites in British (Guy et al., 2004). Moreover, the misuse and abuse of acaricides often result in residual acaricides in the organs and tissues of the laying hens (Marangi et al., 2012).

#### 1.8.2. Emerging and future control strategies

#### 1.8.2.1. Vaccines

A vaccine under development reduced number of *D. gallinae* in mite-infested birds (Bartley et al., 2015; Harrington et al., 2009). There is potential to develop a vaccine against *D. gallinae* based on somatic or recombinant proteins, but their effects is still insufficient.

#### 1.8.2.2. Biological methods

Biological control methods, such as natural enemies and entomopathogenic fungi, have been examined in *D. gallinae.* As candidates of the natural enemies, several predatory mites consuming *D. gallinae* has been confirmed (Ali et al., 2012; Lesna et al., 2009; Lesna et al., 2012). However, field effect was strongly depend on various factors such as temperature, alternative prey, contact frequency between natural enemies and *D. gallinae* (Ali et al., 2012; Lesna et al., 2012). Some entomopathogenic fungus such as *Metarhizium anisopliae* were fetal to *D. gallinae* under laboratory conditions (Kaoud, 2010; Tavassoli et al., 2011). However, their efficacy was insufficient even in semi field condition, because fungi require very high humidity or free waters to act.

#### 1.8.2.3. Physical methods

Heating poultry houses to above 45◦C is commonly applied for controlling *D. gallinae* in the Netherlands and Norway (Mul et al., 2009). The disadvantage of heat-treatment is its high cost and

damage to the equipment in poultry houses (Mul et al., 2009). Therefore, this method is not suitable to poultry houses which are large or have plastic equipment components (Mul et al., 2009).

Inert substances include diatomaceous earth, kaolin, and silicas. Many products of inert substances are widely used (e.g., InsectoSec, BIOFA, Germany, and Decimite+, BASF, Germany). These products absorb cuticular lipids from the surface of mites, resulting in death by dehydration (Vincent et al., 2003). However, these substances are difficult to apply in practical situations. For example, *D. gallinae* becomes active during hot and humid periods in the poultry house (Nordenfors and Höglund, 2000; Othman et al., 2012), but high humidity (>85%) reduces the efficacy of the inert substances (Kilpinen and Steenberg, 2009; Maurer et al., 2009). Their effect also was greatly affected by the quality of the raw materials (Mul et al., 2009; Schulz et al., 2014). In addition, silica products were banned in the Netherlands recently, because they pose serious risk for humans and animal due to irritation of respiratory tract caused by inhaled silica particles (Flochlay et al., 2017).

The acaricidal effects of oils against *D. gallinae* have been reported *in vitro*, but not in the poultry farm (Maurer et al., 2009). In addition, mineral oils, such as diesel, which was more toxic to phytoseiid mites than plant oils (Momen et al., 2006), are not recommended for use in poultry houses because of their unpleasant odor and the risk of egg contamination (Hoop, 2008).

**1.9. The aim of our study**

*Dermanyssus gallinae* can not be easily controlled due to an unclear optional timing for control of this mites in poultry farms. It is difficult for farmers to discover *D. gallinae* in early stages of infestation, because this mites is too small and hides during the daytime. After infestation of *D. gallinae* prevailed in a poultry house, it is practically impossible to control the mites because of its high viability (Nordenfors et al., 1999) with reproductive potential (Maurer and Baumgärtner, 1992). Therefore, to clarify optimal timing of treatment with synthetic acaricidal substances against this mites is important. Moreover, only applications of acaricides for controlling this mites has led a reduction of their effectiveness (Beugnet et al., 1997; Nordenfors et al., 2001) as well as residual acaricides in poultry products resulting in a threat for human health (Marangi et al., 2012). Therefore, alternatives control methods against *D. gallinae* have been required.

In the present study, we developed a “non-parallel board trap” which has high ability for capture of *D. gallinae* to monitor mite population in poultry farms. In order to clarify the indicator of optimal timing for treatment with acaricidal substances, we analyzed the relationship between the damage caused by mites and the mite population monitored using this trap. Furthermore, as an alternative control measure, a mixture of substances derived from food and food additives (SFF), including a polysaccharide extract from laver seaweed, was examined for acaricidal effects on *D. gallinae in vitro* and *in vivo*. Moreover, the mechanism of its acaricidal effect was examined.

# Chapter 2

# Development of a trap for *Dermanyssus gallinae*

## 2.1. Abstract

We have constructed a novel trap based on the behavior of the *Dermanyssus gallinae* and compared it with the other traps. First, we prepared two types of trap made of two overlapping boards. One type had the boards not touching each other (non-parallel board trap) and the other consisted of two boards kept apart along the entire board (parallel board trap). Then we compared the rate of collected mites. As the result, the non-parallel board trap collected more mites than the parallel-board trap. Next, to determine which materials would be most effective, we tested non-parallel board traps were made of cedar, Japanese cypress, bamboo and polyvinyl chloride. The cedar and Japanese cypress traps collected significantly more mites than the other traps. Furthermore, our non-parallel board trap made of cedar collected significantly more mites than the corrugated cardboard and thick card traps. In conclusion, the novel non-parallel board trap could capture the mites efficiently. This trap could be a useful tool for monitoring and exterminating mites and studying on the ecology of the poultry red mite in poultry farms.

## 2.2. Introduction

*Dermanyssus gallinae* sucks blood only at night and hides in cracks, such as the connection between cages, egg channels and conveyor belts for eggs during the daytime (Harrison, 1963; Oshio, 1979). Recently, it has been confirmed that *D. gallinae* parasitizes daytime and night without leaving poultry that is heavily infested by northern foul mite (Nakamae et al., 1997). It is difficult for farmers to discover *D. gallinae* in the early stages of infestation, because it is only about 1.0mm in length and hides during the daytime (Sikes and Chamberlain, 1954). After infestation of *D. gallinae* prevailed in a poultry house, it is practically impossible to control the mites because they increase in the poultry house rapidly due to high reproductive potential (Chuave, 1998). Therefore, Zenner et al. (2009) have suggested to determine the threshold for starting extermination based on population of *D. gallinae* monitored in poultry houses. Furthermore, we should take appropriate and effective measures at the early stages of infestation following to the threshold. In order to determine the optimal timing for control, it is necessary to use traps which have high ability for capture of *D. gallinae* as well as practicality for monitoring mite population. The trap made of corrugated cardboard (cardboard trap) (Nordenfors and Chirico, 2001) has been evaluated to be effective. However, it is impossible to visually count the number of mites in the cardboard trap without disassembling the trap. In addition, the cardboard trap is not suitable for monitoring since it is vulnerable to moisture. None of the other effective traps have been reported, and traps for *D. gallinae* are not generally used in Japan.

In this study, a novel trap with a higher capture rate than previously reported traps was developed.

## 2.3. Materials and methods

### 2.3.1. *Dermanyssus gallinae*

*Dermanyssus gallinae* used in this study were collected from one layer farm. Then we identified them as *D. gallinae* with a stereomicroscope morphologically (Baker et al., 1956; Moss, 1968; Oshio, 1979). Next, in order to breed *D. gallinae*, this mites were kept in a mouse rearing container (46.0cm x 46.0cm x 60cm) along with a young chicken for blood sucking (Boris Brown or Julia between 7 and 30 days old, male) and cedar traps for proliferation of *D. gallinae* (proliferation traps). We confirmed that *D. gallinae* had naturally proliferated in the proliferation traps after regular observation. Furthermore, in order to prevent escape of the mites, the container was sealed with adhesive tape, its inner sides was lubricated with vaseline, and it was soaked in a limonene solution.

### 2.3.2. General test methods

A rearing container with over 50 mites as described above was placed in an incubator set at 28°C and 75% relative humidity (RH). The traps for the test were placed on both sides of the proliferation trap overnight (Fig. 2. 1). Then the number of mites caught in the traps was counted using a magnifying glass. Since the test was conducted simultaneously with proliferation of the mites, the

number of mites in the container fluctuated. Therefore, we evaluated the ratio of the number of mites caught in the test traps to the number of mites in the proliferation trap as the capture rate. For animal welfare, several chickens were prepared and rotated for blood sucking. The time for a single chicken in the rearing container was less than 15 hours.

### 2.3.3. Development of the trap

#### 2.3.3.1. Gap shape

Two traps were made from 2 wood pieces each (4.5cm x 8.5cm x 1.0cm), and a gap was made using a hinge (length 25mm) to the short sides of the wooden pieces and connecting the two pieces. One type was a non-parallel board trap, as the opposite side from the hinge was allowed to touch (Fig. 2. 2 A-a). The other type of trap was inspired by Hagimori et al. (2004) and small wooden dowel was placed in the gap on the opposite end from the hinge, resulting in the wooden pieces being parallel (Fig. 2. 2 A-b). Both traps were made of cedar wood with equal distances between the wood at the hinged end. We then compared the capture rate of *D. gallinae.* Experiments were performed five times.

#### 2.3.3.2. Gap width

In order to determine the gap width of the hinged end, the gap width of 1.0mm, 2.0mm and 3.0mm were examined. The trap with a 3.0mm gap width was used as a standard and traps with the other gap widths were simultaneously examined. Experiments were performed three to five times.

#### 2.3.3.3. Trap materials

Finally, in order to select the best material with high capture rate, non-parallel board traps with a gap width of 3.0mm were made of cedar, Japanese cypress, bamboo and polyvinyl chloride. The cedar trap was used as a standard and the other traps were simultaneously examined. Experiments performed four to five times.

### 2.3.4. Comparison between the reported traps and our developed trap

The other traps were made according to the previous report (Nordenfors and Chirico, 2001) as a base. Corrugated cardboard (3.0 mm thick) was cut into 10cm x 7cm. For another type, we used thick cardboard (210 g/m$^2$, size 20cm x 7cm) folded in half based on the previous report by Levot (1991). Specifically, we put 100 adult mites (male and female) saturated with blood, and the two traps into a rearing container. After a night, we then recorded the number of mites collected in each trap. Experiments were replicated six times.

### 2.3.5. Statistical analysis

The data was analyzed using statistical software R-3.0.1. An F-test for equality of variance was performed after checking the normal distribution using the Kolmogorov-Smirnov verification method. According to these results, a Student's t-test or Welch's t-test were carried out. The significance level was set to $p < 0.05$. The capture rate of the mite was arcsine-transformed and statistically processed.

## 2.4. Results and discussion

Gap shape of the traps did not significantly affect their capture of *D. gallinae*. However, at all gap widths, there was a tendency that the non-parallel board trap had a higher capture rate than the parallel board trap (Table 2. 1). In the parallel board trap, *D. gallinae* developed clusters against the small wooden dowel on the unhinged end of the trap (Fig. 2. 2 B-b). In contrast, the mites congregated along the unhinged side of the trap in the non-parallel board trap (Fig. 2. 2 B-a). This difference may be affected by darkness of the gap in the non-parallel board trap that had less light than the parallel board trap, because *D. gallinae* avoid light. In addition, the non-parallel board trap with a variation in the gap may have easily captured different stage of the mites who prefer a tight fit. Moreover, the non-parallel board trap is more practical than the parallel board trap, because it is simple and stable.

Although the gap width test showed no statistically significant differences, there was a tendency that traps with a 3.0mm gap width captured more mites than the others (data not shown).

As for comparison of trap material, the cedar trap had significantly ($p$ <0.01) higher capture rate of *D. gallinae* than those made of bamboo or polyvinyl chloride (Table 2. 2). The Japanese cypress trap had the same capture rate as well as the cedar trap. Among the test materials in this study, polyvinyl chloride was better than the other materials in reusability. *Dermanyssus gallinae* sometimes develop clusters on the polyvinyl chloride feeding troughs. However the polyvinyl chloride was inferior to cedar in its capture rate, even when wood derived ingredients and scents were added or the inside of the trap was made to be rough (data not shown). In the case of the poultry red mite, protonymphs have the highest survival rate at 70 to 90% RH (Nordenfors et al., 1999). Since cedar and Japanese cypress absorb and retain much more moisture than polyvinyl chloride, mites must prefer the cedar and Japanese cypress traps.

As the result, we chose the non-parallel boards made of cedar, which costs less than Japanese cypress, with a gap width of 3.0mm as our developed trap.

Our developed trap captured significantly ($p$ <0.01) more mites than the cardboard trap or the thick card trap (Table 2. 3). This indicated that our novel developed trap is superior to the previous traps in capturing *D. gallinae*. The thick card trap is similar in design to our developed trap. Therefore, we assumed that the difference in capture rates might be related to properties of the trap surface and ingredients contained in the material.

Mul et al. (2009) described four types of traps for the poultry red mite: ADAS traps, cardboard traps, perch traps and tube traps. Several studies on the dynamics of populations of *D. gallinae* using traps have already been reported (Harrison, 1963; Levot, 1991; Nordenfors and Chirico, 2001; Zener et al., 2009). Of these four traps, the cardboard trap was effective to some extent. Even though this trap is useful for extermination, it is difficult to count number of mites in the trap. For monitoring purposes, we have to take apart its cardboard before counting the mites. In addition, this trap xould not be reused, if this trap was wet (Zenner et al., 2009). In contrast, our developed trap makes it easy to count the number of mites, and its capture ability is not easily affected by moisture. As the result, our developed trap is superior in usefulness and capture ability. Therefore, our trap could be an ideal tool to monitor an accurate mite population in the farm.

In application of mite trap, an exterminating method for *D. gallinae* using cardboard traps containing neem oil or acaricides has been reported (Chirico and Tauson, 2002; Lundh et al., 2005). In addition, using cardboard traps only as a capturing tool by discarding the trap after mites are captured can also be considered. If our developed trap is available, our trap would be comparatively effective due to its higher capture ability.

The ecophysiology of the poultry red mite is unclear. The effective accumulated temperature for *D. gallinae* has been still unknown although appearance of many agricultural pests can be predicted

using effective accumulated temperature. By using our developed trap as observation tool of mites, the effective accumulated temperature for *D. gallinae* must be clarified.

**2.5. Figure legends**

Fig. 2. 1 Schematic diagram of trap development test.

Fig. 2. 2 Overview of two types of traps

A Outer view of traps

B Opened traps (many mites collected inside trap)

a : non-parallel board trap  b : parallel board trap

**Table 2. 1 Comparison of capture rate of *Dermanysus gallinae* between traps with different gap widths**

| Gap width | Capture rate of *D. gallina* [1)] (%) | |
|---|---|---|
| | Non-parallel board trap | parallel board trap |
| 1.0mm | 32.3±36.1 | 7.9±9.8 |
| 2.0mm | 10.1±4.2 | 7.2±9.8 |
| 3.0mm | 16.5±13.2 | 10.1±7.0 |

[1]Capture rate of *D. gallinae* = (number of mites in test trap/number of mites in proliferation trap) x 100

**Table 2. 2 Comparison of capture rate of *Dermanysus gallinae* between traps made of different materials**

| Materials | | | Capture rate of *D. gallinae*[1)] (%) | | |
|---|---|---|---|---|---|
| Cedar | : | Japanese cypress | 22.1±11.6 | : | 18.5±11.0 |
| Cedar | : | Bamboo | 11.6±5.8[A] | : | 0.1±0.2[B] |
| Cedar | : | Polyvinyl chloride | 55.6±9.1 [A] | : | 0.8±1.1[B] |

[1]Capture rate of *D. gallinae* = (number of mites in test trap/number of mites in proliferation trap) x 100

[AB]Different alphabet letters indicate significant difference ($p$ <0.01)

**Table 2. 3 Comparison of number of *Dermanysus gallinae* captured by our developed trap and previous traps**

| Type of trap | | | Number of mites per trap | | |
|---|---|---|---|---|---|
| Developed trap[1)] | : | Cardboard trap | 78±18.6$^{A}$ | : | 6±3.8$^{B}$ |
| Developed trap | : | Thick card trap | 87±9.7$^{A}$ | : | 0±0.4$^{B}$ |

[1]The developed trap is non-parallel with a 3.0mm gap, cedar wood

$^{AB}$Different alphabet letters indicate significant difference ($p$ <0.01)

**Fig. 2. 1**

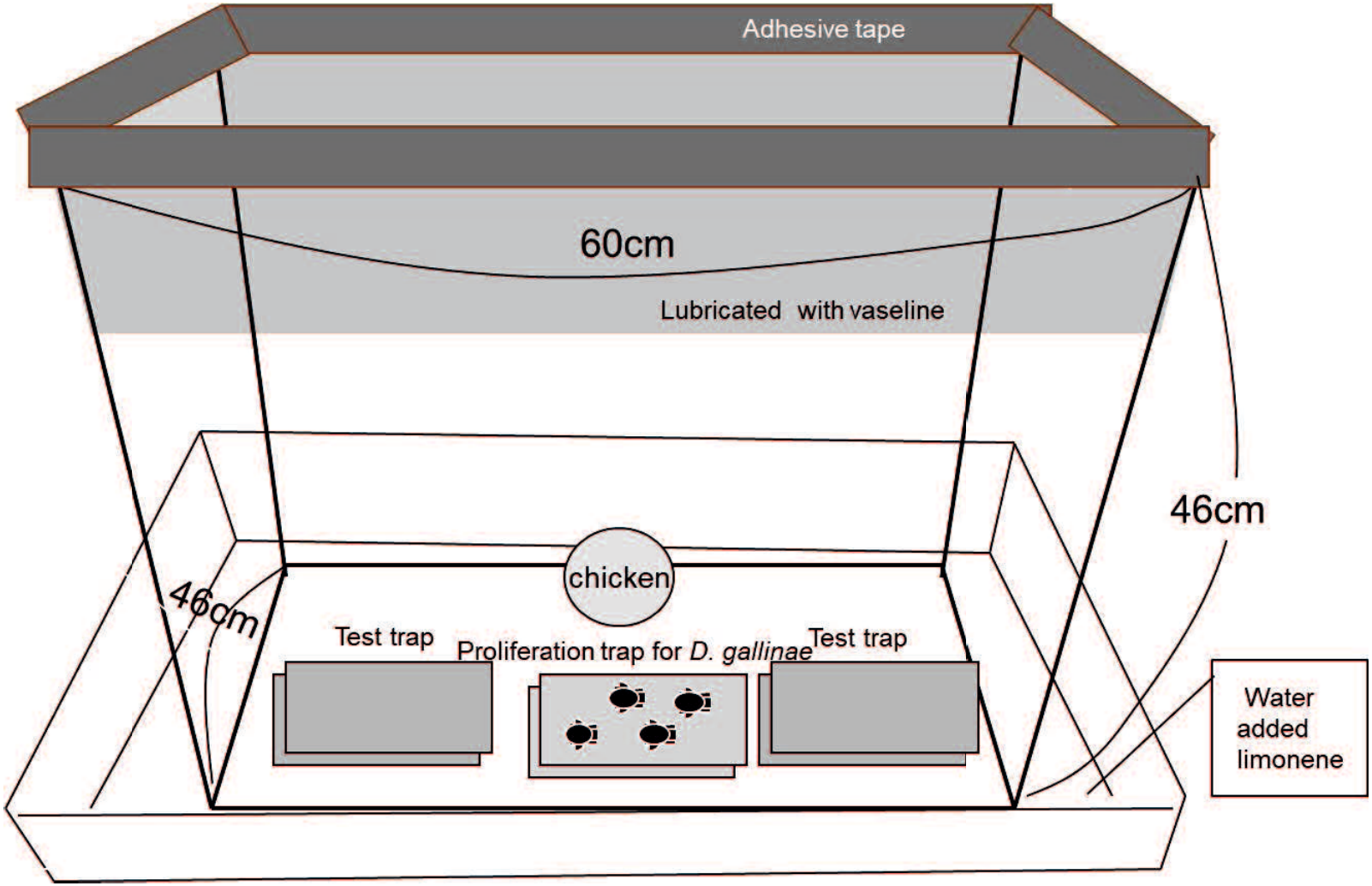
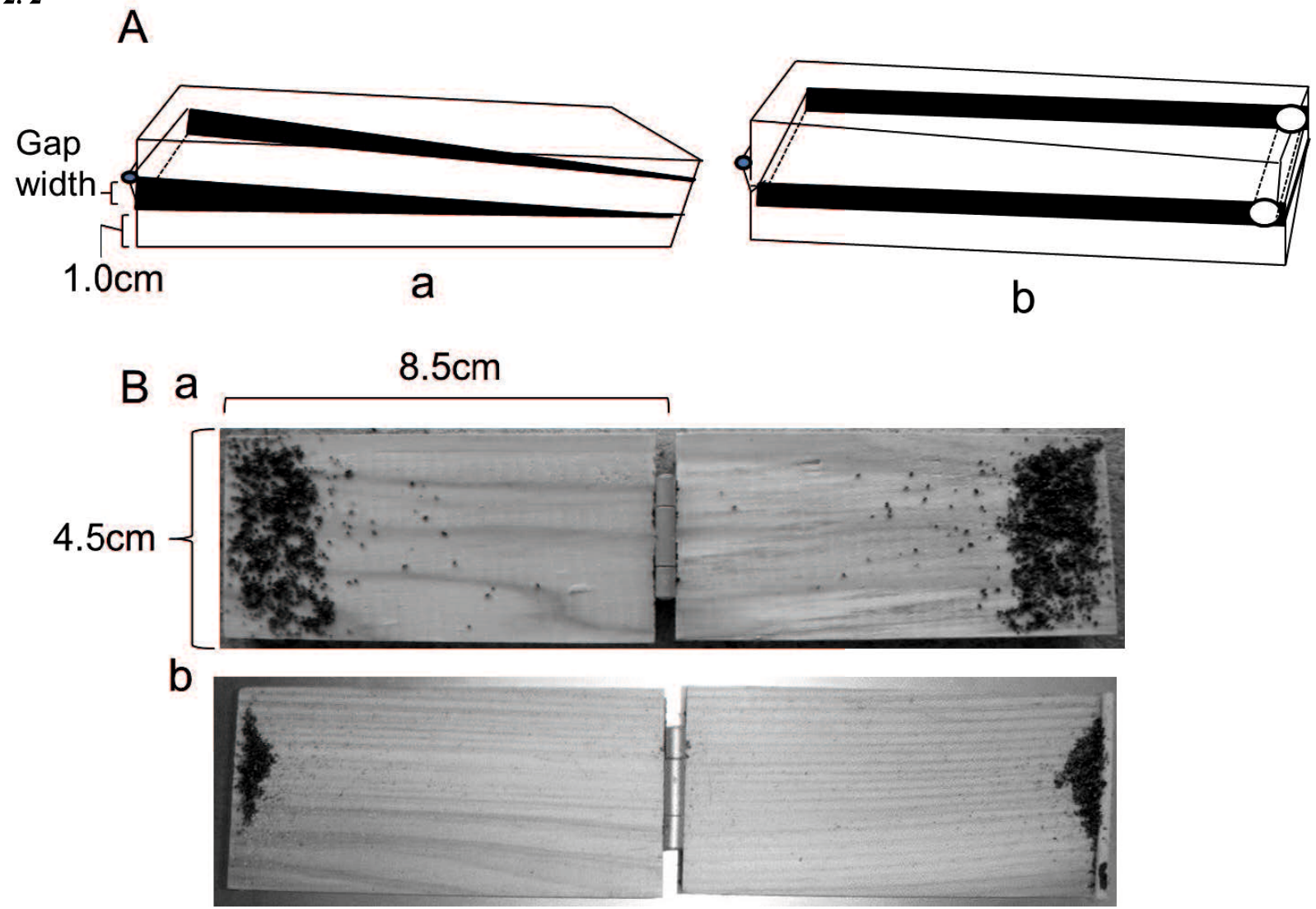

Adhesive tape
60cm
Lubricated with vaseline
46cm
46cm
chicken
Test trap
Proliferation trap for D. gallinae
Test trap
Water added limonene

**Fig. 2. 2**



# Chapter 3

## Correlation between the proportion of stained eggs and the number of mites (*Dermanyssus gallinae*) monitored using a "non-parallel board trap"

### 3.1. Abstract

The poultry red mite (*Dermanyssus gallinae*) is a serious problem for the poultry industry worldwide. However, the relationship between the mite population and the damage that they cause is still unclear. In this study, the mite population in poultry houses was examined using an established trap method, and the risk of blood-stained eggs caused by the mites was assessed. Traps were placed once a week outside the egg channels and/or on the floor in two poultry farms in Fukuoka Prefecture, Japan, from April 2012 to July 2014. The numbers of blood-stained eggs and total eggs were counted at weekly intervals. The results showed that the number of mites increased from April to May, and reached a peak around the beginning of June when the average temperature and humidity were >24°C and 70-90%, respectively. In the segmented model, the correlation between the proportion of blood-stained eggs and the number of mites or temperature was positive over a threshold. In conclusion, our established trap method is useful for monitoring mites and can be used to predict when poultry farms should be treated to prevent appearance of blood-stained eggs.

### 3.2. Introduction

Poultry red mite is not easily controlled and once a poultry house is infested it is very difficult to eradicate them (Chauve, 1998) because they can survive long periods of starvation (Nordenfors et al., 1999). Furthermore, it is almost impossible to avoid damage caused by poultry red mite after infestation has spread in a poultry house because *D. gallinae* has a high reproduction potential (Maurer and Baumgärtner, 1992). *D. gallinae* is small and hides during the daytime which makes it difficult for farmers to observe them. In addition, resistance of poultry red mite to various synthetic products has been reported in many countries (Beugenet et al., 1997; Fiddes et al., 2005; Marangi et al., 2009; Nordenfors et al., 2001; Thind and Ford, 2007). In Japan, resistance against all commercial acaricides has increased recently (Murano et al., 2015). Problems of resistance to synthetic products have likely worsened due to their misuse. Also acaricide residues in laying hens, eggs and muscle have been reported (Aulakh et al., 2006; Marangi et al., 2012).

A sensitive method is therefore required for monitoring populations of *D. gallinae* on farm to understand the problem and to prevent the damage caused by the mite. Also, analysis of the correlation between the damage caused by mites and the mite population can identify indicators which can be used to control infestations by treatment for poultry red mite. Several studies on the dynamics of populations of *D. gallinae* have already been reported (Nordenfors and Chirico, 2001; Nordenfors and Höglund, 2000; Zenner et al., 2009). However, the relationship between the mite population and the

proportion of blood-stained eggs is still unclear.

In this study, the population of poultry red mite in poultry houses was examined using our established trap which is more sensitive than the previously reported traps (Odaka et al., 2014) and the risk of blood-stained eggs caused by mites was assessed. In addition, the relationship between environmental factors in the poultry house and the mite population or the risk of blood-stained eggs was revealed.

## 3.3. Materials and methods

### 3.3.1. Experimental sites and animals

This study was performed from April 2012 to July 2014 in two poultry farms (A and B) in Fukuoka Prefecture, Japan. Farm A is located at the southwestern part of Fukuoka Prefecture and the other farm B is located 44km apart from Farm A. These farms have cage-system poultry houses which are common in Japan. Farm A is a low floor poultry house with approximately 320 hens housed in two rows of two level metal cages (Table 3. 1). Farm B has a high-rise floor poultry house where excretion from hens accumulates on the lower floor with approximately 250 hens housed in two rows of two level metal cages (Table 3. 1). The hens were kept at their farms for approximately 10 or 18 weeks (Table 3. 1) and *D. gallinae* had already been observed at both farms.

All examination in this study were carried out under permission by the farm owners and any special treatment to chicken were not performed.

### 3.3.2. Measurement of the population of mites using traps

Our previously developed “non-parallel board trap” (Odaka et al., 2014) was used for monitoring the population of mites. This trap consisted of two cedar boards (4.5 cm×8.5 cm×1.0 cm each) connected at one end by a hinge.

The traps were placed outside the egg channel using a hook and rubber band (egg channel

traps), and on the floor (floor traps) at intervals of ten cages once a week. Traps were collected after 24 h and the mites in the traps were immediately put into sealed bags. The bags were cooled to 4 °C and images were captured with a digital camera before the mites in the bags were returned to each sampling place. Then, the number of mites in each picture (4,000 × 3,000 pixels) was counted.

The number of traps placed at each farm is shown in Table 3. 1. Traps were placed in only half of the space in the poultry house at farm A in 2013 and only egg channel traps were used for the survey at farm B.

### 3.3.3. Environmental data

Temperature and humidity were recorded hourly by more than one automatic logger (OndotoriTM, T and D corporation, Nagano, Japan) at both farms during the survey except during the middle of April 2012 and March 2014. Average temperature and humidity were calculated daily.

### 3.3.4. The number of blood-stained eggs

To elucidate the effect of *D. gallinae* on egg production, the numbers of blood-stained eggs and total eggs were counted once a week. The proportion of stained eggs was calculated by dividing the number of stained eggs by the total number of eggs.

### 3.3.5. Statistical analysis

The number of mites collected by egg channel traps or floor traps at farm A plus 0.5 per trap was logarithmically transformed. Means were compared between egg channel traps and floor traps separately for each collection using analysis of variance.

The average number of mites collected by egg channel traps or floor traps per collection at farm A plus half of the discrete unit, 0.5/m (m: number of traps), was logarithmically transformed (Yamamura, 1999; Yamamura, 2016). The relationship between the number of mites collected using the two traps was examined by linear regression analysis.

We applied a segmented regression model to examine the relationship between the proportion of stained eggs and the number of mites, assuming that egg staining is negligible when mite abundance is below a certain level. In this analysis, the proportion of stained eggs was arcsine-square-root transformed and the average number of mites per collection at both farms was logarithmically transformed after processing as described above to perform a linear regression analysis.

This model can be formulated as follows.

$y=0$ if $x \leqq b$ (breakpoint)

$y=a(x-b)$ if $x > b$ (breakpoint),

where y is arcsine-square-root transformed proportion of stained eggs, x is the log-transformed (N (average number of mites per collection) + 0.5/m (number of traps)), and a and b are slope and

breakpoint to be estimated.

The segmented regression model was also applied to examine the relationship between the proportion of stained eggs and the average daily temperature when the survey for stained eggs was performed, assuming that egg staining is negligible when the temperature is below a certain level. In this analysis, x was the average temperature as an independent variable instead of the number of mites. Four missing data points of the average temperature at farm A and two at farm B were complemented with predictive values according to the observatory data by the Meteorological Agency. A 95% confidence interval of the breakpoint (b) and the slope (a) was estimated using bootstrapping repeated 10,000 times. The significance level was set to $p < 0.05$. These analyses were performed using the R i386 3.0.1 and Microsoft Excel 2013.

## 3.4. Results

### 3.4.1. Relationship between the number of mites and environmental factors

The fluctuations in mite population are shown in Figs. 3. 1 and 3. 2. The number of mites were shown as logarithmically transformed (N+ 0.5) to avoid problems caused by 0. The number of mites started to increase from April to May. Data from Farm A in 2013 (Fig. 3. 1 b-2013) and Farm B in 2014 (Fig. 3. 2 b-2014) indicate that the number of mites reached a maximum near the beginning of June, after the average temperature was over 24°C and humidity was 70-90%.

### 3.4.2. Comparison between egg channel and floor traps

In 2012, the floor traps collected more mites than the egg channel traps (Fig.3. 1 b-2012). The average number of mites in the floor traps was between 1.3 to 20 times greater than the numbers in the egg channel traps. Significant differences ($p$ <0.01 or $p$ <0.05) in the number of mites were observed between the egg channel traps and the floor traps except on 24th April. In 2013, the egg channel traps collected more mites than the other traps in most sampling periods between February and late May (Fig. 3. 1 b-2013). In June, more mites were collected in floor traps than in egg channel traps. Significant differences ($p$ <0.01 or $p$ <0.05) in the number of mites were observed between the two traps on 19th March, 12th June and 23rd June.

On the other hand, the results of linear regression analysis showed that the number of mites

trapped in the egg channel was positively correlated with the number trapped on the floor ($p$<0.01) (Fig. 3. 3).

### 3.4.3. Relationship between blood-stained eggs and mite numbers

At farm A, the stained eggs were first observed on 2nd May 2012 (Fig. 3. 1 b-2012) and 19th March 2013 (Fig. 3. 1 b-2013). The corresponding average number of mites per egg channel trap on these dates was 68.0 (2012) and 57.8 (2013) and for floor traps was 770.2 (2012) and 14.0 (2013). At farm B, the stained eggs were first observed on 22nd May 2014 and the average number of mites per egg channel trap was 131.9 (Fig.3. 2 b-2014). The proportion of stained eggs then increased as the number of mites increased at both farms.

The segmented model for the relationship between the proportion of stained eggs and the number of mites in the egg channel traps fits the observed data very well with estimated parameters, a=13.18 (95% CI: 10.02-16.72) and b=1.52 (95% CI: 1.32-1.67; $R^2$=0.84, $p$ <0.00001; Fig. 3. 4A). The model indicated that stained eggs occur when mite density in the egg channel trap exceeds a threshold of 101.52, 33.11-0.5/m (m; number of traps), and beyond the threshold the arcsine-square-root transformed proportion of the stained eggs increased by a slope of 13.18 per 10 times increase in mite density. Similarly, the segmented model fits well with the observed relationship between the proportion of stained eggs and the mite density in the floor trap with estimated parameters, a=13.61

(95% CI: 10.86-18.37) and b=1.82 (95% CI: 1.61-2.08; $R^2$=0.87, $p$ <0.00001; Fig. 3. 4B). The model indicated that stained eggs occur when mite density in the floor trap exceeds a threshold of 101.82, 66.07-0.5/m (m; number of traps).

### 3.4.4. Relationship between stained eggs and average temperature

The segmented model also fitted well with the observed relationship between the proportion of stained eggs and the average daily temperature with estimated parameters. The model indicated that the stained eggs occur when the temperature is > 16.95°C (95% CI: 13.01-18.82°C, $R^2$=0.81, $p$ <0.00001; Fig. 3. 5), and beyond the threshold the arcsine-square-root transformed proportion of stained eggs increased by a slope of 2.58 (95% CI: 2.35-3.90) per 1°C increase in temperature.

### 3.5. Discussion

We found an increase in mite numbers during hot and humid periods (between May and June) in accordance with previous reports (Murano et al., 2013; Nordenfors and Höglund, 2000; Othman et al., 2012). In addition, the optimum conditions for growth of *D. gallinae* were reported to be at temperatures between 25°C and 30°C with a humidity of 70-85% (Tucci et al., 2008). Our observation of the mite populations is almost consistent with this previous report. The number of mites decreased gradually because *D. gallinae* is very sensitive to humidity (Chauve, 1998) and the high humidity during the rainy season (end of June/beginning of July) in Japan would have had an effect on the growth of the mites. A similar phenomenon has been reported in Japan previously (Murano et al., 2013).

A difference in the number of mites collected by the egg channel traps and the floor traps was observed between 2012 and 2013 at farm A. Maurer (1993) reported that poultry red mite drops onto the floor after feeding to seek shelter, manure and litter, where they are able to hide. Mites which drop onto the floor may have preferred traps to shelter in because manure was scraped out every day at farm A. Moreover, the temperature in April 2012 was higher than in April 2013. The number of mites in the floor traps would be influenced by temperature because increasing temperature is effective in activating *D. gallinae* (Kilpinen, 2001). However, the number of mites in both traps was positively correlated, indicating that both could be good methods for monitoring the mite population. On the

other hand, the above results suggested that the number of mites in the floor traps may be different depending on the poultry house structure. Therefore, we recommend that the egg channel traps might be more useful for monitoring mites in cage-system poultry houses.

We have focused on blood-stained eggs as a consequence of poultry red mite. In Japan, poultry farm workers sometimes leave their jobs (Murano et al., 2008; Murano et al., 2015) because they have been suffering from transient skin irritation, more serious eczema and even allergic reactions (Cafiero et al., 2011; Potenza et al., 2008; Sahibi et al., 2008). In our preliminary study, workers started to complain about the mites when the stained eggs were first observed at both farms (data not shown). van Emous et al. (2005) reported that substantial infestations by the mites may increase blood-stained eggs. However, the relationship between the stained eggs and the number of poultry red mite in poultry houses was unknown. In this study, we applied the segmented regression model which provided a guideline for mite density and temperatures which can be used for mite control measures. In this model, the correlation between the proportion of stained eggs and the number of mites was positive over a threshold. In particular, the number of mites using the egg channel trap indicated a clear minimum mite density when stained eggs occur. The number of mites using floor traps and temperature did not present clear thresholds, but below these thresholds the proportion of stained eggs was small anyway.

The proportion of stained eggs in Farm A were higher than in Farm B. Furthermore, the average temperature around farm A was about 0.4°C higher than those around Farm B from April to

July. Therefore, the rapid increase in the proportion of stained eggs in Farm A might have been influenced by average temperatures. Further analysis is required to clarify the relationship between the average temperature in a poultry house and the proportion of stained eggs.

In conclusion, we confirmed that monitoring mites with our established traps is a useful method for identifying the time periods where application of mite control measures may be implemented to prevent the appearance of stained eggs. By monitoring poultry red mite, the economic damage caused by them and the resistance to synthetic acaricides may be reduced and the risk of poultry product residues could be minimized. Control of poultry red mite under the average number of mites (N), 33.11-0.5/m (m; number of traps) using egg channel traps, is likely to be effective.

### 3.6. Figure legends

Figure3. 1 Environmental data (a) and dynamics of mites and the proportion of blood-stained eggs (b) at farm A. Horizontal dotted line indicates the average temperature of 24°C. Asterisks (* and **) indicate significant differences ($p$ <0.05 and $p$ <0.01, respectively) between egg channel traps and floor traps. N: the average number of mites collected by egg channel traps or floor traps.

Figure 3. 2 Environmental data (a) and dynamics of mites and the proportion of blood-stained eggs (b) at farm B. Horizontal dotted line indicates the average temperature of 24°C. N: the average number of mites collected by egg channel traps.

Figure 3. 3 Comparison of the number of mites in egg channel traps and floor traps. Values of $R^2$ and $p$ were obtained from linear regression analysis. N: the average number of mites collected by egg channel traps or floor traps, m: number of traps

Figure 3. 4 Relationship between the proportion of stained eggs and the average number of mites collected by the egg channel traps (A) or the floor traps (B). Segmented linear models were plotted after re-transformation from arcsine-square-root values. Values of $R^2$ and $p$ were obtained from linear

regression model in which the proportion of stained eggs was arcsine-square-root transformed. N: the average number of mites collected by egg channel traps or floor traps, m: number of traps

Figure 3. 5 Relationship between the proportion of stained eggs and average temperature. Segmented linear model was plotted after re-transformation from arcsine-square-root values. Values of $R^2$ and *p* were obtained from linear regression model in which the proportion of stained eggs was arcsine-square-root transformed.

**Table 3. 1 Hens and mite traps used in this study**

| | Farm A | Farm B |
|---|---|---|
| Number of hens | 320 (2012)<br>160 (2013) | 250 (2014) |
| Age of hens (weeks old) | 55-64 (2012)<br>53-70 (2013) | 48-65 (2014) |
| Commercial layers | Hy-Line Brown, Lohman LSL | Hy-Line Brown |
| Trap durance | 24 hr | 24 hr |
| Number of traps | 32 egg channel traps (2012)<br>16 floor traps (2012)<br>16 egg channel traps (2013)<br>8 floor traps (2013) | 24 egg channel traps (2014) |

**Fig. 3. 1**

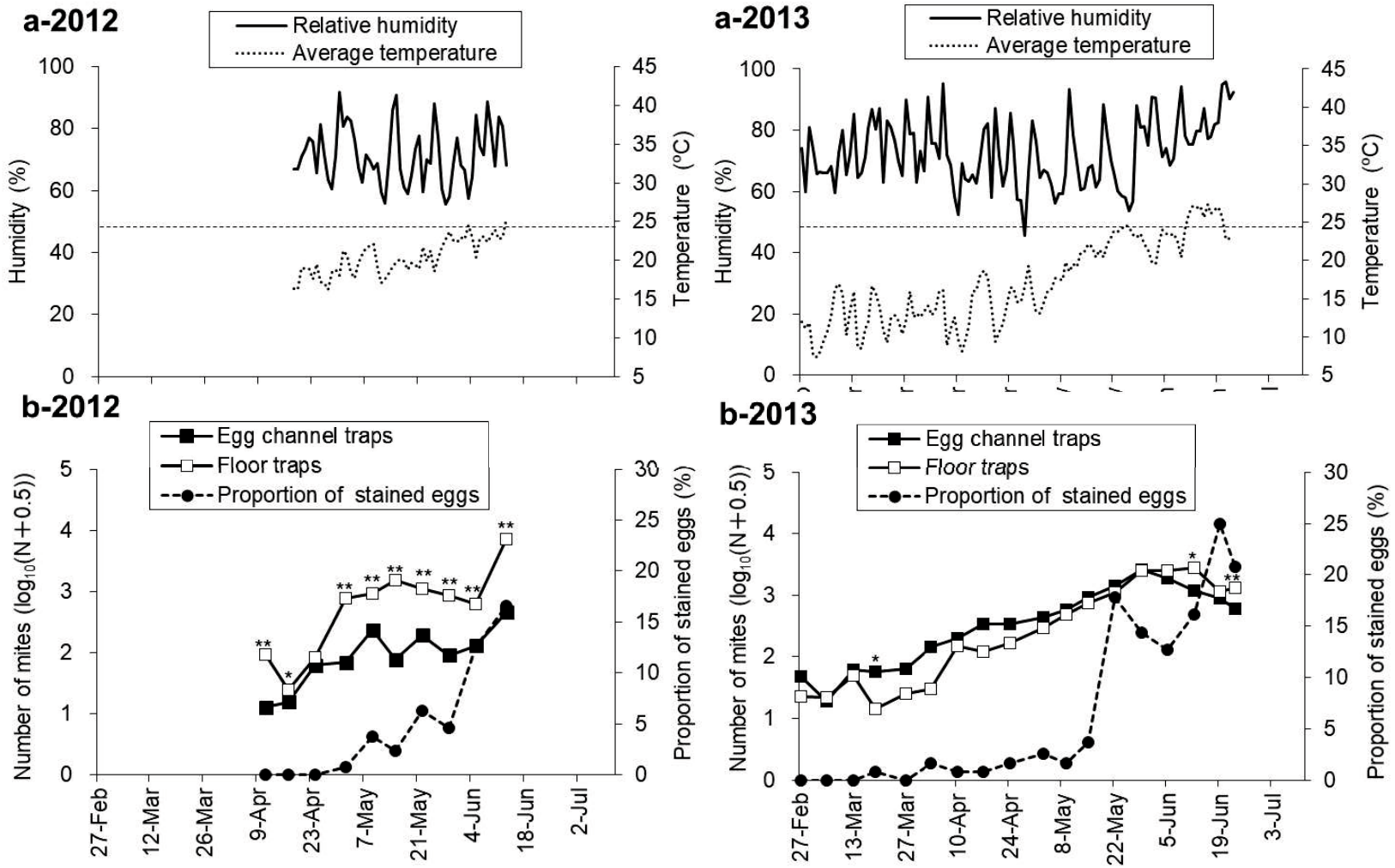

a-2012
Relative humidity
Average temperature
Humidity (%)
Temperature (°C)
a-2013
Relative humidity
Average temperature
Humidity (%)
Temperature (°C)
b-2012
Egg channel traps
Floor traps
Proportion of stained eggs
Number of mites (log10(N+0.5))
Proportion of stained eggs (%)
27-Feb
12-Mar
26-Mar
9-Apr
23-Apr
7-May
21-May
4-Jun
18-Jun
2-Jul
b-2013
Egg channel traps
Floor traps
Proportion of stained eggs
Number of mites (log10(N+0.5))
Proportion of stained eggs (%)
27-Feb
13-Mar
27-Mar
10-Apr
24-Apr
8-May
22-May
5-Jun
19-Jun
3-Jul

**Fig. 3. 2**

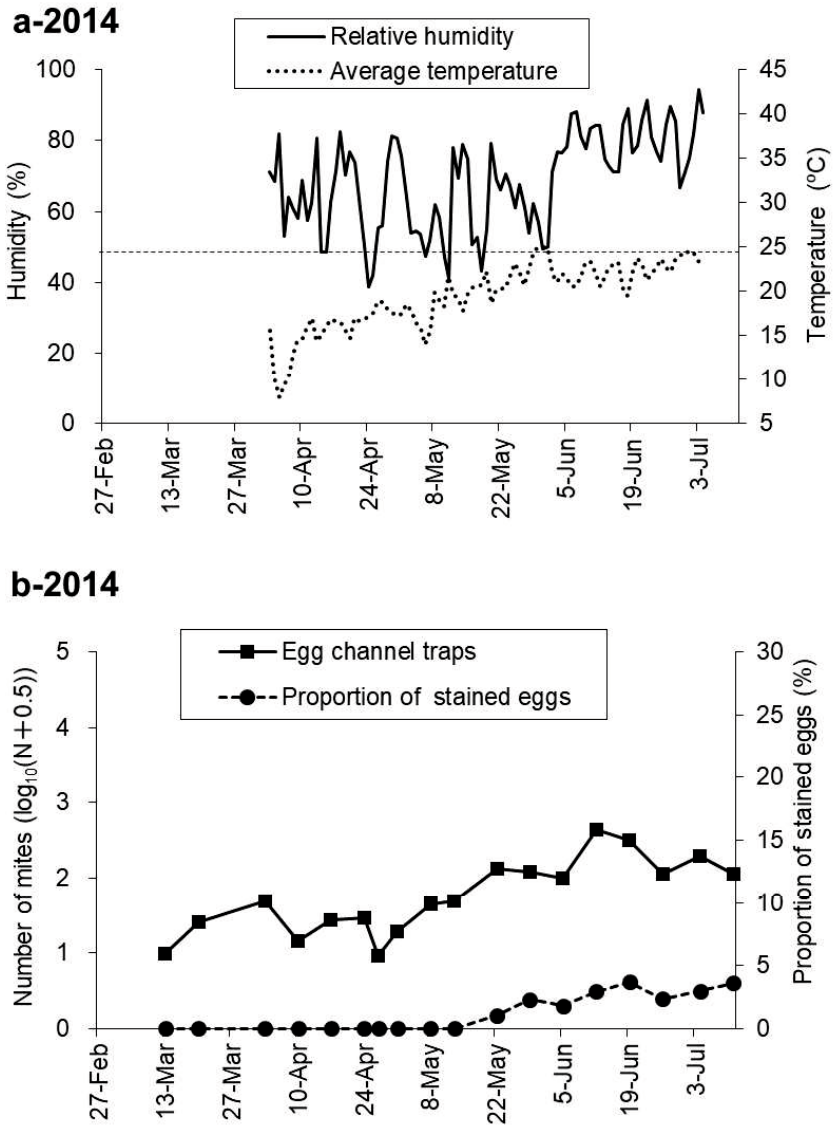

a-2014
Relative humidity
Average temperature
Humidity (%)
Temperature (°C)
100
80
60
40
20
0
45
40
35
30
25
20
15
10
5
27-Feb
13-Mar
27-Mar
10-Apr
24-Apr
8-May
22-May
5-Jun
19-Jun
3-Jul
b-2014
Egg channel traps
Proportion of stained eggs
Number of mites (log10(N+0.5))
Proportion of stained eggs (%)
5
4
3
2
1
0
30
25
20
15
10
5
0
27-Feb
13-Mar
27-Mar
10-Apr
24-Apr
8-May
22-May
5-Jun
19-Jun
3-Jul

**Fig. 3. 3**

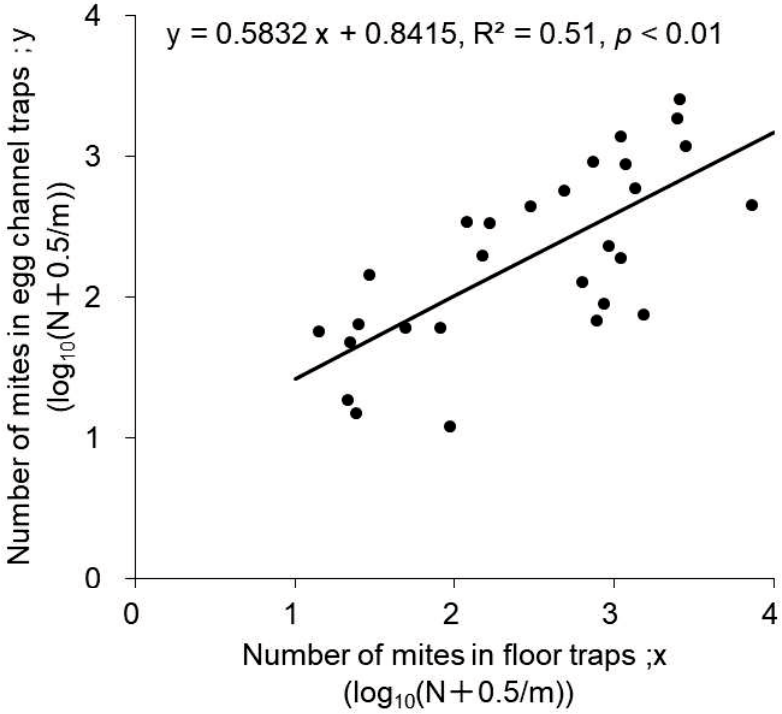

y = 0.5832 x + 0.8415, R² = 0.51, p < 0.01
Number of mites in egg channel traps ;y
(log10(N+0.5/m))
Number of mites in floor traps ;x
(log10(N+0.5/m))
0
1
2
3
4

**Fig. 3. 4**

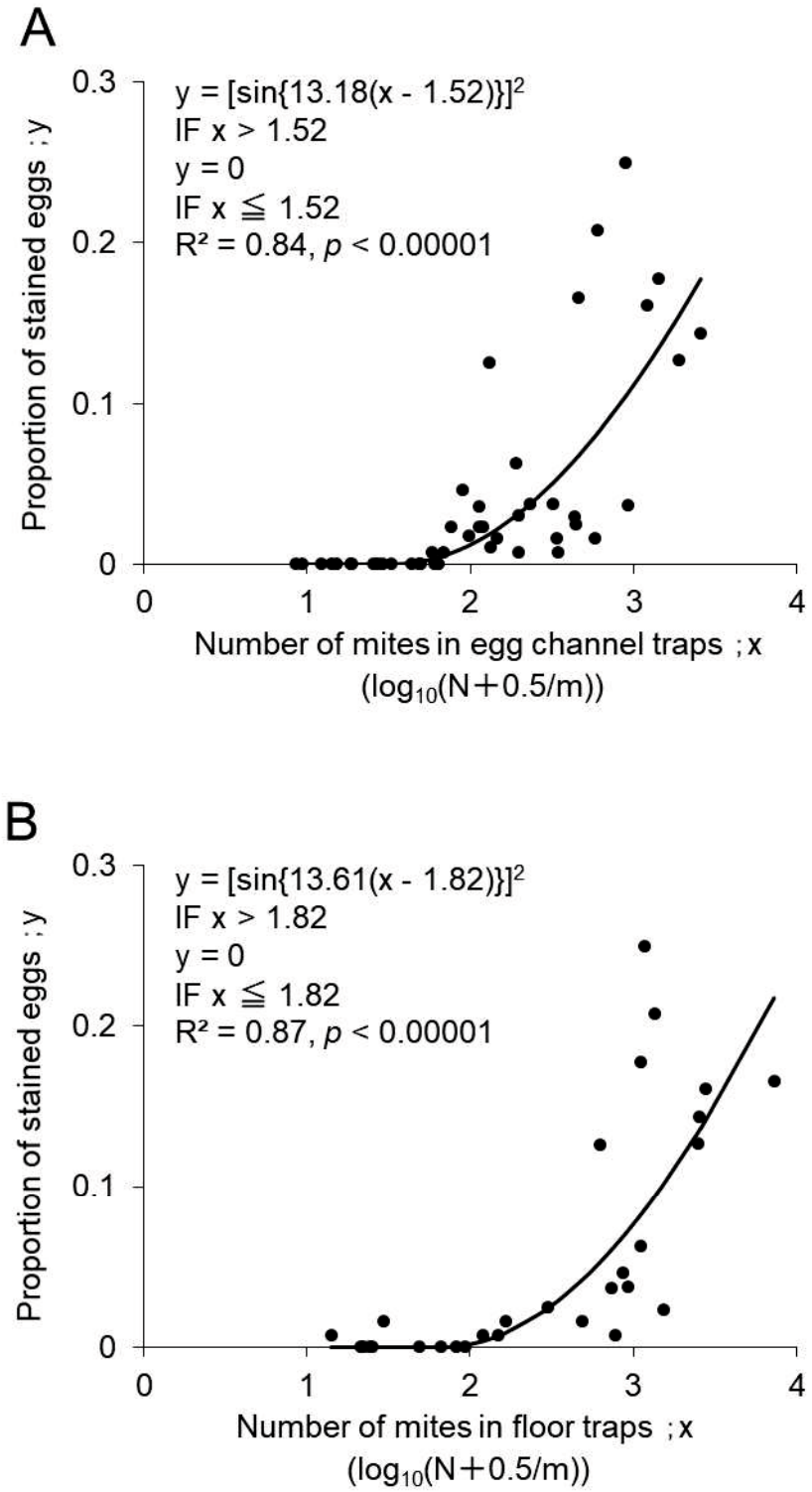
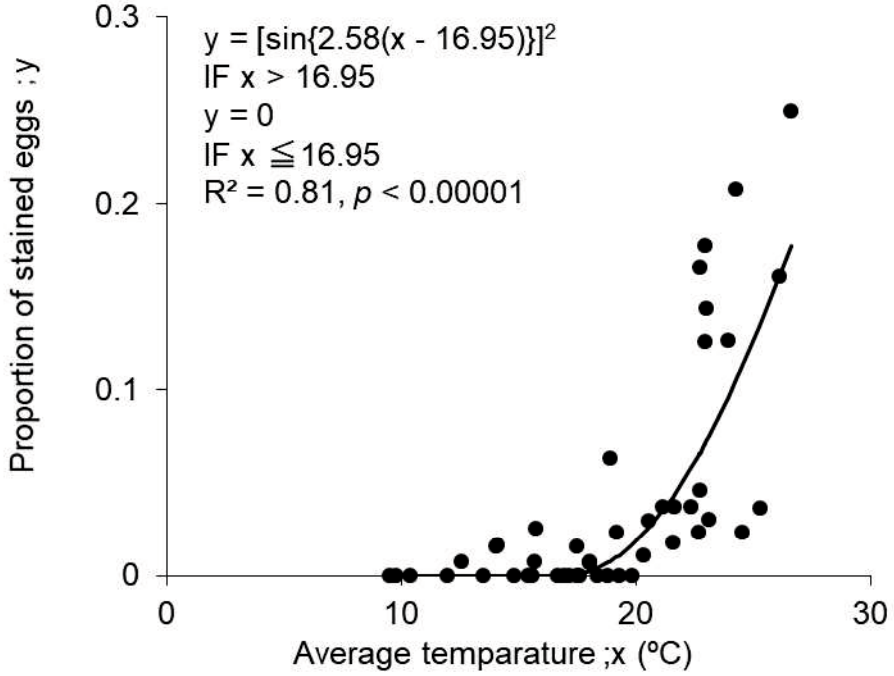

A
y = [sin{13.18(x - 1.52)}]²
IF x > 1.52
y = 0
IF x ≦ 1.52
R² = 0.84, p < 0.00001
Proportion of stained eggs ; y
Number of mites in egg channel traps ; x
(log10(N+0.5/m))
B
y = [sin{13.61(x - 1.82)}]²
IF x > 1.82
y = 0
IF x ≦ 1.82
R² = 0.87, p < 0.00001
Proportion of stained eggs ; y
Number of mites in floor traps ; x
(log10(N+0.5/m))

**Fig. 3. 5**


y = [sin{2.58(x - 16.95)}]²
IF x > 16.95
y = 0
IF x ≦16.95
R² = 0.81, p < 0.00001
Proportion of stained eggs ;y
0.3
0.2
0.1
0
0
10
20
30
Average temparature ;x (ºC)

# Chapter 4

# Efficacy of a novel mixture of substances derived from food and food additives for controlling *Dermanyssus gallinae* (Mesostigmata: Dermanyssidae)

**4.1. Abstract**

*Dermanyssus gallinae* De Geer (Mesostigmata: Dermanyssidae), also known as poultry red mite, causes serious economic losses to the poultry industry worldwide. In this study, a mixture of substances derived from food and food additives (SFF), including a polysaccharide extract from laver seaweed, *Pyropia yezoensis* (Bangiaceae), was examined for acaricidal effects. Treatment with SFF was more effective in killing mites by blocking their respiratory organs than SFF without the polysaccharide extract. In a field trial, treatment with SFF reduced the density of mites in a poultry farm as well as the proportion of blood-stained eggs. This SFF appears useful for controlling the population of *D. gallinae* in poultry farms.

## 4.2. Introduction

The control of *D. gallinae* has relied on the application of acaricides (Marangi et al., 2012; Thind and Ford, 2007). However, the extensive and repeated usage of acaricides has resulted in a reduction of their effectiveness due to the acquisition of resistance by poultry red mite (Beugnet et al., 1997; Nordenfors et al., 2001). Such resistance to acaricides has been reported in many countries, including Japan (Fiddes et al., 2005; Marangi et al., 2009; Murano et al., 2015; Thind and Ford, 2007). In addition, the misuse and abuse of acaricides often result in residual acaricides in the organs and tissues of the laying hens (Marangi et al., 2012). Therefore, we focused on physical control as an alternative control measure against *D. gallinae*. Some inert substances and oils have been reported to have physical control activity against *D. gallinae* under laboratory conditions (Maurer et al., 2009). However, previous studies have not identified substances that could exert stable acaricidal effects under field conditions (Kilpinen and Steenberg, 2009; Maurer et al., 2009).

In this study, we focused on a mixture of substances derived from food and food additives (SFF). The SFF consisted of vegetable oil, organic acid, lecithin, and polysaccharide. Among these constituents, oil has been reported to have a blocking effect on the spiracles of several plant pest insects and mites (Davidson et al., 1991; de Ong et al., 1927; Stadler and Buteler, 2009). In addition, Katumoto et al. (2012) reported that lecithin may enhance the acaricidal effect of vegetable oil on aphids. In particular, we focused on a polysaccharide extract from *Pyropia yezoensis* (Ueda) M. S. Hwang & H.

G. Choi, a laver seaweed called “nori” in Japanese, comprising mainly porphyran with approximately 0.83 mg/mg of glucose. Porphyran is a sulfated polysaccharide from the hot water-soluble portion of the algal cell wall (Morrice et al., 1983), and it has a relatively high viscosity (Bhatia et al., 2010; Sugano et al., 2009). In addition, Bhatia et al. (2010) showed that porphyran had amphiphilic properties and might be useful as a new polysaccharide surfactant. Therefore, this polysaccharide was expected to function as an emulsifier in the SFF, similar to other surfactants in common insecticides.

In this study, the effects of SFF on the mortality of *D. gallinae* was examined in vitro and in vivo. Moreover, the mechanism of its acaricidal effect was examined.

## 4.3. Materials and methods

### 4.3.1. *Dermanyssus gallinae*

Adult and immature *D. gallinae* were collected from one commercial layer farm in Fukuoka Prefecture, Japan. The collected mites were kept in a plastic cage (46 cm × 46 cm × 60 cm) at 28°C, 70% to 90% relative humidity (RH), and 12-h light:12-h dark photoperiod conditions without blood-sucking. Some non-parallel board traps (Odaka et al., 2014) were placed in the plastic cage to pick up active mites. Trapped mites were used for the laboratory experiments within 3 days of collection.

### 4.3.2. Test substances

The SFF (Porufui; MMO Co., Ltd., Fukuoka, Japan) contained vegetable oil, organic acid, lecithin, and 0.25% of polysaccharide extracted from *P. yezoensis* as described previously (Sumi et al., 2005). Briefly, dried powder of *P. yezoensi* was mixed with water and heated at 100°C and 0.1 MPa for 180 min. The soluble fraction was separated by centrifugation, and debris was removed by ultracentrifugation. The supernatant was concentrated using an ultrafiltration system (Noritake Concentrating System 7M4-2P; Noritake Co., Ltd., Aichi, Japan) equipped with ceramic membranes (Membralox® P19-40; nominal filtering accuracy of 50 nm; Pall Corporation, New York, NY, USA). After filtration with a synthetic adsorbent, the concentrated solution was dried and crushed. SFF without the polysaccharide extract (SFFWP) was also obtained from MMO Co., Ltd. As controls, two

insecticides, carbamate (Barizon®; Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and organophosphate (Premium Sumithion® 10% emulsifiable concentrate for animal use; SC Environmental Science Co., Ltd., Osaka, Japan) were diluted to the median values according to the prescription by the manufactures before use.

### 4.3.3. Acaricidal efficacy test

The acaricidal efficacy of SFF was examined as described previously (Busvine and Barnes, 1947; Hemingway et al., 1993; Koehler et al., 1993) with a minor modification. Although the previous studies used filter papers or vinyl tiles to expose insects to pesticides, we used basswood plywood boards that would maintain the humidity of the test area. Diluted solutions of the test substances, i.e., SFF, SFFWP, carbamate, and organophosphate, were sprayed onto the basswood plywood boards (6.0 cm × 8.0 cm × 0.4 cm) with a sprayer (Canyon® HI-SPRAYER 500 ml; Asaka Industrial Co., Ltd., Osaka, Japan) at a constant pressure 5 cm from the boards. The boards were tilted gently several times so that the solution would adhere uniformly to them at about 50 μl/cm$^2$. Then, 159 to 598 *D. gallinae* adults and nymphs were transferred onto the plywood boards with a paintbrush without drying process as described previously in the acaricidal efficacy test of oils (Maurer et al., 2009). Each plywood board was covered with a petri dish (5.2 cm in diameter × 1.4 cm in height) and the space between the dish and the board was sealed with adhesive bonding tape so that the mites inside could not escape. This

apparatus was set in the dark at 28°C and 70% to 90% RH. After 24 h the numbers of surviving mites and dead mites were recorded under a stereomicroscope. Mites were counted as dead if they did not respond to a gentle touch with a paintbrush. The mortality rate of *D. gallinae* was calculated by dividing the number of dead mites by the total number of mites. Five replicates were performed for each treatment.

#### 4.3.4. Scanning electron microscopic observation

To observe the effect of the SFF, nine to 15 mites that died after SFF exposure and 9 control mites that were exposed to distilled water were treated with osmium tetroxide vapor at room temperature and then observed with a scanning electron microscope (SEM; TM3000; Hitachi, Tokyo, Japan).

#### 4.3.5. Poultry farm and experimental design of the field experiment

The field experiment was performed from April to July, 2016, in one poultry farm in Fukuoka Prefecture, Japan. This farm had a cage-system poultry house, which had metal cages arranged in two rows, and 426 hens were bred. This poultry house was divided into three sections: a test section for treatment with SFF; an untreated control section without any treatment; and a buffer section containing 20 cages that was located between the two other sections for preventing mite contamination (Fig. 4.

1a). The hens used in the experiment are described in Fig. 4. 1b. Before this experiment, the farm was already infested with *D. gallinae*.

To examine the effect of SFF on *D. gallinae* in the poultry farm, we started spraying SFF when the average number of mites per trap exceeded 33.11-(0.5/m) (m; the number of traps placed in the poultry house) as proposed by Odaka et al. (2017). In dilution of the SFF solution, dilution of 100-fold or less caused adverse effects on the driving parts of the equipment in the poultry house in our preliminary experiment. Therefore, the dilution rate of the SFF was decided to be 200-fold or more. The SFF was sprayed once a week in June and once every 2 weeks in July. Cationic surfactant (Cleakil®-100; Tamura-Seiyaku Corp., Tokyo, Japan) was sprayed to clean the equipment on the 20th of June, 2016. The SFF solution was mixed with a Wire Mixer ADL-3LR (Asaba Manufacturing, Inc., Nagano, Japan). The spray solution was applied directly onto the surfaces of the cages, cage wires, feed troughs, and accompanying equipment using a power sprayer with a tank and a nozzle (Round Nozzle® 25 N-KA-055SB; Yamaho Industry Co., Ltd., Wakayama, Japan). The amount of spray solution used was 0.5 l/$m^2$.

#### 4.3.6. Density of *D. gallinae*

To monitor the population of *D. gallinae,* non-parallel board traps were attached to the egg channel at every 10 cages (Fig. 4. 1a, b) and collected after 24 h. The trapped mites were immediately

put into sealed bags, and then stored at 4°C. Images were captured with a digital camera to record the number of mites in each sealed bag.

### 4.3.7. Proportion of blood-stained eggs

The numbers of blood-stained eggs and total eggs were recorded every week. The proportions of blood-stained eggs among the total number of eggs were calculated.

### 4.3.8. Statistical analysis

All analyses were carried out using R 3.3.2 (R Development Core Team, 2016). The effects of SFF and SFFWP on the mortality rate of *D. gallinae* was analyzed by a generalized linear model (GLM). To examine the effects of SFF, which was diluted to different concentrations, and the two insecticides, Tukey's test was conducted using the multcomp package (version 3.2.2). Analyses by a generalized linear mixed model with a Poisson error distribution were carried out using the lme4 package (version 3.2.2) to compare the numbers of mites between the test and control sections. The explanatory variables were ‘Treatment’, ‘Preapply’, and ‘Day’ as the fixed effects, and ‘Subject’, where the cage traps were placed, as a random effect. ‘Preapply’, the number of mites on each cage before the first treatment, was added as a parameter that accounted for the correlational structure of the repeated measurements. Analyses by GLM were also conducted to evaluate the differences in the

proportion of blood-stained eggs between the test and control sections. The best-fitting models were selected based on the Akaike information criterion.

## 4.4. Results

### 4.4.1. Effect of the polysaccharide extract on the mortality rate of *D. gallinae*

With the application of 200-fold diluted SFF and SFFWP, the mortality rates of *D. gallinae* were 85.7% ± 4.5% and 65.0% ± 13.1%, respectively (Fig. 4. 2a). SFF was significantly more effective in killing mites than SFFWP ($p < 0.01$) (Fig. 4. 2a).

### 4.4.2. Concentration-dependent effect of SFF on the mortality of *D. gallinae*

The mortality rates of *D. gallinae* after the application of 100-fold, 200-fold, 400-fold, and 800-fold diluted SFF were 97.2% ± 1.6%, 85.6% ± 6.3%, 45.4% ± 14.1%, and 22.2% ± 10.9%, respectively (Fig. 4. 2b). The lower diluted SFF was significantly more effective at killing mites than the higher diluted SFF ($p < 0.01$). The mortality rates with the application of 750-fold diluted carbamate and 150-fold diluted organophosphate were 6.5% ± 4.2% and 34.2% ± 22.7%, respectively.

### 4.4.3. SEM observation of SFF-treated mites

SEM observation showed that the respiratory organs of *D. gallinae*, i.e., the peritreme and spiracle, were blocked after SFF treatment. The states of blocking of the peritreme and spiracle differed among the individual mites. We classified the states of blocking into three categories: blockage of both the peritreme and spiracle (Fig. 4. 3a); blockage of only the peritreme (Fig. 4. 3b);

and no blockage of the peritreme and spiracle (Fig. 4. 3c). The ratios of mites with blockage of both the peritreme and spiracle after the application of 100-fold, 200-fold, 400-fold, and 800-fold diluted SFF were 100%, 88.9%, 80.0%, and 50.0%, respectively (Fig. 4. 3d). There was a trend that the ratio of mites with blockage of the peritreme and spiracle increased in a SFF concentration-dependent manner.

#### 4.4.4. Effect of SFF application on the number of *D. gallinae*

Application of 200-fold diluted SFF to the poultry farm started on the 3rd of June, when the average number of mites per trap was 84.6 in the test section. Significant differences ($p < 0.01$) in the numbers of mites were observed between the test and control sections (Fig. 4. 4a).

#### 4.4.5. Effect of SFF application on the proportion of blood-stained eggs

The proportion of blood-stained eggs in the test section remained below 1.0%, while that in the control section increased from 4.3% (June 10) to 12.9% (June 29) (Fig. 4. 4b) during the observation period. This demonstrated that SFF application had a statistically significant effect on the proportion of blood-stained eggs ($p < 0.01$).

**4.5. Discussion**

In this study, it was evident that SFF was more effective in controlling *D. gallinae* in farms with reduction of effect of conventional insecticides, i.e., carbamate and organophosphate. We demonstrated that the application of SFF in a poultry farm could decrease the number of mites as well as the damage caused by the mites. In the poultry farms around the world, reduction of effect of the commercial insecticides against *D. gallinae* has been reported (George et al., 2015). In British, more than 60% of poultry farms had experienced infestations of acaricide-resistant mites (Guy et al., 2004). Murano et al. (2015) reported that 18.7% of poultry farms in 29 prefectures had infested by *D. gallinae* which acquired resistance against all conventional insecticides (three carbamates, one pyrethroid and three organophosphates) in Japan.

Both the acaricidal effect of SFF against *D. gallinae* and the ratio of mites with blockage of the peritreme and spiracle depended on the concentration of SFF (Figs. 4. 2 and 4. 3). These results indicated that the mites died of suffocation as a result of the blockage of their respiratory organs. The vegetable oil in SFF was rich in glycerides of fatty acids, which are also known as spiracle-blocking pesticide for agricultural mites and aphids (Miyata and Masuda, 2006a, b).

SFF was significantly more effective in killing mites than SFFWP, confirming the acaricidal effect of the polysaccharide extract in SFF. Dried “nori” consists of two main components, i.e., proteins and carbohydrates (Kayama et al., 1983; Mumford and Miura, 1988), and approximately 80%

of the carbohydrate component is porphyran (Kitano et al., 2012; Sugano et al., 2009). In the extraction process of this polysaccharide extract from “nori”, hot water extraction and ultrafiltration are performed to prevent the extraction of proteins and to remove the low molecular weight portion from the extracted substances. Therefore, the polysaccharide extract in SFF consists mainly of high molecular weight porphyran, which has a mass of approximately 400 kDa (weight-average molecular weight), and small impurities, such as denatured proteins. Porphyran was reported to have high emulsifying activity and high emulsion stability, because it was presumed to adsorb to the surface of oil droplets (Takahashi et al., 2000). In addition, higher molecular weight porphyran preparations had higher emulsifying effects (Bhatia et al., 2010). Therefore, the dilution of SFF may have improved the uniformity of the solution, because porphyran was able to coat the surface of oil droplets in the SFF solution. Solutions with high dispensability may facilitate the deposition of active ingredients, such as the glycerides of fatty acids, onto mites. After being mixed with insecticides, some emulsifying substances enhance the activities of the insecticides due to improvements to the viscosity, penetration, or tension of the solution (Hartzell and Wilcoxon, 1960; Imai et al., 1994; Matsubara, 1968; Wolfenbarger et al., 1967). However, the mechanism of the effect of the porphyran in SFF on the mortality of *D. gallinae* remains unclear.

Inert substances (e.g., diatomaceous earth, kaolin, and silicas) and oils (e.g., mineral oils and plant oils) have been reported to be physical acting substances against *D. gallinae* (Maurer et al., 2009;

Sparagano et al., 2014). However, these substances are difficult to apply in practical situations. For example, *D. gallinae* becomes active during hot and humid periods in the poultry house (Nordenfors and Höglund, 2000; Othman et al., 2012), and we also previously reported that the population of *D. gallinae* reached a peak after the average temperature exceeded 24°C and the humidity was 70% to 90% (Odaka et al., 2017), but high humidity (>85%) reduces the efficacy of the inert substances (Kilpinen and Steenberg, 2009; Maurer et al., 2009). As for oils, the acaricidal effects of oils against *D. gallinae* have been reported in vitro, but not in the poultry farm (Maurer et al., 2009). In addition, mineral oils, such as diesel, which was more toxic to phytoseiid mites than plant oils (Momen et al., 2006), are not recommended for use in poultry houses because of their unpleasant odor and the risk of egg contamination (Hoop, 2008). Although our 200-fold diluted SFF solution smelled a little, the application of SFF was effective even under an environment of high humidity from June to July, when the population of *D. gallinae* reached a peak.

The timing of treatment with acaricidal substances is important. After infestation of *D. gallinae* prevailed in a poultry house, it is practically impossible to control the mites because of their high reproductive potential (Maurer and Baumgärtner, 1992). Therefore, we previously proposed using non-parallel board traps to monitor the population level of the mites and to implement control measures while the population is low (Odaka et al., 2017). In this study, we applied this method in the poultry house and succeeded in controlling the population of the mites. As such, we recommend

monitoring of the mite population prior to the application of control methods.

In conclusion, SFF had an acaricidal effect by blocking the respiratory organs of *D. gallinae* and was effective in controlling *D. gallinae* in the field. This SFF appears useful for controlling *D. gallinae* in poultry farms.

**4.6. Figure legends**

Fig. 4. 1 The poultry farm used in this study and the design of the experiment.

**a** The arrangement of the test, control, and buffer sections in the poultry house is shown. Treatment with a mixture of substances derived from food and food additives (SFF) covered half of the buffer section as indicated by thick arrows. **b** Information on the hens and traps used in the poultry house.

Fig. 4. 2 Mortality rates of *Dermanyssus gallinae*.

**a** Mortality rates of *D. gallinae* with the application of SFF and SFF without the polysaccharide extract (SFFWP). Asterisks indicate a significant difference (generalized linear model (GLM), $p < 0.01$). **b** Mortality rates of *D. gallinae* with SFF, which was serially diluted with distilled water, and two insecticides, i.e., carbamate (Barizon®) and organophosphate (Premium Sumithion®). The dilution rates are provided in parentheses. The mortality rate of mites exposed to distilled water is also shown. Different alphabet letters indicate significantly different mortality rates (Tukey's test, $p < 0.01$).

Fig. 4. 3 Scanning electron microscopic (SEM) observation of the respiratory organs of *D. gallinae* after treatment with SFF.

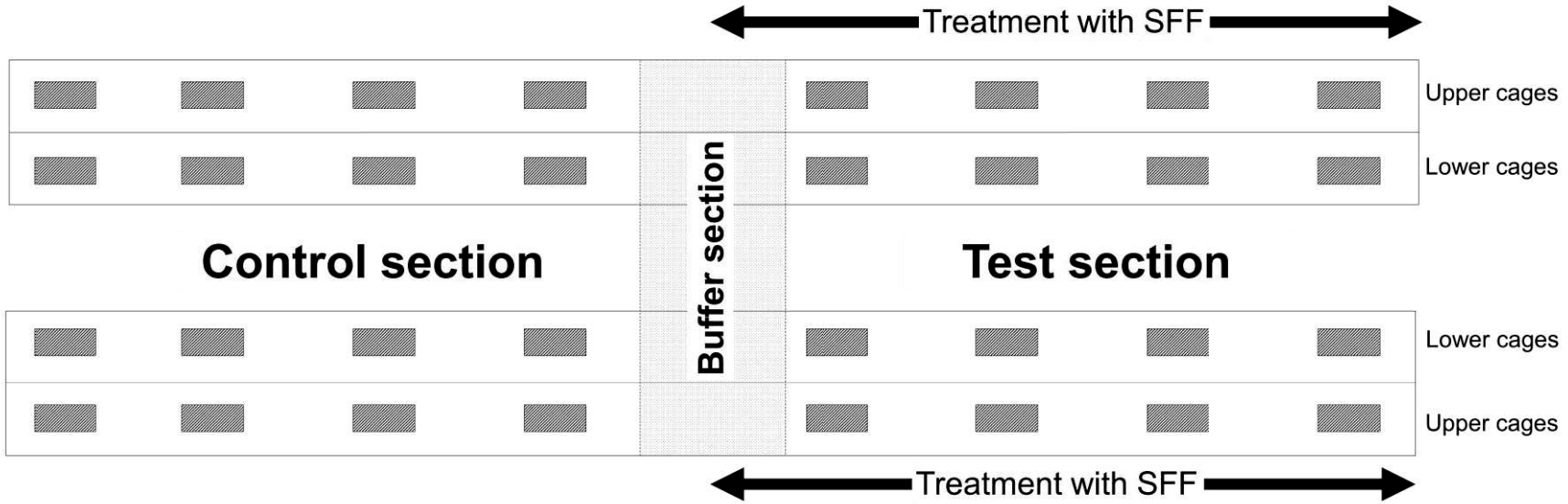
**a** Blockage of both the peritreme and spiracle after treatment with SFF (1:200). **b** Blockage of only the peritreme after treatment with SFF (1:400). **c** No blockage of the peritreme and spiracle after treatment with distilled water. **d** Ratios of *D. gallinae* with blockage of respiratory organs.

Fig. 4. 4 Changes in *D. gallinae* numbers and the proportions of blood-stained eggs during the field trial.

**a** Changes in the numbers of *D. gallinae* per trap. The numbers of mites differed significantly between the test and control sections (generalized linear mixed model, $p < 0.01$). **b** Changes in the proportions of blood-stained eggs. The proportions of blood-stained eggs differed significantly between the test and control sections (GLM, $p < 0.01$).

**Fig. 4. 1**

a




b

| | Commercial layers | Age of hens (weeks old) | Number of hens | Number of traps | Trap durance (h) | Distance between traps |
|---|---|---|---|---|---|---|
| Test section | Hy-Line Brown | 41~56 | 46 | 16 | 24 | 10 cages |
| | | 54~69 | 142 | | | |
| | Lohman LSL | 54~69 | 10 | | | |
| Control section | Hy-Line Brown | 41~56 | 47 | 16 | 24 | 10 cages |
| | | 54~69 | 135 | | | |

**Fig. 4. 2**

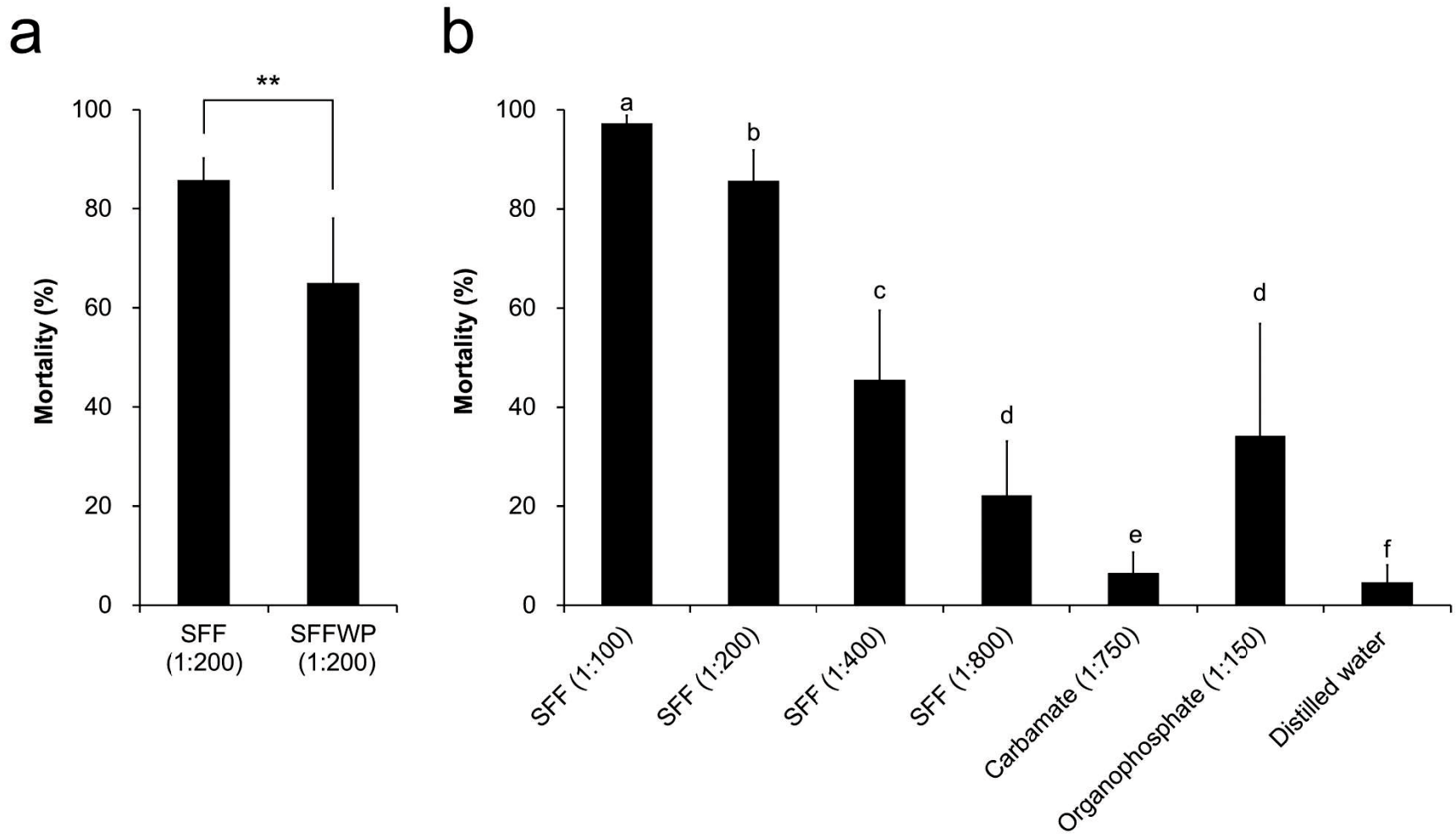

a
**
100
80
60
40
20
0
Mortality (%)
SFF (1:200)
SFFWP (1:200)
b
a
b
c
d
e
d
f
100
80
60
40
20
0
Mortality (%)
SFF (1:100)
SFF (1:200)
SFF (1:400)
SFF (1:800)
Carbamate (1:750)
Organophosphate (1:150)
Distilled water

Fig. 4. 3

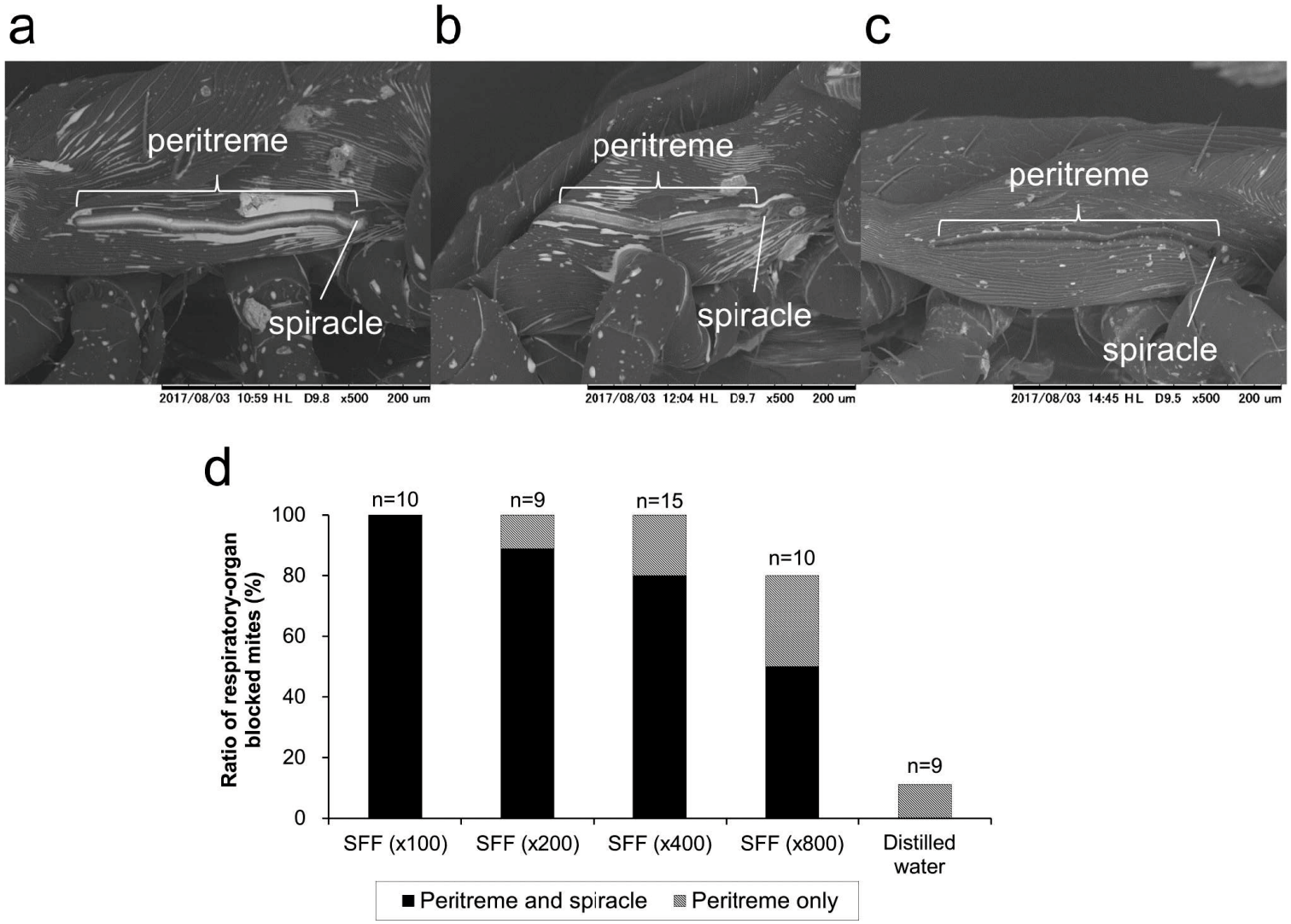
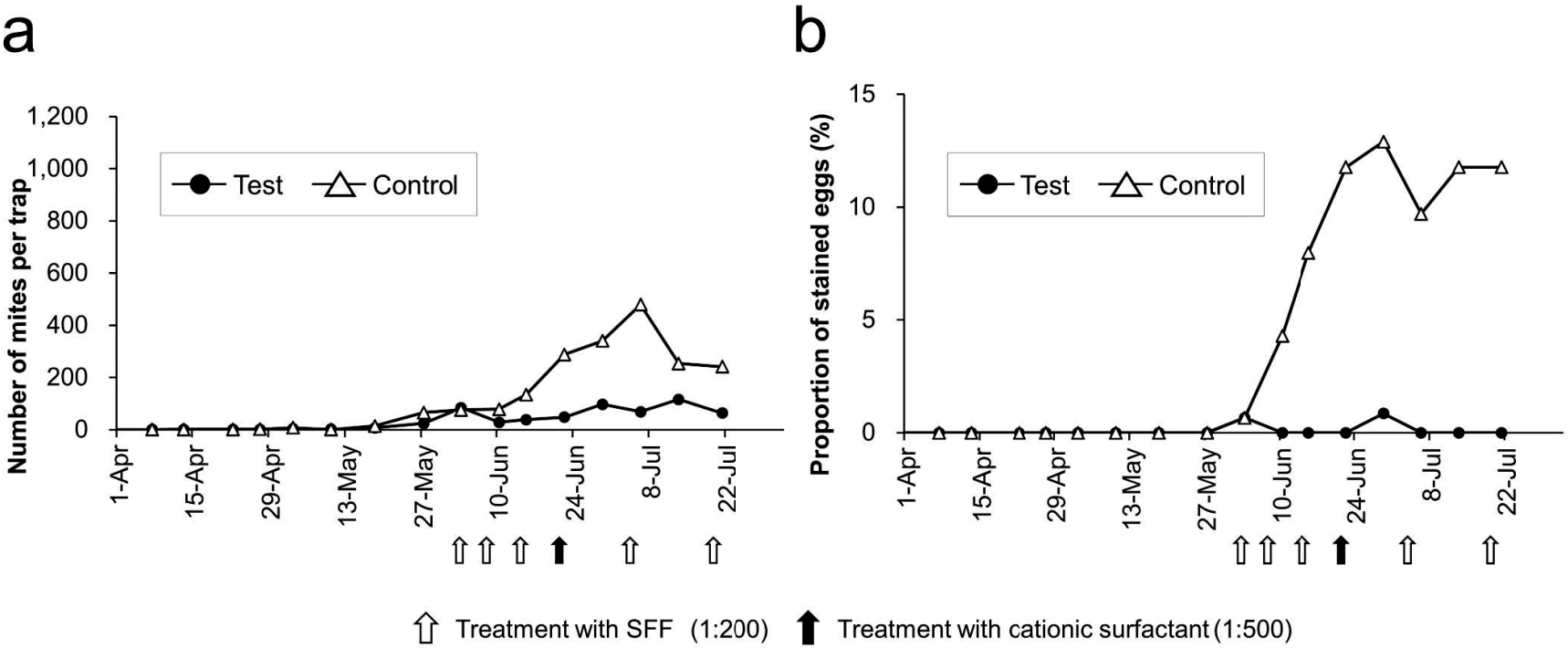

a
b
c
peritreme
spiracle
peritreme
spiracle
peritreme
spiracle
2017/08/03 10:59 HL D9.8 x500 200 um
2017/08/03 12:04 HL D9.7 x500 200 um
2017/08/03 14:45 HL D9.5 x500 200 um
d
Ratio of respiratory-organ blocked mites (%)
100
80
60
40
20
0
n=10
n=9
n=15
n=10
n=9
SFF (x100)
SFF (x200)
SFF (x400)
SFF (x800)
Distilled water
Peritreme and spiracle
Peritreme only

**Fig. 4. 4**


a
b
Number of mites per trap
1,200
1,000
800
600
400
200
0
Test
Control
1-Apr
15-Apr
29-Apr
13-May
27-May
10-Jun
24-Jun
8-Jul
22-Jul
Proportion of stained eggs (%)
15
10
5
0
Treatment with SFF (1:200)
Treatment with cationic surfactant (1:500)

# Chapter 5

# General conclusion

In my PhD course, I established the good tool for monitoring *D. gallinae,* clarified the relationship between number of mites and the damage, and developed effective substance for controlling mites.

**Subject: Development of a trap for *Dermanyssus gallinae***

Sensitive monitoring method of the population of *D. gallinae* in the poultry house has been required, because it is difficult for farmers to observe them visually and to understand the optimal timing for control them. Therefore, we have constructed a novel trap, non-parallel board trap, based on the behavior of the *D. gallinae*. The novel non-parallel board trap could capture more mites than the previous reported traps. This trap could be a useful tool for monitoring *D. gallinae* and studying on the ecology of this mite in poultry farms.

**Subject: Correlation between the proportion of stained eggs and the number of mites (*Dermanyssus gallinae*) monitored using a "non-parallel board trap"**

In this study, to clarify the indicator of optimal timing for treatment with acaricidal substances, the mite population in poultry houses was examined using an established trap method, and the risk of blood-stained eggs caused by the mites was assessed. The results showed that the number of mites increased from April to May, and reached a peak around the beginning of June when the

average temperature and humidity were >24°C and 70-90%, respectively. In the segmented model, the correlation between the proportion of blood-stained eggs and the number of mites was positive over a threshold. In conclusion, our established trap method is useful for monitoring mites and can be used to predict when poultry farms should be treated to prevent appearance of blood-stained eggs.

**Subject: Efficacy of a novel mixture of substances derived from food and food additives for controlling *Dermanyssus gallinae* (Mesostigmata: Dermanyssidae)**

In this study, a mixture of substances derived from food and food additives (SFF), including a polysaccharide extract from laver seaweed, *Pyropia yezoensis* (Bangiaceae), was examined for acaricidal effects as an alternative control measure. Treatment with SFF was more effective in killing mites by blocking their respiratory organs than SFF without the polysaccharide extract. In a field trial, treatment with SFF reduced the density of mites in a poultry farm as well as the proportion of blood-stained eggs. This SFF appears useful for controlling the population of *D. gallinae* in poultry farms.

**Conclusion**

In my PhD study, there are some novel findings.

1. The novel “non-parallel board trap” with high ability for capture of *D. gallinae* to monitor mite population was developed.

2. Monitoring mites by “non-parallel board trap” is a useful method for determination of the periods when application of mite control measures may be implemented to prevent the appearance of blood-stained eggs.
3. The novel mixture of substances derived from food and food additives (SFF) was effective in controlling *D. gallinae* as a physical control substance.

These findings must be available for development of a further control system of *D. gallinae*.

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