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RESEARCH PAPER

Litter size of Japanese flying squirrels *Pteromys momonga* varies seasonally: an intermediate pattern between arboreal and ground squirrels

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Abstract. In most mammalian species, there is a general principle known as the 'one-half rule', in which litter size is half the number of mammaries. However, arboreal squirrels deviate from this rule and small litter size (mainly two to three), whereas ground squirrels adhere to the 'one-half rule' in their litter size patterns. In this study, we showed that spring litter sizes of Japanese flying squirrels were typical of arboreal squirrels, while summer litter sizes were comparable to those of ground squirrels, according to the conventional rule. Thus, Japanese flying squirrels have characteristics of both arboreal and ground squirrel species in terms of litter size. This finding provides novel insights that challenge the classical notion that arboreal squirrels have small litter sizes independent of the number of mammaries.

Key words: mammary number, one-half rule, predation, reproduction, Sciuridae

Introduction

The relationship between the number of mammaries and litter size has long been discussed since the time of Aristotle (Pearl 1913, Aristotle 1937, Stewart et al. 2020), with observations suggesting that species with more mammaries generally have larger litter sizes. While various factors, such as adaptation to the environment, play a role in determining litter size, it is considered that the number of mammaries most strongly limits litter size (Stewart et al. 2020). This phenomenon, termed the 'one-half rule', was first studied in small mammals belonging to Rodentia, of which the mean litter size of species appears positively correlated with mammary number and is often half the number of mammaries (Gilbert 1986). Later, it was considered that this rule was extended to other medium- to large-sized mammals (Diamond 1987). However, certain mammals occasionally deviate from this rule, and the underlying causes of these deviations remain largely unknown; no clear hypothesis has been cited, except for some species, such as the eusocial naked mole-rat (*Heterocephalus glaber*, Rüppell, 1842) (Sherman et al. 1999).

Arboreal (tree and flying) squirrels are a typical animal that deviates from this rule. A notable distinction arises in the relationship between litter size and mammary number between arboreal and ground squirrels (Gilbert 1986). Although the litter size of ground squirrels follows the 'one-half rule', those of arboreal squirrels are usually one to three, independent of the number of mammaries. Although it has been hypothesised that differences in reproductive investment between arboreal and ground squirrels influence differences in litter size,

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no clear conclusions have been drawn (Hayssen 2008). Moreover, even if the hypothesis was correct, it remains questionable why arboreal squirrels maintain excessively high mammary numbers (usually eight) despite having small litter sizes.

To better understand the coevolution of mammary gland number and litter size, it is crucial to identify the factors that led to deviations from the rule among phylogenetically close species (Stewart et al. 2020). However, these questions are difficult to answer because changes in litter size closely match phylogeny (Hayssen 2008), and no species has been found with both arboreal and ground litter sizes within a single lineage. Because the discovery of intermediate species often fills the evolutionary gap (Zhou & Zheng 2003, Warren et al. 2008, Phillips et al. 2009), finding squirrels with both types of reproductive characteristics would be the beginning of a solution to this problem.

We consider the Japanese flying squirrel (*Pteromys momonga*, PM) with ten mammaries to be an intermediate species that fills the evolutionary gap in litter size in the family Sciuridae. More specifically, PM give birth in tree cavities twice each year, in spring (March to April) and in summer (August to September) (Kakuta 2006, Kobayashi 2012, Suzuki 2023), and litter sizes in summer reaches eight (Kobayashi 2012). On the other hand, the litter size in spring is around two (Suzuki 2023). Here, as a first step to clarify the evolution of litter size in the family Sciuridae, we clarify the position of PM as a species with litter size intermediate between that of arboreal and ground squirrels and discuss the evolution of litter size in PM, taking into the phylogenetic relation of the squirrel species. Specifically, we collected information on spring and summer litter sizes for PM from the literature. These litter sizes were compared to those reported for other arboreal and ground squirrels. If PM litter size characterises both arboreal and ground squirrels, PM litter size would be predicted to vary between seasons. Specifically, the litter size of PM in spring is expected to be smaller than in summer and comparable to that of other arboreal squirrels. In contrast, in summer, the litter size will not only be approximately half the number of mammaries, following the 'one-half rule' but also comparable to ground squirrels with similar mammary numbers.

Material and Methods

We conducted an exhaustive search of the literature related to the litter size of squirrels using multiple search engines, including Google Scholar, PubMed, and CiNii Research. We employed the combination of two words: 'squirrel', along with terms such as 'litter size', 'offspring', 'yearling', 'juvenile', 'birth', 'reproduction', 'pups', or 'nest box' for searching pertinent literature. In addition, we also searched for the litter sizes of PM in NDL DIGITAL COLLECTIONS and CiNii Research using the same keywords in Japanese. We set the following four criteria for data collection. 1) We included literature that explicitly stated the original litter size in the text or accompanying figures. 2) Literature that only showed averaged litter size and SD was excluded from the dataset. 3) As body size affects litter size in flying squirrel species (Hayssen 2008), we chose as many flying squirrel species as possible that are comparable in weight to PM (100.0-173.0 g, Suzuki 2023). 4) Given the positive correlation between litter size and the number of mammaries in ground squirrels, we chose a species with a number close to ten (Moor 1961, Micherner & Koeppl 1985, Bartels & Thompson 1993), the PM mammary number. These criteria resulted in data being available for 13 species of 11 genera: three species in two genera in flying squirrels (*Pteromys Volans* – PV, 131.3-137.5 g; *Glaucomys sabrinus* – GS, 120.8-141.9 g; and *Glaucomys Volans* – GV, 53.2-70.0 g), four species in three genera in tree squirrels (*Callosciurus erythraeus* – CE; *Tamiasciurus hudsonicus* – TH; *Sciurus carolinensis* – SC; and *S. aureogaster* – SA), and six species in six genera in ground squirrels (*Callospermophilus lateralis* – CL; *Tamias sibiricus* – TS; *Urocitellus richardsonii* – UR; *Ammospermophilus harrisii* – AH; *Xerospermophilus tereticaudus* – XT; and *Cynomys gunnisoni* – CG). According to Thorington et al. (2012), the weights of flying squirrels were. Finally, we collected 2,363 litter data from 51 literature sources for the 14 squirrel species, including PM. The number of litter sizes in each species is shown in Table 1. Details of the data used and a list of the references in the analysis are given in Table S1.

Firstly, to estimate the phylogenetic relationship of squirrels treated in this study, molecular phylogenetic analysis based on the complete coding sequence cds (except for KC737847, which is partial cds) of the cyt*b* gene was conducted. The nucleotide sequences of fifteen species of Sciuridae were obtained from GenBank. The accession numbers of each species are shown in Table 1. That of PM was AB097682. Alignments were performed using the online version of MAFFT 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013) with the default setting. Aligned sequences were confirmed with BioEdit

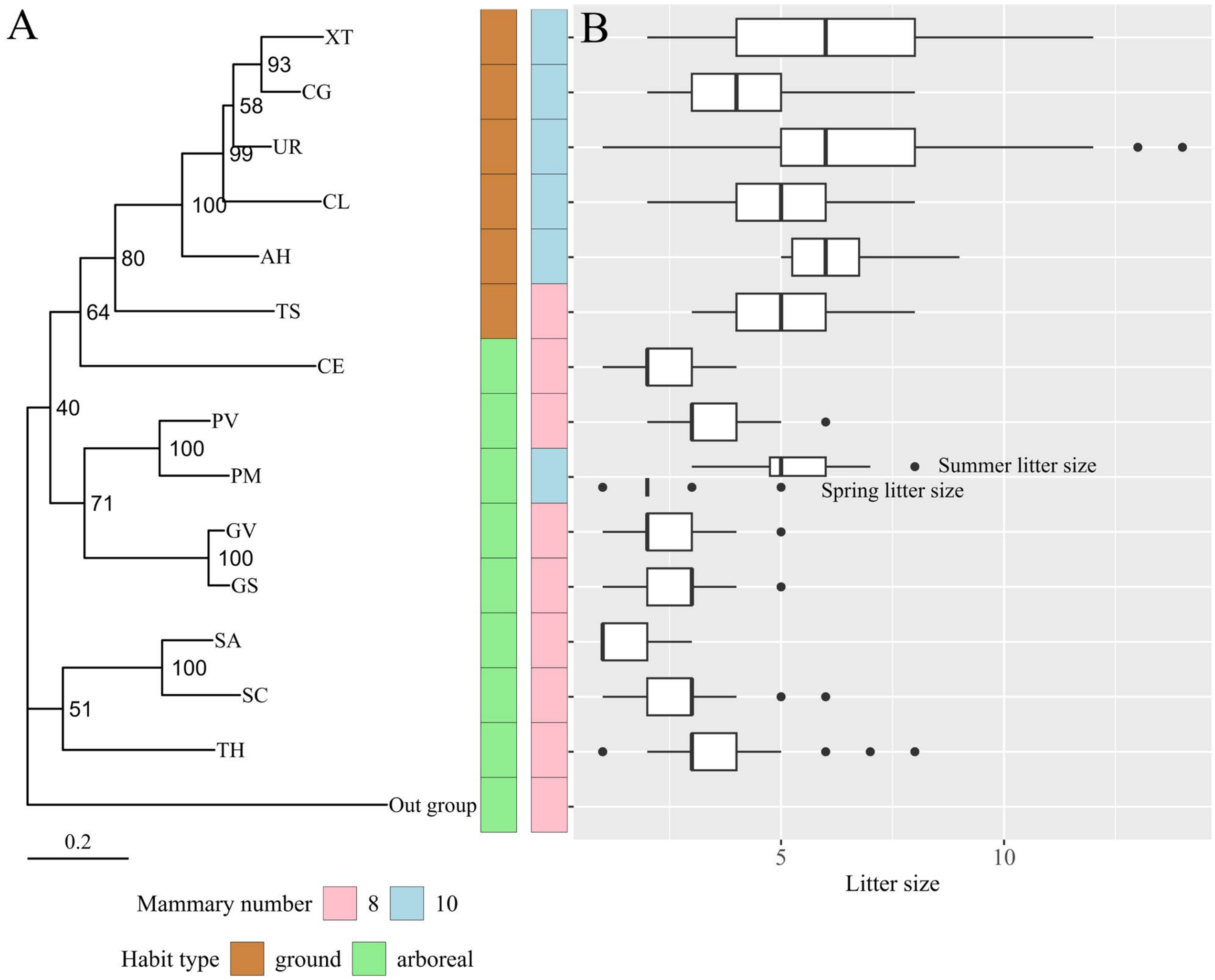

Fig. 1. Maximum likelihood phylogenetic tree of squirrels used to analyse litter size based on cyt*b* gene with habits and mammary number (A) and boxplot of their litter sizes (B). A) the dataset of the phylogenetic tree is composed of 1,134 sites (the number of constant sites is 639, the number of invariant sites is 639, and the number of parsimony informative sites is 421). The TIM2 + F + I + G4 was chosen as the best-fit model according to the Bayesian information criterion. Branch support values by bootstrap analysis are shown at the node. PM – Japanese flying squirrel *Pteromys momonga*; PV – *Pteromys volans*; GS – *Glaucomys sabrinus*; GV – *Glaucomys volans*; CE – *Callosciurus erythraeus*; TH – *Tamiasciurus hudsonicus*; SC – *Sciurus carolinensis*; SA – *Sciurus aureogaster*; CL – *Callospermophilus lateralis*; TS – *Tamias sibiricus*; UR – *Urocitellus richardsonii*; AH – *Ammospermophilus harrisii*; XT – *Xerospermophilus tereticaudus*; CG – *Cynomys gunnisoni*. *Graphiurus murinus* was used for the outgroup. B) the bold lines and the lower and upper hinges indicate the median and first and third quartiles of the data in box plots. The whiskers indicate the most extreme data points. Dots are outliers.

ver. 7.2.5 (Hall 1999) and the first six sites that were missing data for KC737487 were manually removed, and both ends were arranged. The phylogenetic tree was generated by maximum likelihood (ML) analysis using the software IQ-TREE v2.1.3 (Minh et al. 2020) with the ML + rapid bootstrap setting with 1,000 replicates. The consensus tree was viewed and arranged with MEGA version 11 (Tamura et al. 2021). *Graphiurus murinus* (Accession number: AJ225115) was used for the outgroup.

Secondly, we compared the litter sizes of PM with those of other squirrel species. Given the need for multiple comparisons to show differences in litter size between species and that litter size is count data, we first constructed a generalised linear model with a Poisson distribution. Litter size in each litter was treated as the dependent variable. Species was included as an independent variable in the model. However, PM litter sizes were divided between spring and summer based on the month described in the original paper. Tukey's multiple comparison test was used to evaluate the differences in litter sizes between PM in spring or summer and other squirrel species. All analyses were run in R 3.6.2 (R Core Team 2019). The code for the analysis is shown in Appendix S1.

Table 1. Average litter sizes and summary of the generalised linear model results and Tukey's multiple comparison test. Accession number refers to the gene bank.

Species	No. of Litter	Average litter size (SD)	*Pteromys momonga* in spring *vs.*				*Pteromys momonga* in summer *vs.*				Accession number
			Estimate	Standard error	*Z*	*P*	Estimate	Standard error	*Z*	*P*	
Pteromys volans	59	3.27 (0.93)	–0.46	0.18	–2.59	0.308	0.473	0.131	3.619	0.017	AB164674
Glaucomys sabrinus	50	2.60 (1.25)	–0.23	0.19	–1.26	0.992	0.703	0.140	5.020	< 0.001	AF030392
Glaucomys volans	60	2.32 (0.79)	–0.12	0.18	–0.65	1.000	0.818	0.138	5.920	< 0.001	AF157921
Callosciurus erythraeus	66	2.14 (0.76)	–0.04	0.18	–0.21	1.000	0.899	0.138	6.523	< 0.001	MK256836
Tamiasciurus hudsonicus	425	3.15 (0.90)	–0.43	0.17	–2.56	0.324	0.511	0.112	4.540	< 0.001	FJ200743
Sciurus carolinensis	170	2.67 (1.02)	–0.26	0.17	–1.53	0.956	0.676	0.119	5.691	< 0.001	FJ200744
Sciurus aureogaster	7	1.57 (0.79)	0.27	0.34	0.78	1.000	1.206	0.321	3.762	0.010	KC737847
Callospermophilus lateralis	143	5.06 (1.19)	–0.90	0.17	–5.35	< 0.001	0.036	0.115	0.315	1.000	AF157950
Tamias sibiricus	53	5.06 (1.05)	–0.90	0.18	–5.13	< 0.001	0.038	0.125	0.300	1.000	MT109533
Urocitellus richardsonii	999	6.48 (1.87)	–1.15	0.16	–6.97	< 0.001	–0.211	0.110	–1.920	0.789	KX278589
Ammospermophilus harrisii	14	6.29 (1.33)	–1.12	0.20	–5.70	< 0.001	–0.180	0.153	–1.180	0.996	AF157926
Xerospermophilus tereticaudus	21	6.24 (2.64)	–1.11	0.19	–5.96	< 0.001	–0.172	0.140	–1.234	0.994	FJ965334
Cynomys gunnisoni	262	4.19 (1.13)	–0.71	0.17	–4.26	0.001	0.226	0.113	1.998	0.738	AF157930

Results

A phylogenetic tree is shown in Fig. 1. Extant terrestrial squirrel species clearly evolved from arboreal ancestors. Apart from PM, the arboreal squirrels treated here had eight mammaries. On the other hand, for ground squirrels, TS, the most closely related to arboreal squirrels among the target species, had eight mammaries, while those of other species had ten.

A box plot of litter size in each squirrel species was shown in Fig. 1. Firstly, litter sizes of PM varied between seasons (Estimate = 0.938, Standard error = 0.197, Z = 4.752, and $P < 0.001$), with spring litter sizes (averaged was 2.1, SD was 0.94, and sample size was 18) being significantly smaller than those in summer (5.3, 1.39, and 18). Next, the spring litter size of PM was comparable to that of arboreal squirrels but smaller than that of the ground squirrels (Table 1). In contrast, the summer litter size of PM was significantly larger than those of arboreal squirrels, but similar to ground squirrels (Table 1). In addition, the summer litter size was similar to the general litter size based on the 'one-half rule', as shown by the following equation (Gilbert 1986): $L = 0.39 + 0.46M$

L is litter size and M is the number of mammaries. Since the number of mammaries in PM is ten, the litter size is estimated at 4.99 when applied to this formula. In contrast, the spring litter size was less than half the estimated value.

Discussion

This study demonstrated that litter sizes of PM differ between seasons, and PM in spring have a reproductive output comparable to other arboreal squirrels, while during summer, it shares similarities with ground squirrels, conforming to the conventional 'one-half rule' (Gilbert 1986). In other words, PM has characteristics of both arboreal and ground squirrel species in terms of litter size. In the present study, we analysed only available original data from species that share similar weight and mammary numbers with PM. As a result, only 13 species were comparable with the PM. This number corresponds to approximately 5% of the family Sciuridae (Mercer & Roth 2003). Many papers on squirrel breeding have been published (Hayssen 2008), but only some contain detailed original data, which explains why so few species were used in this study. Nevertheless, these 13 species can be considered to reflect the litter size of each taxon. The average litter size of the seven arboreal squirrel species with eight mammaries compared in this study ranged from 1.57 to 3.27 (Table 1). Previous studies have shown average litter sizes for 64 arboreal squirrel species (Hayssen 2008), of which 72% fall within this range. Also, 28% of the species outside this range were giant flying squirrels, genus *Petaurista*, which were not covered in this study. The average litter size of ground squirrels with eight to ten mammaries treated in this study ranged from 4.18 to 6.49, broadly in accordance with the 'one-half rule' (Gilbert 1986). These findings suggest that similar results would be obtained even if more species were compared. In addition, few studies have examined seasonal changes in litter size in the family Sciuridae. The only study of which we are aware is the seasonal variation in litter size of GV, but there is little difference between spring (2.9) and summer (3.5) litter sizes (Stapp & Mautz 1991). It is rare for arboreal squirrels like PM to have such a substantial variation in litter size between seasons.

We first consider the phylogenetic position of PM, the number of mammaries, and litter size. The phylogenetic tree we have presented shows that the three flying squirrels (PV, GS and GV) species, which are more closely related to PM, have eight mammaries. The number of mammaries of other arboreal squirrels is also eight. In contrast, the number of mammaries of PM is ten. This finding indicates that the number of mammaries in PM increased after the speciation of PM and PV. If there were no relationship between mammary number and litter size in arboreal squirrels, then selection would not favour an increase in the number of mammaries, with selection for large litter size the most likely driver of this trait.

What has contributed to the increase in the PM litter size? Differences in reproduction within Sciuridae reflect geographic constraints (Hayssen 2008). Genus *Pteromys* is thought to have colonised Japanese islands via the Korean Peninsula during the Middle Pleistocene, and it is plausible that speciation occurred, giving rise to the endemic species PM (Kawamura 1988). A possible geographical constraint is the difference in the amount of powerful predators – snakes – in the habitats of the two species. In small mammals, small litter size is often associated with a low predation risk (Garbino et al. 2021). Ground squirrels are more susceptible to predation than arboreal squirrels, particularly from snakes that are significant predators of ground squirrels (Swaisgood et al. 1999). Consequently, in areas lacking arboreal snakes, it is plausible that arboreal squirrels have a smaller litter size than ground squirrels. In the northern Eurasian continent, which is the original habitat of PV (Oshida et al. 2005), there are few arboreal snakes compared to Asia (Harrington et al. 2018). The only predatory snake (*Vipera berus*, Linneaus, 1758) of rodents (Luiselli 2006) is terrestrial and very rarely forages in arboreal regions (Groen et al. 2020). In contrast, the Japanese rat snake (*Elaphe climacophora*, Boie, 1826), an arboreal snake (Harrington et al. 2018), is widely distributed in mountainous areas in Honshu, Shikoku, and Kyushu Islands, which coincides with the range and habitat of PM. This snake is endemic to Japan and can reach up to 2 m or more. It is a predator of offspring, especially for animals that breed in tree cavities (Hayashi et al. 1996, Shigeta et al. 2020). The evolution of parental alarm calls by the Japanese great tit (*Parus minor*, Temminck & Schlegel, 1848) against the Japanese rat snake provides strong evidence of high predation pressure on chicks in cavities by the snake (Suzuki 2011, 2018). So, this snake can be an important predator of young PM. As the Japanese rat snake usually emerges from hibernation in April (Fukada 1983), during the PM spring birthing season in March to April (Suzuki 2023), the snakes are still in torpor or less active due to low temperatures, and it is believed that the predation risk of young PM is low. In contrast, predation risk will be higher in summer, when the snakes are most active. Thus, PM may flexibly change litter size in response to differences in predation risk.

We propose that predatory snakes strongly influence the large litter size of PM, but several issues need to be resolved to strengthen this hypothesis. First, it remains to be seen whether the relationship between optimal litter size and predation pressure, as suggested by PM, applies to all arboreal squirrels. For example, in tropical rainforests, where arboreal snakes are more abundant, although arboreal squirrels consistently maintain small litter size to mammary number (Muul & Liat 1974, Hayssen 2008), they can reproduce throughout the year (Tamura et al. 1989) and give birth more frequently (Santicchia et al. 2015) because the rainforest is rich in food through the year. Rainforest arboreal squirrels appear to express a different adaptation than PM, which can only give birth twice yearly due to limited winter food resources. In contrast, the Japanese squirrel *Sciurus lis*, with a distribution range similar to that of PM, may be subject to the same one-half litter rule. Although little is known about the litter size of the Japanese squirrel, the maximum litter size of this species, which has eight mammaries, is six (Tamura 2009), a relatively large litter size for arboreal squirrels. In addition, the average litter size is 4.5 (Hayssen 2008), the largest litter size in the genus *Sciurus*. In addition, the arboreal snake *E. schrenckii* (Strauch, 1873) is also found in northern China and the Korean Peninsula (Harrington et al. 2018). Interestingly, in an area of northern China, PV with ten mammaries was found (Imaizumi 1975). Therefore, a detailed study of the litter size of Japanese squirrels and PV with ten mammaries is essential to show that our hypothesis applies more widely.

The second problem is that the reasons for the small litter size of PM in spring need to be clarified. What factors limit the spring litter size in PM with the potential for a large litter size? In rodents, maternal body condition is an important factor affecting litter size (Neuhaus 2003). Thus, the quality and quantity of available foods may limit spring litter size. Although little is known about the detailed food contents of PM, it is considered folivorous, foraging mainly on leaves, flowers (pollen), and buds (Suzuki 2023). As the mating season of PM for spring birth is during the harsh winter months of January and February (Kikuchi et al. 2022), food resources for PM are expected to be limited and of low quality. Elucidating the detailed food resources of PM and examining the relationship between maternal body condition and litter size is the next important task.

Almost 40 years since it was recognised that arboreal squirrels had extremely small litter sizes compared to mammary numbers (Gilbert 1986), no arboreal squirrels with litter sizes commensurate with teat mammary number have been found. The finding of this study provides novel insights that challenge the classical notion that arboreal squirrels have small litter sizes independent of the number of mammaries (Gilbert 1986). Also, as far as we know, the causes of the excessive mammary numbers of arboreal squirrels were unclear and not even hypothesised. Despite several limitations, we consider that PM possess a large mammary number that allows them to accommodate variable litter sizes in response to environmental changes, including predatory snakes.

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Author Contributions

K.K. Suzuki: conceptualisation, data collection, statistical analysis, methodology, discussion, project administration, software, supervision, validation, visualisation, writing the original draft. Y. Ando: phylogenetic analysis, discussion. We have no competing interests. We received no funding for this study.

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Supplementary online material

Table S1. Litter size data used in the analysis (https://www.ivb.cz/wp-content/uploads/JVB-vol.-73-2024-Suzuki-K.K.-Ando-Y.-Table-S1.csv).

Appendix S1. R script for litter size analysis (https://www.ivb.cz/wp-content/uploads/JVB-vol.-73-2024-Suzuki-K.K.-Ando-Y.-Appendix-S1.pdf).