Final Report:

Relation Between Body Size/age and Fecal Pellet Size in Sika Deer (*Cervus nippon yakushimae*) of Yakushima Island

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1 Introduction

Recent conflicts between humans and wildlife, such as Asian black bear (Ursus thibetanus), wild boar (Sus scrofa) and sika deer (Cervus nippon), have recently received attention in Japan [Honda and Sugita 2007; Koda et al. 2008; Oi and Suzuki 2001; Watanabe 1980]. These conflicts have been reported in many places in Japan, and Yakushima Island is one of the places [Takatsuki 1990]. Yakushima Island is registered as the first World Natural Heritage (since 1993) in Japan [ UNESCO] [Agetsuma 1996]. However, the natural environment of Yakushima Island is changing because of overgrazing by Sika deer (Cervus nippon yakushimae) [Tsujino and Yumoto 2004]. The following problems are recognized in Yakushima: (1) Loss of seedlings and saplings because of grazing and browsing [Takatsuki and Gorai 1994; Tsujino and Yumoto 2004], (2) A decrease in the number of mature trees due to bark stripping [Akashi and Nakashizuka 1999; Yokoyama et al. 2001]. (3) And a decrease of rare species of plants [樋口浩 2013].

The population of Sika deer has increased since 1950 due to a lack of predation pressure and severe winters, expansion of artificial grasslands, suspension of deer hunting since ca 1970, and a restriction on logging since 1964. Tsujino and Yumoto [Tsujino and Yumoto 2004] suggested that currently the density of Sika deer in Yakushima is high (1.5–63.8 head/km²). Hence, they predicted that the damage will soon occur on Yakushima Island similar to that observed on Kinkazan Island [Koda et al. 2008; Tsujino and Yumoto 2004].

Therefore, it is necessary to monitor the increase-decrease rate of the deer population and potential reasons for variation, to understand the effect of the deer population on the vegetation of Yakushima Island. Age and sex structure of a population is essential for exploring trends in recruitment population growth, mortality and reproduction status. However, a method for the accurate estimation of age and sex at the population level is uncertain. Here, we present the three methods; fecal collection with direct observation, DNA analysis, and hormonal analysis, for the estimation of age and sex class of the deer population in Yakushima Island. The DNA analysis and hormonal analysis are the two new methods for the purpose of examine the age-sex class of the deer population and we also examine how those two methods are reliable by comparing with the result of direct observation.

2 Material and Methods

2.1 Fieldwork

2.1.1 Study Site and Subjects

The study was conducted in the primary and secondary forests in the vicinity of Seibu Rindo in the western shore of Yakushima Island (Kagoshima Prefecture, Japan). Study subjects were wild Yaku Shika deer (Cervus nippon yakushimae). Fifty-three samples were collected from 12 adult males, 34 adult females, and 7 juveniles. Sex of the individuals was determined by the presence of horns for males and absence for females. Individuals were considered as juveniles if the horns were extremely short and the legs were thin and long compared to the body size.

2.1.2 Data Collection

Data were collected from October 19th to October 21st, 2015, and from around 07:45 to 15:00. Researchers were divided into three teams. As soon as a deer or a group of deer was found,
it was followed until defecation. Once a deer defecated, one researcher recorded the focal individual using a video camera, and wait, without moving, for the focal animal to leave. When the deer had moved away, a second researcher went to the exact same place where the deer was and posed with a scale, then the first researcher video recorded him/her. Body size of the individuals was estimated by analyzing the videos. Body size corresponded to the length between the shoulder and the hoof of the individual. Body size was measured by comparing the size of the individual (in the first video) with the size of the scale that the researcher was holding (in the second video).

DNA samples were collected by swabbing the surface of the fecal pellets with a cotton bud and put in a tube filled with lysis buffer containing 10% SDS. The size of the longer and the shorter axis was calculated for all fecal pellets of all the study subjects using a caliper (0.00 mm precision).

DNA samples and fecal pellets were kept at room temperature for about a week before genetic and hormonal analyses.

### 2.1.3 Statistical Analysis on the fecal pellet size

A GML test was used to compare (i) the length of the long axis between adults and juveniles, (ii) the length of the short axis between adults and juveniles, and (iii) the body size between adults and juveniles.

A linear model fitting/ANOVA tests was used to compare the size of the longer and shorter axis of the fecal pellet with the body size of the deer (all individuals).

All the GML tests and linear model fitting/ANOVA tests were done with R 3.1.3. The significant level used for the tests was $\alpha = 0.05$.

### 2.2 DNA Analyses

#### 2.2.1 DNA Extraction

Fecal samples generally serve as low DNA concentration. QIamp® Fast DNA Stool Mini Kit was employed in extracting DNA from fecal samples following the protocol. Always spin down to remove drops from the inside of the tube lid. All extracted fecal DNA per sample was quantified via Nano drop machine (DNA concentration ranging from -1.7 ng/µL to 2.4 ng/µL).

#### 2.2.2 Amplification and Genetic Analyses

Two primers, ZFY/X and SRY were chosen to amplify with feces DNA from deer. The nucleic acid sequence of a Y chromosome genomic fragment encoded an open reading frame whose predicted amino acid sequence was a tandem repeat of 13 "zinc-finger" domains. The Y chromosome gene encoding this zinc-finger protein was called ZFY, and the homologous sequence on the normal X chromosome was called ZFX. These genes encodes a zinc finger-containing protein that may function as a transcription factor. SRY is an intronless sex-determining gene on the Y chromosome in placental mammals and marsupials. ZFY/X exit in both males and females, but sex-determining gene SRY was only exited in males (see Table 1 for the primer sequence). Reaction mixture of PCR and PCR condition were shown as Table 2 and Table 3. PCR products were observed by electrophoresis with 1% TAE gel (1% Agarose gel in Buffer TAE with GelGreen (5µL in 100mL gel)).
Table 1 PCR primer sequences and amplification conditions

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequence</th>
<th>Annealing temperature(℃)/Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZFXY</td>
<td>ATAATCACATGGAGAGCCACAAGCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCACCTTCTTTGGATCTGAGAAAGT</td>
<td>62℃/45 cycles</td>
</tr>
<tr>
<td>SRY</td>
<td>CCCATGAACGATTCAATTGTGTGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATTTTAGCCTTCCGACGAGGTCGATA</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Reaction mixture of PCR (25µL).

<table>
<thead>
<tr>
<th>Volume [µl]</th>
<th>10× Ex Taq Buffer</th>
<th>2.5 mM dNTPs Mixture</th>
<th>10 µM Forward Primer (SRY)</th>
<th>10 µM Reverse Primer (SRY)</th>
<th>10 µM Forward Primer (ZFXY)</th>
<th>10 µM Reverse Primer (ZFXY)</th>
<th>T4 gene 32 protein</th>
<th>TaKaRa ExTaq</th>
<th>Template DNA</th>
<th>Water</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.1</td>
<td>0.125</td>
<td>2</td>
<td>16.275</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 3 PCR condition. Duration and temperature of each phase

<table>
<thead>
<tr>
<th>Phase</th>
<th>Temperature(℃)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot start</td>
<td>94</td>
<td>∞</td>
</tr>
<tr>
<td>First Denaturtion</td>
<td>94</td>
<td>10 min</td>
</tr>
<tr>
<td>Denaturatation</td>
<td>94</td>
<td>10 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>62</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1 min</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>10 min</td>
</tr>
<tr>
<td>Cooling Down</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

* 45 cycles from denaturation to extension.

2.3 Hormonal Analyses

2.3.1 Fecal Steroid Hormone Extraction and Enzyme Immunoassay

After freeze vacuum drying for approximately two and a half hours, samples were crushed with two zirconia beads (3800 rpm, 60 sec) and centrifuged (9,000 G, 1 min). Fecal samples were extracted an aliquot of 0.01 g with 500 µl of 80% methanol by vortex-mixing for 30 min. After centrifugation of fecal suspensions (9,000 G, 1 min), we decanted 120 µl of the supernatant into tubes. We analyzed fecal extracts for concentrations of immunoreactive testosterone (T),
estradiol-17β (E2), and progesterone (P4) using enzyme immunoassays (EIA). Fecal extracts were diluted with EIA buffer (T: 30 fold dilution, E2: 10 fold dilution, P4: 20 fold dilution) and duplicate 20 µl aliquots of this solution were added to 96-well plates bound with the 2nd antibody. To obtain a standard curve, standard of each sex steroid hormones in the range 0.195-100 ng/ml were diluted serially, and were also dispensed into the well in duplicate. Immediately after the addition of 100 µl 1st antibody, and an equal volume of horseradish peroxidase each sex steroid hormones, the plats were incubated in the dark for overnight at 4 degree C. Free-bound separation was achieved by emptying the plate and washing four times. A mixture of 75 µl substrate buffer A and 75 µl of solution B was added to each well, followed by incubation for 10 min at 37 deg C in the dark. The reaction was terminated by the addition of 4 N-H2SO4 (50 μl) and the absorbance at 450 nm was measured using a microplate reader (SUNRISE, BIO-RAD Laboratories Inc.).

The following formula was applied to each hormone results:

Correct hormone concentration (ng/g) = Raw hormone concentration (ng/ml) × 0.5 ml × 1/0.01 g dry fecal sample weight

The 1st antibody, 2nd antibody, enzyme linked antigen and standard are as follows, respectively:

1st antibody: each sex steroid hormone (testosterone, estradiol-17β, or progesterone) antiserum
2nd antibody: anti-rabbit IgG (H+L)
Horseradish peroxidase (HRP): each sex steroid hormone (testosterone, estradiol-17β, or progesterone) antigen
Standard: each sex steroid hormone (testosterone, estradiol-17β, or progesterone)

The cross-reactivity of the 1st antibody is as follows:

**Testosterone**
Testosterone (T): 100%, 5α-Dihydrotestosterone: 10%, 4-Androstenedione: 0.5%, 5α-Androstanediol: 0.25%, 5-Androstene-3β, 17β-diol: 0.2%, 5α-Androstane-3α, 17β-diol: 0.1%, 5β-Androstane-3α, 17β-diol: 0.07%, Cortisol: 0.01% Corticosterone: 0.01%, Progesterone: 0.03%, Pregnenolone: <0.01%, 17α-Hydroxypregnenolone: <0.01%, Aldosterone: <0.01%, Dehydroepiandrosterone: <0.01%, Estradiol: <0.01%

**Estradiol-17β**
Estradiol-17β (E2): 100%, Estrene: 0.8%, Estriol: 0.5%, Estradiol-3-sulfate: 26.8%, Estradiol-3-glucuronide: 56.3%, Estrone-3-sulfate: 0.86%, Estrone-3-glucuronide: 1.20%, Testosterone: 0.05%, Pregnenolone: 0%, Progesterone (P4): 0%, Cortisol: 0%, Cortisone: 0%, 4-Androstenedion: 0%, Dehydroepiandrosterone: 0%, Dihydroteestosterone: 0%

**Progesterone**
Progesterone (P4): 100%, 5α-Pregnanedione: 12.6%, 11α-OH-progesterone: 5.3%, 20α-OH-progesterone: 0.2%, Pregnenolone: 2.0%, Deoxy cortisolone: 0.01%, 17α-OH-progesterone: 0.01%, Corticosterone: 0.01%, Cortisol: 0.01%, Aldosterone: 0.01%
3 Results

3.1 Fieldwork

3.1.1 Result of Fieldwork

In total, we collected 53 samples (12 males, 34 females and 7 juveniles) but we had to exclude 2 adult females from the analysis because we weren’t able to measure body size from the video record.

Statistical analysis gave us no correlation between the body size and the size of the pellet. When compared the long axis with the measured body size of the animal, we found no correlation (Figure 1 A, p=0.51). The same happened when comparing short axis and body size (Figure 1 B, p=0.12). However, we found significant differences in long (p<0.01), short axis of pellet (p<0.01), and body size (P=0.03) between adults and juveniles. Long and short axis were longer in adults compared to juveniles.

Figure 1 The relationship between body size and fecal pellet size.
A: Long axis; B: Short axis.

3.1.2 Discussion of Fieldwork

Our study diverges from another research in Nara, using the same methodology. In Nara’s study,
researchers found indeed a correlation between pallet short axis and body size, as predicted. However, both studies have similar results concerning pellet size and age, supporting our prediction that pellet size is affected by age.

We can attribute some of our discrepancies to our own study limitations. For example, the method used to determine animals’ body size is not 100% accurate and may induce some errors. Moreover, we have a small sample, biased to females, in only one region of Yakushima and with little detail concerning age range – the only age classes were adult vs. juvenile. Also to take into account is the possibility of sample contamination and differences between group measures (group inter-reliability), factors that may have influenced our results.

### 3.2 Genetic Analyses

#### 3.2.1 Gene Based Sex Identification

We conducted gene based sex identification for all 54 samples sampled during the field work. The target genes were SRY which locates only on Y chromosome, and ZFXY which locates on both X and Y chromosomes. The expected amplicon were 224 bp and 445 bp for SRY and ZFXY respectively. After conducted PCR to both genes, electrophoresis were used for showing results.

We treated the lanes with two bands around 200 to 455 bp as a sign of female, and the lanes only showed one band around 400 bp as a sign of male. We also got lanes only showed one band around 200 bp, which meant that only the gene on Y chromosome were amplified, and lanes didn’t showed any band (Figure 2). Besides we can’t assure the sex of 2 juvenile individuals. We treat these three situations as unusable data.
Out of all 54 samples, we got 36 usable data. In 18 unusable data, 3 lanes only showed band around 200 bp. The usable rate of all results was 66.67%. By gene based sex identification, we got 12 males and 24 females.

Compared with observation data, only 1 usable result showed different sex identity, individual No.42 was observed as female but identified as a male by gene based method (Table 4). In total the correlation rate in usable data was 97.22%.

Table 4 Comparison of observation result and gene based result

| No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|-----|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|
| O   | F | M | F | F | JM| F | F | F | M | F | M | F | F | M | JM| F | F | JF|
| G   | F | M | F | F | M | F | F | F | M | N | M | N | F | M | N | N | F | N |
| No. | 19| 20| 21| 22| 23| 24| 25| 26| 27| 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
| O   | F | F | JF| F | F | M | F | JM| F | F | F | F | F | M | F | J |   |
| G   | N | F | F | N | M | N | N | F | F | F | F | F | N | F | N | F | F |
| No. | 37| 38| 39| 40| 41| 42| 43| 44| 45| 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 |
| O   | F | F | M | M | F | M | F | F | M | F | M | J | M | F | F | M | F |
| G   | F | N | M | M | F | M | N | F | F | N | M | M | F | F | N | N |

*O refers observation result; G refers gene based result.

** Orange means male, blue means male, gray mean unusable data

3.2.2 Discussion of Genetic Analyses

Except unusable data, only one result was different from observation result. It suggested that the gene based sex identification is a very accurate method for sex identification. And we can estimate that the two juvenile whose sex were unknown during field work were female (No. 36) and male (No. 49), respectively.

The only uncorrelated result may cause by contamination. The target genes we used exist in all mammals including human. On the one hand, human gene contamination can occur. Although we used masks and gloves during sampling as well as conducting experiments, it was difficult to pervert all possible contamination. On the other hand, contamination from other feces was possible. During sampling, we sometimes found old fecal around our target sample. The cells from those old fecal may contaminate the target sample.

About one third of the results were unusable. Such poor success rate was the biggest problem for relying on gene based sex identification. Several weak points of this method appear in different
stages. During sampling, we swabbed the surface of fecal. Colon cell on the surface were expected to be transferred to the buffer. However if the fecal were swabbed too hard, other ingredient of fecal may also transferred to the buffer, which may destroy DNA or interfere the DNA extraction, then lead to unusable samples. Another difficulty occurred in the lab. After extraction we test the concentration of DNA. For most samples, the concentration is lower than 1 ng/µl (Figure 3). It is normal for DNA sampled from fecal. Such low DNA concentration can easily lead to unsuccessful PCR.

![Figure 3 DNA concentration in buffer after extraction and purification.](image)

### 3.3 Hormonal Analyses

Our results for the hormonal analysis are represented in Figure 4. Estradiol-17β, Figure 5. Progesterone, and Figure 6. Testosterone, respectively.

For the steroid hormone estradiol we found a higher content for males (253.78 ng/g) in the non-breeding season but there were no statistical significant differences between sexes. However, we did find a significant different between breeding and non-breeding seasons for both sexes (females 141.02 ng/g and males 149.89 ng/g in the Breeding Season, vs. females 192.16 ng/g and males 253.78 ng/g in the Non-Breeding season).

From our samples for Progesterone (Figure 5), our highest value was that of females in the breeding season (5483.69 ng/g) compared to both values for males in both Breeding and Non-Breeding seasons (3,444.40 ng/g and 3696.69 ng/g). Even with this higher value, we still did not find any statistically significant difference between sexes or seasons.

Testosterone results were the most interesting and surprising of our results. Our highest value found was that of females (2958.09 ng/g) during breeding season, surpassing values for males in
the same season at 2684.84 ng/g. We did find higher content for both males and females in the Breeding Season compared to the Non-Breeding season, however; no statistical significance was found between either of the two sexes or between seasons. These findings did not support our hypothesis that higher levels for testosterone would be found for males than females, but we suspect various variables, that we will give further details in our discussion.

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Figure 4 Average content of estradiol (ng/g *y-axis), and breeding/non-breeding Season

![Graph showing estradiol content for breeding and non-breeding seasons for females and males](attachment://estradiol_graph.png)
(*x-axis), found in our collected samples for both male and female

Figure 5 Average content of progesterone (ng/g *y-axis), and breeding/non-breeding Season (*x-axis), found in our collected samples for both male and female deer in breeding and non-breeding seasons.

Figure 6 Average content of testosterone (ng/g *y-axis), and breeding/non-breeding Season (*x-axis), found in our collected samples for both male and female deer in breeding and non-breeding seasons.

3.3.2 Discussion of Hormonal Analyses

Our results for the hormonal part of our project did not support our original hypotheses that males should have significantly higher values of testosterone (Figure 6) than females and that it should also be higher in breeding season compared to that of non-breeding season for mate competition and reproductive success. As well as the idea that females should have significantly higher values progesterone (Figure 5) than males or in breeding season than in non-breeding season, was also not supported. Estradiol was the only sex-steroid hormone which our results did show a significant difference between seasons, but we did not see a higher concentration in females as we expected, as no significance was found (Figure 4).

Many possibilities for these unexpected results could have happened, and most likely more
than one did occur. First there were some human errors in the lab that gave us high values in the standard deviation, for example we did use a smaller sample size than we had originally planned (0.1 g vs 0.001 g that we used from our original sample), which made us modify some of our methods, such as increase cycles which might have given us lower values than expected. We also cannot cross out potential cross contamination in the lab, as much as we used proper protective gear (gloves and masks), common mistakes such as forgetting pipetting changes, and confusing wells in the dilution process, skin contact, among others were very possible.

Also, our sample size between breeding season (around 50 usable samples) vs. 11 samples during non-breeding season made some of our statistical analysis very difficult to conduct. It is quite possible that there were significant differences between seasons but we would need to collect more samples and equal amount of samples during both seasons. As well as our samples should be more representative of the actual population of Yakushima, which was not possible in just a few days of collection for each season.

One of our biggest limitations from our fieldwork was the possibility of contamination. The island is so overpopulated with deer that many of the samples that were collected had already fallen in other animals’ fecal, which might have been of a different age, sex, or reproductive status. Potentially, for future experiments it would be better to be more critical and not collect samples that were close to any other fecal. Alternatively, we could explore other ways to discriminate between old and new fecal in the field to avoid cross-contamination.

Another option for improvement and for further continuation of these type of studies is to use other types of hormonal analysis in the lab, simultaneously, to compare the efficacy with deer pellet fecal. Some of these other options could include Solid Phase Extraction (SPE) and/or Column Chromatography.

4 General Discussion

In conclusion, by using direct observation, DNA analysis and hormonal analysis, we were able to more accurately estimate the sex and age of deer. Additionally, from pellet size, age (adult or juvenile) can be estimated. We expect that those methods can be applicable to understand about the following in detail. 1. Sex ratio in population. By direct observation, it was difficult to recognize the sex of young individual. However, by using DNA analysis, more reliable data can be obtained for those age classes. Additionally, collection of sex ratio data of each year will help us to develop an appropriate hunting policy, such as the number culled for each sex. 2. Behavioral and ecological studies on each age-sex class, in breeding season and in non-breeding season. Until now, we poorly know if there is any behavioral or ecological difference between age-sex class or in seasons for Sika deer living in Yakushima Island. Female caribou (*Rangifer tarandus*) with calves living on small islands, have been known to select relatively safe habitats in the summer. On the other hand, males selected those habitats that had the greatest number of forbs species and also selected habitats where there were more ferns, the most important forage group, and where there was more white spruce (Ferguson, 1988). Therefore, it might be possible that Sika deer in Yakushima Island may show such ecological and behavioral patterns, like the caribou. We should combine direct observation with DNA analysis or hormonal analysis to see if females with fawns prefer to use different area compared with nulliparous females, or if males prefer to forage at a different area from females or juveniles. This knowledge will improve culling and population
census methods of deer.

However, still we need to consider about the future improvements regarding our three methods. (1) During the field study, there were many old fecal pellets on the ground. The old feces might not be appropriate for DNA analyses and currently we can not recognize sex from fecal pellets. If we will be able to find some cue to determine sex from fecal pellets, even old feces from unidentified individuals could be used. In that case, sample number would increase drastically and focal observations would not be necessary. (2) We could not identify the sex of 16 out of 54 samples, even by DNA analysis. Fecal pellets of deer were much harder and drier in comparison to macaque feces. Therefore, we need to establish a specific DNA collection protocol for ungulates. For example, use different buffers, swab more fecal pellets, or swab as soon as possible after defecation in order to prevent the samples from drying too quickly. (3) Hormonal values expected for each sex class were not accurately reported from our analysis. Our values did not support breeding seasonality or were indicative of whether females were in estrus. Therefore, we need to consider the utilization of alternate methods such as, Solid Phase Extraction (SPE) or column chromatography, to quantify hormonal levels with more precision.

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