Individual and Sexual Differences in Time to Habituate to Food-stimuli Presentation of Potential Prey in *Hyla japonica*

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Abstract: The traits of many animal species exhibit individual and sexual differences. Individuals who repeatedly receive a stimulus without harm become habituated to it. However, few studies have been conducted on individual and sexual differences in the process of habituation to unexperienced food stimuli. Therefore, we hypothesized that individual differences or sexual differences would be observed in reaction to a food-stimuli presentation at a laboratory of potential prey (after that "food stimuli"). We tested the hypothesis using the Japanese tree frog *Hyla japonica*, and conducted statistical analyses with the results. A generalized linear model (GLM) showed individual and sexual differences in time to get used to the food stimuli. Females habituated more rapidly to food stimuli than males. The reasons for the difference between sexes are discussed in view of two ultimate and one proximate reason.

Main text

INTRODUCTION

Individual and sexual differences in traits have been known in many animal species.

Sexual differences have often been observed in some behavioral patterns of animals; for

example, males compete with other males for mates, but females do not (Wong and

Candolin, 2005). Also, males usually have weapons to compete for females, but females

do not (Emlen, 2008). A sexual difference in whether the túngara frog, *Physalaemus*

pustulosus, takes a defensive or aggressive response to an auditory stimulation has been

confirmed (Bernal et al. 2007). In this way, sexual differences are observed in the

reaction when animals receive stimulation.

Individual variations in behavioral traits are also well observed as animal personality

(e.g., Stamps and Groothuis, 2010; Sih et al., 2015). For example, individual variation has

been observed in the Japanese tree frog, *Hyla japonica*, in which variation of boldness

was reported (Borzée et al. 2018). Animal individuals get used to stimuli; that is,

habituation to stimuli is often observed when a similar stimulation is provided

repeatedly to an animal (Yilmaz and Meister, 2013). In same animals including

amphibians, habituation in stimuli has been reported (Peeke and Herz 1973, Megela and

Capranica 1983). Also in *Homo sapiens*, sexual differences are recognized in individual

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reactions to novel stimuli (Buchsbaum and Pfefferbaum, 1971; Maccoby and Jacklin, 1974).

When an individual animal repeatedly receives a similar stimulation without harm or profit to the individual, the responses of the individual gradually adjust to the stimulation. This phenomenon is observed in many taxa, and it is called the "habituation process" (Thompson and Spencer, 1966; Carew et al., 1972). For an individual, habituating to meaningless food stimuli can eliminate unnecessary movement, and thus it can conserve energy because of increasing situation of stopping (not moving), leading to an increase in fitness, i.e., the increase of his/her offspring. For instance, California sea slugs, *Aplysia californica*, retract their gills when the gills are stimulated. However, the reaction intensity of the gills gradually decreased when the stimulation was given repeatedly (Castellucci et al., 1970). This is due to decreasing transmission efficiency of the synapse. Such habituations have been investigated in many taxonomic groups including mammals (Doran-Sheehy et al., 2007; Deecke et al., 2002; Wong et al., 2010).

Animal individuals often do not recognize novel objects as food (Rozin, 1976), and thus they do not eat during a certain period of time. After the period, the animal recognizes a novel object as food and then eats it. Therefore, we expect that habituation would occur in response to food-stimuli presentation. However, few studies have shown the

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process of habituation to unexperienced food stimulation or investigated individual and sexual differences in the process.

Because there are also sexual differences in reactions to stimuli (Bernal et al., 2007), it is interesting to investigate whether sexual differences exist in habituation to an unexperienced food. We assumed, here, as a null hypothesis that there are no sexual differences in processes of getting used to a presentation of potential prey (after that, we express it as "food stimuli"), and also are no habituation to feeding experience in animals.

To evaluate this hypothesis, we used the Japanese tree frog *Hyla japonica* as a model animal.

MATERIALS AND METHODS

Animals. Hyla japonica was collected at the farm of Okayama University (latitude: 34.69, longitude: 133.91) from August to September in 2012 and from September to October in 2013 (For details on the clade sampled, please see Dufresnes et al., 2016). The numbers of individual were 43 in 2012 (20 females and, 23 males), and 105 in 2013 (69 females and 36 males). The frog is widely distributed common species in Japan, and the individual rearing is easy. *Hyla japonica* has been recently changed to *Dryophytes*

japonicus (Duellman et al., 2016). They eat many of insect species including ants (Formicidae), beetles (Coleoptera), dipterans, catapillars (Lepidoptera larvae), bugs (Hemiptera), orthopteran species spiders, and isopoda (Hirai and Matsui 2000).

In the present experiment, frogs were reared in a transparent plastic dish (15cm diameter, 10 cm height; Figure 1) in a thermostatic chamber (Sanyo, Tokyo) maintained at 25°C and 16L8D (light-dark condition). In the plastic dish which was covered by the lid made by polyethylene, sterilized gravel was spread with a slope, and tap water was added to a depth of 5 mm from the bottom (130 ml). The cylinder was divided in half in the middle, and frogs could soak in the water on one side and get out of the water on the opposite side. We put artificial turf (length: 4 cm, width: 4 cm, height: 2 cm) in the center of the cylinder. Frogs often used it as a shelter and spent much time hiding under it or looking around on it.

We fed a larvae (instar not specified) of a moth, *Ephestia kuehniella* (body length: 10–15 mm), and third instar nymphs of a cricket, *Gryllus bimaculatus* (length: 8–12 mm), to frogs in 2012 and 2013, respectively. In 2013, the rearing stock of the moth has been discontinued, and thus we used the nymphs of cricket. All frogs were fed once a day; that is food (a moth in 2012 and a cricket in 2013) was thrown into each rearing dish. Okayama University for the Animal Care and Use Committee approved all procedures.

Feeding experiment.   We provided a prey every three days to frogs for more than one month and measured the time delay before eating the food, and finally tested whether the habituation process differed between sexes and among individuals. This experiment was done apart from the daily feeding.

We picked up a live insect with a soft aluminum tweezer and placed it in front of a frog 1 cm from its eyes. The time from exhibiting to consuming the insect was measured by a stopwatch (feeding experiment). Feeding intervals were at least three days. This artificial providing method might be unfamiliar for frogs, because they had been spent fields freely and thus they never meet such preys moving unnaturally. Additionally, the food used the present study may be unfamiliar for the frogs, because moths and crickets are familiar for the frog in their natural habit (Hirai and Matsui 2000). Therefore, in the present study, we defined these preys with artificial feeding as "food stimuli". We successively reared each frog by this feeding method more than 69 days; i.e., we fed each frog a food stimulus more than 19 times. The time limit on consuming was 180 seconds in 2012 and 300 seconds in 2013. Frogs that did not eat the food before the time limit were recorded as 180 and 300 seconds in 2012 and 2013, respectively. During the experiment, frogs were fed the prey every three days.

Statistical analysis. We analyzed the data using the following two methods. Firstly, we divided the data visually into six categories (see Figure 2, Figure 3 for 2012 and Figure 4 for 2013, supplementary materials) as according the logical criteria as follows.

1.　A frog ate the prey in less than half the time limit at the first feeding.

2.　A frog did not eat before the time limit at the first experience but then gradually habituated to the prey by the tenth experiment.

3.　A frog did not eat before the time limit at the first experience, and then became habituated to the prey after the tenth experiment.

4.　A frog ate the food in more than half the time limit at the initial experience.

5.　Feeding times became longer than at the initial experiment (more than half of the time limit).

6.　Others (Those that do not fall from 1 to 5).

We analyzed these patterns by using Fisher's exact test to confirm whether sexual differences existed or not.

Secondly, we considered the time to acclimate to the prey (i.e., the slope of the graphs) as the response variable and the number of experiments, individual

identification number (IDF) as nominal scale, and sexes as explanatory variables.

Thereafter, the data was analyzed by generalized linear models (GLM) (1) quadratic; glm

(family = Gaussian) or (2) logarithm function; glm (family = Gaussian (link = "log")).

$$y＝β1x2+β2x+β3*IDF \quad …(1)$$

$$\log（y）＝β1x+β2*IDF \quad …(2)$$

The statistics were analyzed the two years separately because of many of difference

in factors between the two years, including the frogs used and the prey used. All data

were analyzed using R Core Team (2015).

RESULTS

Firstly, the duration from offering food to eating decreased as the experiment was

repeated. There were several patterns of decreasing times: See Figs. 3 - 6 for the

individual graphs for all frogs tested in 2012 and 2013 (Fig. 3: females in 2012, Fig. 4:

males in 2012, Fig. 5: females in 2013, Fig. 6: males in 2013). The numbers of frogs

exhibiting each of the six patters are shown in Table 1. No significant difference was

found between males and females by Fisher's exact test (2012: p = 0.963, 2013: p =

0.9777) in the pattern classification.

Secondly, we performed generalized linear model analyses on the data. Figure 3

(females) and 4 (males) shows the habituation process for results in 2012, and Figure 5

(female) and 6 (male) shows the results in 2013. The mean duration from providing food

to eating was shortened as the experiment was repeated in all graphs (Figure 2).   In

GLM (quadratic), there were significant differences between individuals in habituation

to prey (2012: linear term F = 2.6744, $d.f.$=42, 730, p <0.001. quadratic term was not

significant F = 1.0617, $d.f.$=42, 688, p = 0.37; 2013: linear term F = 22.785, $d.f.$=107, 4513,

p <0.001, quadratic term F = 12.541, $d.f.$=107, 4406, p <0.001). Moreover, there were

significant differences between males and females (2012: χ^2 = 67.302, $d.f.$ = 2, p <0.001,

2013: χ^2 = 46.234, $d.f.$ = 2, p <0.01, the number of the frogs was 74 in females and 38 in

males). Females habituated faster than males.

When we used GLM (logarithm function; equation (2)), there was also a significant

difference between individuals (2012: F = 8.3541, $d.f.$ = 42, 731, p <0.001; 2013: F =

9.9609, $d.f.$ = 107, 2342, p <0.001) and between males and females (2012: χ^2 = 71.736,

$d.f.$ = 1, p <0.01, 2013: χ^2 = 85.426, $d.f.$ = 1, p <0.001).

DISCUSSION

The present results clearly showed individual and sexual differences in the time to

habituate to presentation of potential prey in *Hyla japonica*. Although habituation times

varied among individuals in males and females, females habituated more quickly to food stimuli than males. We classified the patterns of the habituation process into six types, but no significant differences were found between sexes by the statistics using Fisher's exact test, probably because of the small sample size. However, GLM analysis revealed a significant difference between males and females in habituation to food stimuli. When we analyzed these data by quadratic or logarithm function in GLM, females habituated earlier than males except in 2012 using a logarithmic function. Furthermore, there was a significant difference between males and females when analyzed by a linear equation in GLM.

A sexual difference in habituating times has not been addressed in previous studies concerning animal habituation (Doran-Sheehy et al., 2007; Deecke et al., 2002; Wong et al., 2010). Therefore, to our knowledge, this is the first report of a sexual difference in habituation time to food-stimuli presentation of potential prey. In other words, habituation time to prey is also included in a trait for animal personality, relating to sexual difference.

Why do females habituate to food stimuli more quickly than males? Two ultimate reasons (reason 1 and 2) and one proximate reason (reason 3) may explain the sexual difference. The first (ultimate factor) is to exposure to different sexual selection

pressures for males and females. For example, in *Xenopus tropicalis*, males are bolder

than females, and thus different selective pressures may exist between males and

females, e.g., male competition or territorial fights (Videlier, et al. 2015). If boldness

affects habituation, individuals of the bolder sex may habituate faster than those of the

other sex. In *H. japonica*, a male's calls indicate his geographical position to other males

to compete them and to females to lure them (Aihara et al., 2007), but the calling

behaviors are unknown in females, and therefore this might relate to differences

between sexes in many behavioral traits via correlated response in sexual difference of

calling system. However, this hypothesis cannot explain why females habituate more

quickly to food stimuli than males. Also, it is revealed that this species show boldness

more than a related species and variation in the boldness (Borzée et al. 2018a). It is

known that there is variation in behavior among populations in some anuran species

(Borzée et al. 2018b). Therefore, it is required to compare the level of boldness in

geographical populations in the species in future.

The second (ultimate factor) is a difference in reproductive strategies between males

and females. For example, sexual differences were reported in the range of food

searching in the Northern giant petrel, *Macronectes halli* (González-Solís et al., 2000). In

generally, females must eat more than males because they have to produce as many

eggs as possible. In the species which females have larger body size than males as most

amphibian species (Shine, 1979; Kupfer 2007), females have to eat prey more than males

to grow faster. Also in *Hyla japonica*, females are larger than males in their body size

(Hirai and Matsui, 2000). This size difference might relate to the sexual difference in the

habituation. In the present frog, females may be bolder and without fear of food stimuli

and thus may get used to ¥food stimuli faster than males. Also, there is a possibility that

energetic requirements for growth and reproduction are greater in females than males.

In the present study, we did not measure body mass of each individual. We reared all

frogs individually, thus we predict that each female do not have eggs during the

experiment in the chamber, however we did not know the reproductive status of males

and females. Further experiments are needed to clarify the relationship between

habituation to food stimuli and reproductive growth of frogs in future.

The third (proximate factor) is a difference in effects of hormones between the sexes:

males absorbed more water than females in *Hyla japonica* (Kohno et al., 2004). It is

considered that the sexual difference in water intake may alter the effect of the sex

hormones (Kohno et al., 2004). Therefore, in this study, there is a possibility that the

sexual difference in habituation to food stimuli was affected by hormonal level effects,

although the mechanism is unknown.

To clarify the reasons for the present finding of sexual difference in habituation to consuming food stimuli in this frog, further experiments are required. We also found an individual difference in the time to habituate to food stimuli presentation of potential prey. As studies for animal personality, the trait "habituation to food-stimuli presentation of potential prey" may focus in the future researches. It is also needed to clarify what triggers to habituate to unfamiliar food stimuli in the future studies.

There are substantial differences in habituation between years (much faster in 2012 compared to 2013). The reason why the difference was found might be caused by some uncontrolled environmental conditions including humidity, the difference in frogs used, or different prey items used in the two years, moth in 2012 and cricket in 2013, although it is needed further experiments in future.

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Table 1. Numbers of individual divided by visual classification

Table 1. Numbers of individual divided by visual classification

Year	Sex	Group					
		1	2	3	4-1	4-2	5
2012	female	14	3	0	1	1	1
2012	male	17	3	0	0	1	2
2013	female	22	17	16	7	6	1
2013	male	13	8	9	2	3	1

Legends of Figures

Figure 1 Plastic dish for rearing frogs, *Hyla japonica*.

Figure 2 Mean time to get used to prey in the Japanese tree frog *Hyla japonica*. a. females in 2012, b. males in 2012, c. females in 2013, and d. males in 2013.

Figure 3 Individual data (females) for time to get used to food stimuli of the Japanese tree frog *Hyla japonica* collected in 2012.

Figure 4 Individual data (males) for time to get used to food stimuli of the Japanese tree frog *Hyla japonica* collected in 2012.

Figure 5 Individual data (females) for time to get used to food stimuli of the Japanese tree frog *Hyla japonica* collected in 2013.

Figure 6 Individual data (males) for time to get used to food stimuli of the Japanese tree frog *Hyla japonica* collected in 2013.

Figure 1 Plastic dish for rearing frogs, *Hyla japonica*. The plastic dish was covered by
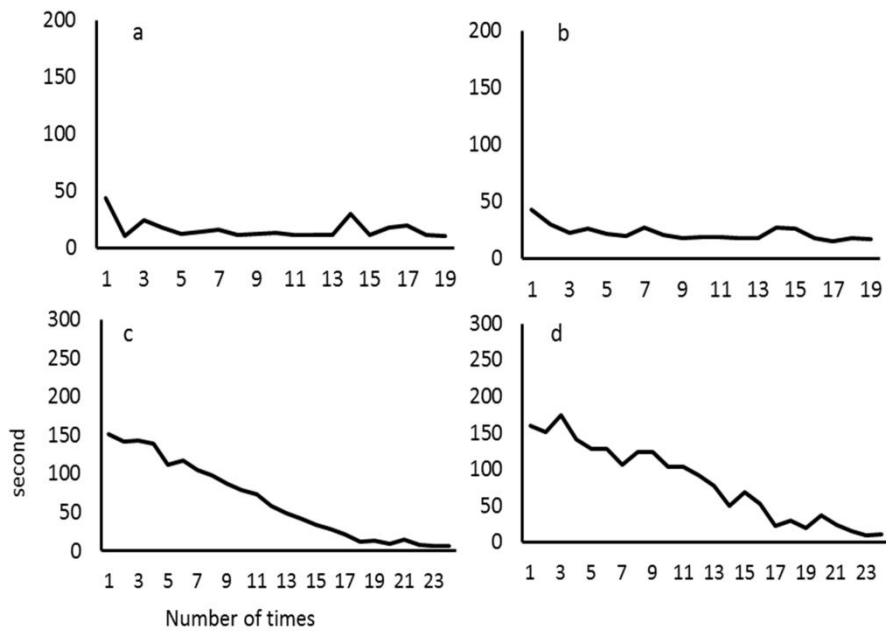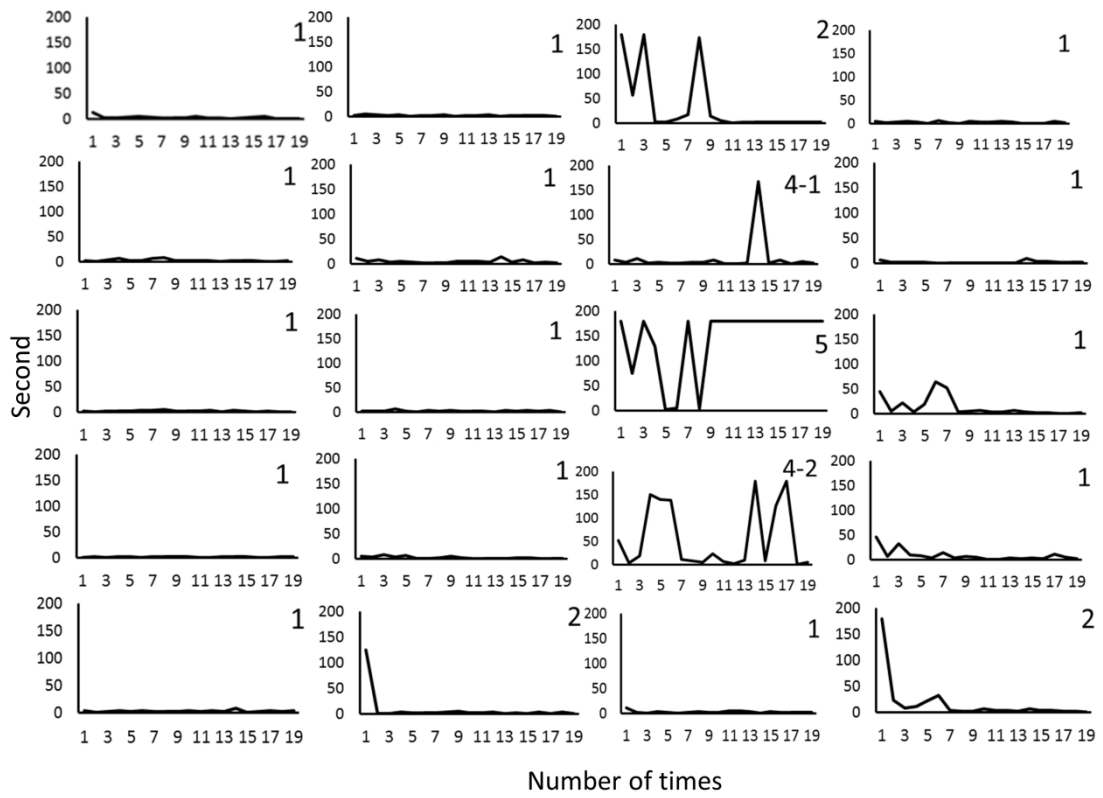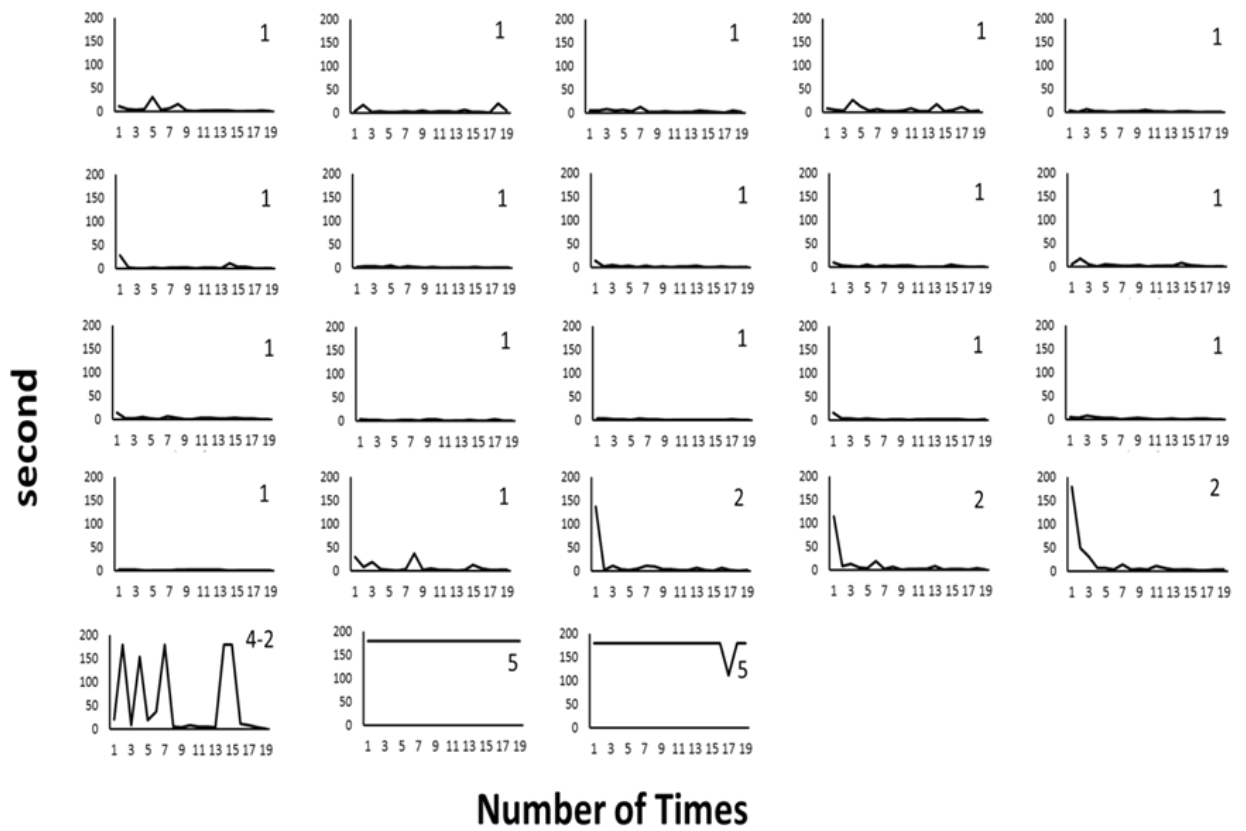
the lid made by polyethylene.

Figure 2 Mean time to get used to novel prey in the Japanese tree frog *Hyla japonica*. a.

females in 2012, b. males in 2012, c. females in 2013, and d. males in 2013.

Figure 3 Individual data (females) for time to get used to food stimuli of the Japanese

tree frog *Hyla japonica* collected in 2012.

Figure 4 Individual data (males) for time to get used to food stimuli of the Japanese tree frog *Hyla japonica* collected in 2012.
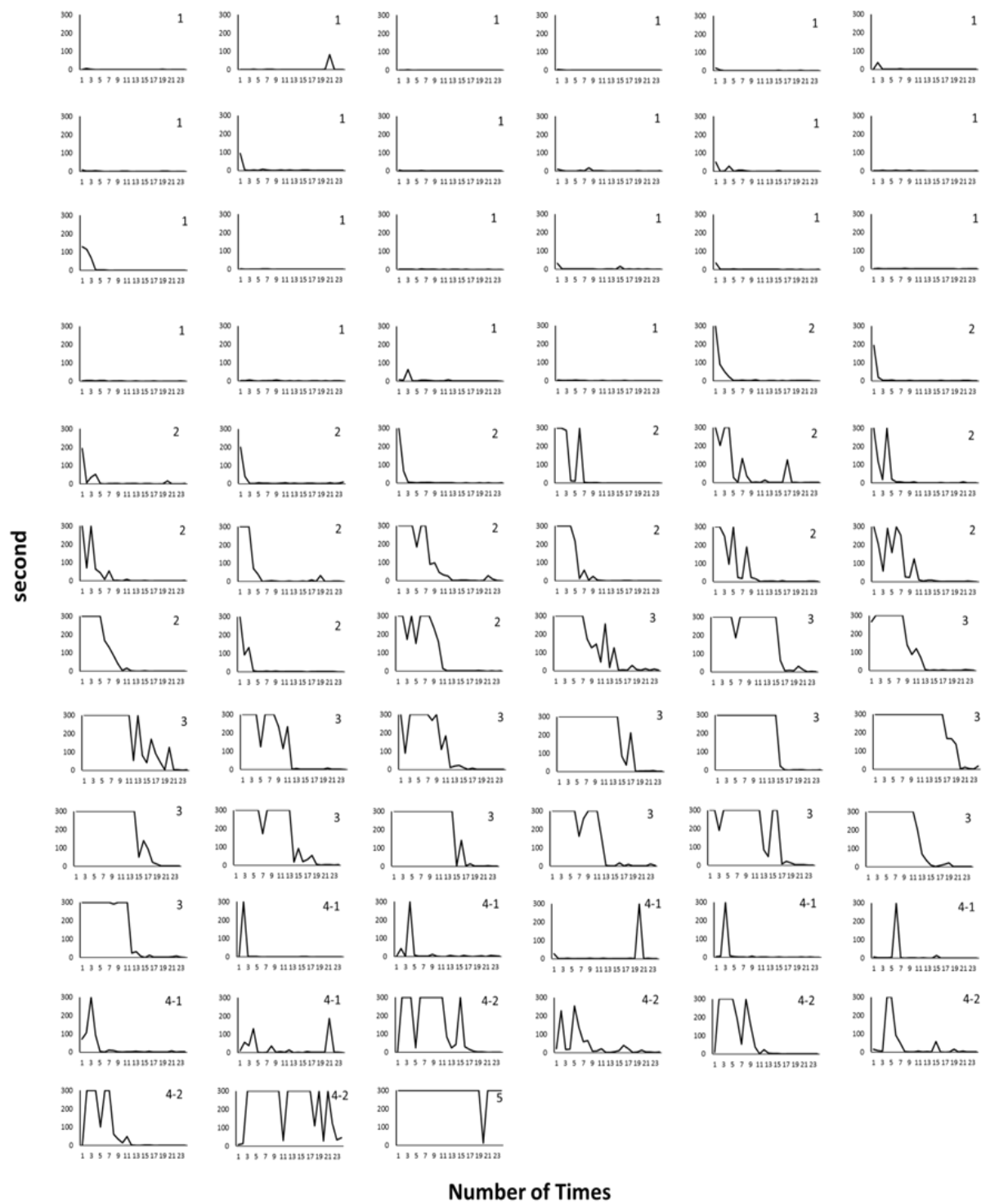
Figure 5 Individual data (females) for time to get used to food stimuli of the Japanese

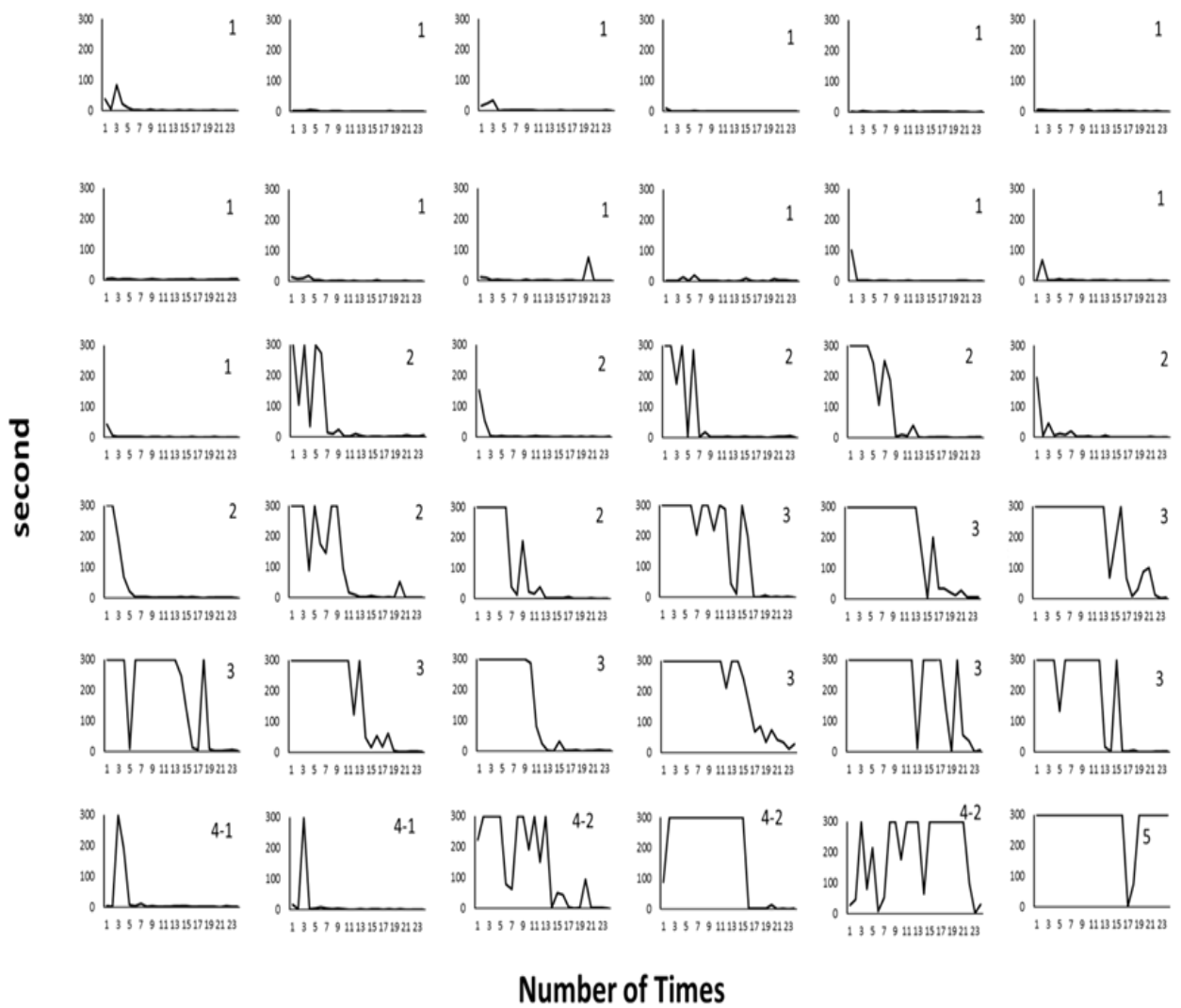tree frog *Hyla japonica* collected in 2013.

Figure 6 Individual data (males) for time to get used to food stimuli of the Japanese tree frog *Hyla japonica* collected in 2013.