

1 Amplitude of circadian rhythms becomes weaker in the north,
2 but there is no cline in the period of rhythm in a beetle

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4 Masato S. Abe¹, Kentarou Matsumura², Taishi Yoshii³, Takahisa Miyatake²

5 ¹ *Center for Advanced Intelligence Project, RIKEN, Tokyo, Japan*

6 ² *Graduate School of Environmental and Life Science, Okayama University,*

7 *Okayama, Japan*

8 ³ *Graduate School of Natural Science and Technology, Okayama University,*

9 *Okayama, Japan*

10

11 **Corresponding author:**

12 Takahisa Miyatake E-mail: miyatake@okayama-u.ac.jp

13 Telephone number: +81-86-251-8339

14 Postal address: Evol Ecol Lab, Graduate School of Environmental and Life Science,

15 1-1-1 Tsushima-naka, Kita-ku, Okayama, 700-8530, Japan

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17 **Running head**

18 Amplitude, not period, has cline in circadian rhythm

19

20 **Abstract**

21 Many species show rhythmicity in activity, from the timing of flowering in
22 plants to that of foraging behaviour in animals. The free-running periods and
23 amplitude (sometimes called strength or power) of circadian rhythms are
24 often used as indicators of biological clocks. Many reports have shown that
25 these traits highly geographically variable, and interestingly, they often
26 show latitudinal or altitudinal clines. In many cases, the higher the latitude
27 is, the longer the free-running circadian period (i.e., period of rhythm) in
28 insects and plants. However, reports of positive correlations between latitude
29 or longitude and circadian rhythm traits, including free-running periods, the
30 power of the rhythm and locomotor activity, are limited to certain taxonomic
31 groups. Therefore, we collected a cosmopolitan stored-product pest species,
32 the red flour beetle *Tribolium castaneum*, in various parts of Japan and
33 examined its rhythm traits, including the power of the rhythm and period of
34 the rhythm, which were calculated from locomotor activity. The analysis
35 revealed that power was significantly lower for beetles collected in northern
36 areas compared with southern areas in Japan. However, it is worth noting
37 that the period of circadian rhythm did not show any clines; specifically, it

38 did not vary among the sampling sites, despite the very large sample size (n
39 = 1585). We discuss why these cline trends were observed in *T. castaneum*.

40 **Keywords:** biological clock, circadian rhythm, cline, latitude, longitude,
41 *Tribolium castaneum*, power of rhythm

42

43 **Introduction**

44 Latitudinal clines are of evolutionary interest because they indicate the
45 action of natural selection [1]. Many traits correlate with latitude, for
46 example, body size [2,3] and life history traits [4]. Circadian rhythm traits
47 are also correlated with latitude.

48 Circadian rhythms are particularly important for timing or regulating key
49 biological events in insects [5]. The free-running period and power of the
50 rhythm (i.e., sometimes called the amplitude or strength of the rhythm) are
51 often used as indicators of circadian rhythms [6,7], and there is much
52 evidence that the free-running periods of circadian rhythms exhibit
53 latitudinal or longitudinal clines at the phenotype to molecular levels in
54 many taxonomic groups [8-12].

55 Additionally, in insect species other than *Drosophila*, many studies on the
56 relationships between free-running periods and latitude have been conducted.
57 In the linden bug, *Pyrrhocoris apterus*, higher-latitude populations are
58 reported to have longer free-running periods [13]. Similar positive relations
59 between latitude and circadian rhythms have been reported in a parasitic
60 wasp, *Nasonia vitripennis* [12], and also in plant species [14].

61 In *Drosophila* species that evolved in tropical regions and then expanded
62 their distribution to temperate regions, rhythm traits vary depending on
63 where the flies live [15]. For example, pupal-adult eclosion rhythms in the
64 far north were more arrhythmic than those in the south among *Drosophila*
65 *littoralis* populations [16]. Circadian locomotor rhythms of *D. melanogaster*
66 derived from Africa had a stronger power of rhythm than those of more
67 northern *Drosophila* species in Europe [17]. However, pioneer studies
68 revealed a negative relation between latitude and these traits [18]. Overall,
69 these studies reveal that this topic remains controversial.

70 Given these discrepancies, we need more biological models. The red flour
71 beetle *Tribolium castaneum* (Herbst) [Tenebrionidae] is a cosmopolitan
72 stored-product pest [19], and hence, it can serve as an insect model species.
73 Its biology and behaviour are well studied [20,21]. Furthermore, genome
74 sequences are available for *Tribolium castaneum* [22].

75 In Japan, this species is distributed in most areas except Hokkaido, in the
76 northern parts, meaning that it can be collected from a wide range of latitudes
77 [23]. It provides us with a novel, fascinating model with which to examine
78 the relations between latitude/longitude and circadian rhythm traits. Hence,

79 we studied the relationships between latitude or longitude and circadian
80 rhythm parameters, including the period of circadian rhythms, power of the
81 rhythm, and locomotor activity, in *T. castaneum*.

82

83 **Materials and Methods**

84 **Insects**

85 The geographical populations of *Tribolium castaneum* used in the present
86 study were collected from 38 different fields in Japan (Figure 1). Table S1
87 shows the latitude and longitude of the collection points, along with the
88 number of samples. The northernmost and southernmost points where the
89 insect could be collected were Aomori Prefecture (north latitude 40.89, east
90 longitude 127.69) and Kumamoto Prefecture-C (north latitude 32.57, east
91 longitude 130.66), respectively. The collection of insects was conducted
92 during 2016 and 2017. We collected beetles from each rice pearling mill in
93 Japan that was set up in a village or town with rice fields. More than twenty
94 beetles were collected from each mill. The collected beetles were reared with
95 a mixture of whole meal (Yoshikura Shokai, Tokyo) enriched with brewer's
96 yeast (Asahi Beer, Tokyo) and maintained at 25°C with a 16 h photoperiod

97 (lights on at 07:00, lights off at 23:00). Each collected group was kept in a
98 separate plastic Petri dish (90 mm in diameter, 15 mm in height). Since this
99 species is a stored-grain pest, these laboratory conditions were chosen to
100 closely mirror the native environment of these beetles. Before the
101 experiments, each beetle population was reared for more than two
102 generations in incubators (MIR-153, Sanyo, Osaka, Japan). We used beetles
103 reared for a few generations in the incubator in the experiment.

104

105 Locomotor activity

106 To assess circadian rhythm, we maintained beetles under 16L:8D conditions
107 for more than 20 days in an incubator kept at 25°C before the measurement
108 of locomotor activity, and we then measured the locomotor activity of *T.*
109 *castaneum* for 10 days in darkness. A beetle from each population was placed
110 in a clear plastic Petri dish (30 × 10 mm) in an incubator (MIR-153, Sanyo,
111 Osaka, Japan) maintained at 25°C under complete darkness (DD). The
112 locomotor activity of each individual was monitored using an infrared
113 actograph. An infrared light beam was passed through a clear Petri dish, and
114 the beam was projected onto a photomicrosensor (E3S-AT11; Omron, Kyoto,

115 Japan) that detected all interruptions of the light beam. Signals of
116 interruption of the infrared light beam were recorded every 6 min [24]. The
117 sample size of each population is shown in Table S1.

118 Statistical analysis

119 To determine the circadian rhythm, the locomotor activity data collected for
120 10 days in constant dark conditions were analysed. The free-running period
121 of circadian rhythms was established using a χ^2 periodogram test [25] for
122 data on locomotor activity between 20 and 28 h [26]. Circadian rhythmicity
123 was determined using χ^2 periodogram analysis, and “power” was used as an
124 index of the strength of rhythms. The power of circadian rhythms was defined
125 as the maximum difference between the χ^2 value and the significance
126 threshold line at $P = 0.05$, that is, the size of the peak above the 5%
127 threshold; see Figure 1 in [6]. Power is high when the rhythm is clear and
128 strong, and a power of less than 0 indicates a statistically arrhythmic state.
129 Moreover, total activity was calculated as the total number of interruptions
130 of the infrared light over 10 days. To analyse the effects of temperature,
131 latitude and sex on the period and power of the circadian rhythms and total

132 activity, we used GLMs with Gaussian link functions. All statistical analyses
133 were performed in R version 3.4.3 [27].

134

135 **Results**

136 First, we analysed the relationship between the geographical area and the
137 rhythm of power. To avoid multicollinearity between latitude and longitude
138 (the correlation coefficient (r) between them was 0.83), we used one of the
139 two as an explanatory variable.

140 The GLM results revealed a significant relationship between latitude and
141 power (Table 1, Figure 2A), while sex was not significantly associated with
142 power (Table 1). The estimated coefficient for latitude in the model was
143 negative, suggesting that the higher the latitude was, the weaker the rhythm
144 was. For longitude, we did not find a significant relationship (Table 1, Figure
145 2B).

146 Second, we investigated the relationship between geographical area and the
147 estimated period of circadian rhythms. The statistical results yielded no
148 significant relationships between them (Table 1, Figure 3).

149 Finally, we investigated the relationship between geographical area and total
150 activity (Figure 4). As the total activity had some outliers, we used log-
151 transformed values. The results showed that total activity was associated
152 with latitude and sex (Table 1, Figure 4B).

153

154 **Discussion**

155 In the present study, the period and power of circadian rhythms and
156 locomotor rhythm varied among geographical populations of *T. castaneum*.
157 Circadian periods seemed to vary evenly between 20 h and 28 h (Figure 3).
158 Circadian rhythm variation occurs in various organisms [5]. The present
159 results showed that the power of circadian rhythms was significantly lower
160 for beetles collected in northern areas compared with southern areas (Figure
161 2). This result suggests that beetles collected from different parts of Japan
162 have genetically different characteristics. In this study, we reared
163 individuals collected from the fields for a few generations in a chamber
164 under the same environmental conditions in the laboratory before measuring
165 their traits. Therefore, it is unlikely that the present results were influenced
166 by any maternal effects. On the other hand, no clines have been observed in
167 the length of circadian rhythms.

168 The trend of weaker circadian rhythms in northern populations has also been
169 observed in other insect species. Specifically, a clear rhythm was shown at
170 lower latitudes whereas no rhythmic activity was shown at higher latitudes
171 in *Hymenoptera* and *Drosophila* species [28,29]. Therefore, the present
172 results are consistent with the results of these previous studies. Why is the
173 rhythm weakened at higher latitudes? One answer may be that in more
174 extreme environments, it may be easier to survive with less restriction of
175 activity by the clock and more control by direct environmental responses,
176 namely, masking of circadian activity [30,31]. To the best of our knowledge,
177 clines in the power of the rhythm have been observed in only a few species.
178

179 On the other hand, positive relations between latitude and the length of
180 circadian rhythms have often been reported; higher-latitude populations are
181 reported to have longer free-running periods [12-14]. However, in the
182 present study, no significant relationship was observed between the period
183 of circadian rhythms and latitude or longitude, despite the very large sample
184 sizes.
185

186 The cline in the amplitude (i.e., power) of the circadian rhythm in *T.*
187 *castaneum* clearly suggests geographic variation. This result is very
188 interesting considering the history of the controversy surrounding the
189 dispersal distance of this insect, specially, that studies do not agree on its
190 dispersal characteristics. Some studies suggest that this beetle disperses very
191 well [32] suggested very high levels of active dispersal through adult flight
192 in *T. castaneum* based on microsatellite genotypes. Drury et al. [33] reported
193 that although these beetles have wings, dispersing by flying is rare at 25°C.
194 On the other hand, Arnold et al. [34] suggested that this beetle usually travels
195 by walking more often than it flies. Semeao et al. [35] showed that
196 populations of *T. castaneum* collected from mills show spatial genetic
197 structure, indicating the occurrence of a recent bottleneck in each mill. The
198 present results clearly showed geographical variation in the amplitude of
199 circadian rhythms among local populations in each mill.

200

201 We considered two few hypotheses regarding why no clines in the length of
202 circadian rhythms were found in *T. castaneum*, as follow: bottlenecks and
203 local adaptation. A small number of individuals or one fertilized female can
204 enter and settle in individual rice mills scattered in the countryside of Japan

205 [36]. A small number of *T. castaneum* will form each population within each
206 mill. Predation pressures, including that from predator insects [36], and the
207 differences due to human cleaning will differ among mills. These pressures
208 can cause differences in traits, especially in the activity traits, of *T.*
209 *castaneum*. Such selection pressures (local adaptation) and founder effects
210 (bottlenecks) would cause a large degree of variation among *T. castaneum*
211 populations. *T. castaneum* cannot fly under stable conditions. Indeed, as
212 described above, although these beetles have wings, dispersing by flying is
213 rare at 25°C [33], with walking being the most frequent mode of travel [34],
214 and walking is the mechanism by which males locally search for females [37].
215 Additionally, Semeao et al. [35] showed that populations of *T. castaneum*
216 collected from mills showed spatial genetic structure, indicating the
217 occurrence of a recent bottleneck in each mill. Therefore, each mill used in
218 the present study may be an ideal environment if without human cleaning,
219 and thus, the beetles may not need to disperse from the rice bran in each mill.
220
221 On the other hand, adults of *T. castaneum* are known to fly well above 28°C
222 [38]. There may be individuals flying beyond the area in the summer season
223 in Japan. If gene flow is greater than expected, it might explain the lack of

224 latitudinal and longitudinal clines shown in the present study. Ridley et al.
225 [32] estimated the dispersal distance of *T. castaneum* using microsatellites
226 and found that adults could fly at least 1 km per year in fields. They reported
227 that *T. castaneum* is predominantly aggregates around areas of grain storage
228 but actively disperses by flight between these spatially separated resources
229 [32].

230
231 Another study showed that genetic distance was not significantly correlated
232 with geographic distance among *T. castaneum* populations in mills in the
233 United States [35]. Semeao et al. [35] provided evidence that populations of
234 *T. castaneum* collected from mills showed spatial genetic structure, but the
235 poor ability to assign individuals to source populations and the lack of
236 isolation by distance suggested lower levels of gene flow than originally
237 predicted. Konishi et al. [36] also suggested that different anti-predator
238 strategies have evolved in each storehouse with and without predatory
239 insects. These studies suggest that the dispute between gene flow or the
240 possibility of evolution in individual storehouses in field mill systems will
241 continue. The present result of a cline in amplitude suggests that gene flow
242 is not occurring at the area scale that we examined. However, estimation of

243 the degree of gene flow between rice mills and phylogenetic relationships
244 between populations within the species in Japan is required.

245

246 Notably, the results of the present study showed significantly smaller P -
247 values, even for small effect sizes, due to the very large sample size ($n =$
248 1585, total sample size). This suggests that the power of circadian rhythms
249 is smaller at higher latitudes, but the change is not large. Moreover, it is
250 worth noting that the period of circadian rhythms does not change, even with
251 the very large sample size, in this beetle species. Further studies on circadian
252 rhythms at the molecular level using this model beetle, which shows different
253 phenotypic phenomena than other insect species, are needed in the future.

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266

267 **Authors' contributions**

268 The experiments were designed by TM, and these were performed by KM. The data
269 were analyzed by MSA. The manuscript was written by MSA, TY and TM. All
270 authors approved the final version prior submission.

271

272 **Ethical Note**

273 The populations of *T. castaneum* used in this study were collected from each mill
274 located at 38 different fields in Japan. These populations have been maintained on
275 wholemeal flour enriched with yeast at 25 °C under a 16:8 h light:dark cycle. These
276 laboratory conditions closely resemble natural conditions of this stored product
277 pest. All individuals in the experiment were handled with care and handling time
278 was kept to an absolute minimum. The use of these beetles conforms to the
279 Okayama University's Animal Ethics Policy.

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Table 1. Statistical test results. Coefficients and standard errors obtained from GLM analysis are shown.

	Response variable					
	Power of rhythm		Period of rhythm		log(Total activity)	
Latitude	-2.356*		-0.572		0.068**	
	(-1.083)		(-0.435)		(-0.023)	
Longitude		-1.126		-0.288		0.023
		(-0.629)		(-0.253)		(-0.014)
Sex (male)	-23.145	-1.303	3.441	30.986	2.408*	3.239
	(-54.072)	(-118.738)	(-21.737)	(-47.701)	(-1.159)	(-2.549)
Latitude × sex (male)	0.753		-0.133		-0.057	
	(-1.519)		(-0.611)		(-0.033)	
Longitude × sex (male)		0.037		-0.238		-0.021
		(-0.876)		(-0.352)		(-0.019)
Constant	54.626	123.33	257.799***	276.420***	4.523***	3.884*
	(-38.616)	(-85.312)	(-15.524)	(-34.273)	(-0.828)	(-1.832)

417 * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

418

419 **Figure captions**

420

421 Figure 1. Collection locations of wild *T. castaneum* populations in Japan.

422

423 Figure 2. Relationship between the power of the rhythm and latitude (A) or
424 longitude (B). The blue line represents the statistically significant regression line.

425

426 Figure 3. Relationship between the period of the rhythm and latitude (A) or
427 longitude (B).

428

429 Figure 4. Relationship between total activity and latitude (A) or longitude (B).
430 The blue and red lines represent the statistically significant regression lines of
431 males and females, respectively.

432



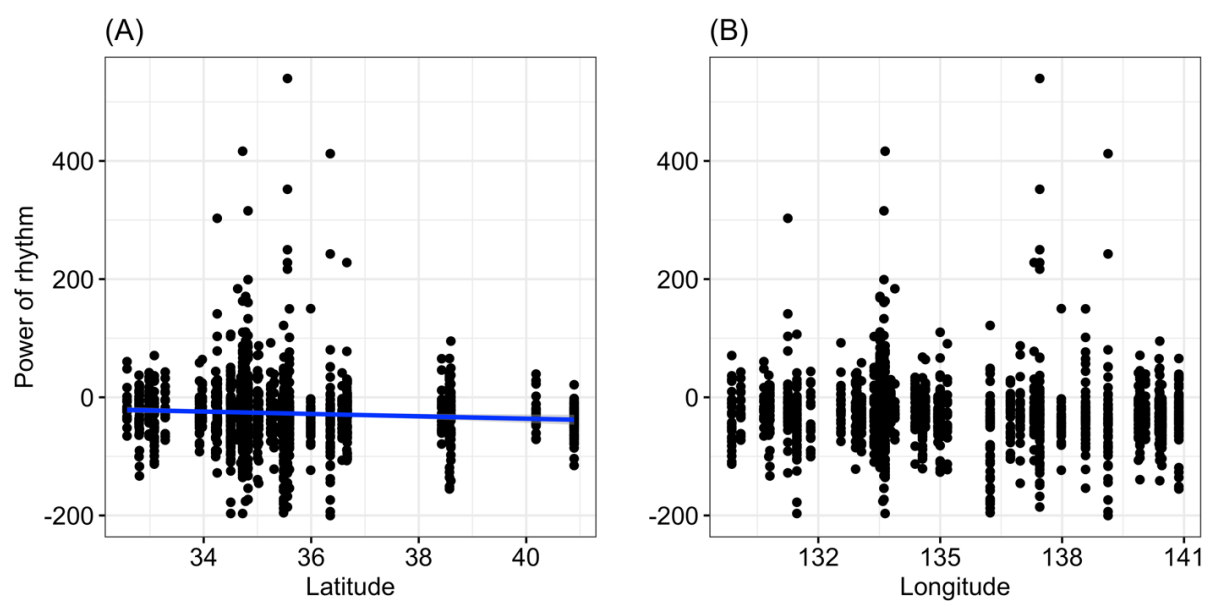
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435 Figure 1

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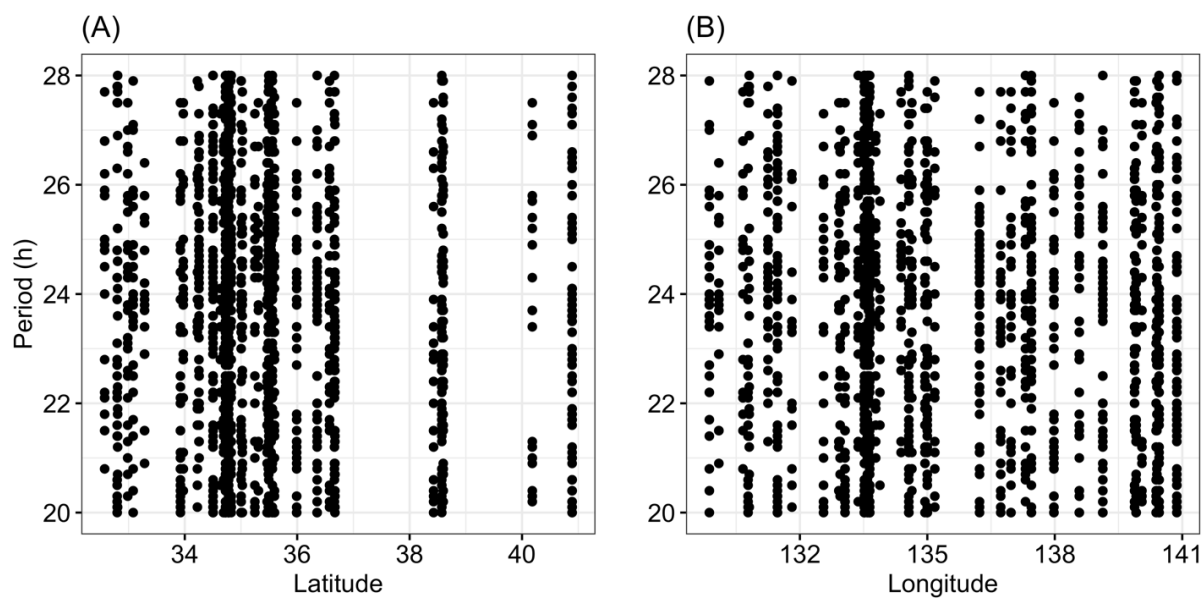


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439 Figure 2

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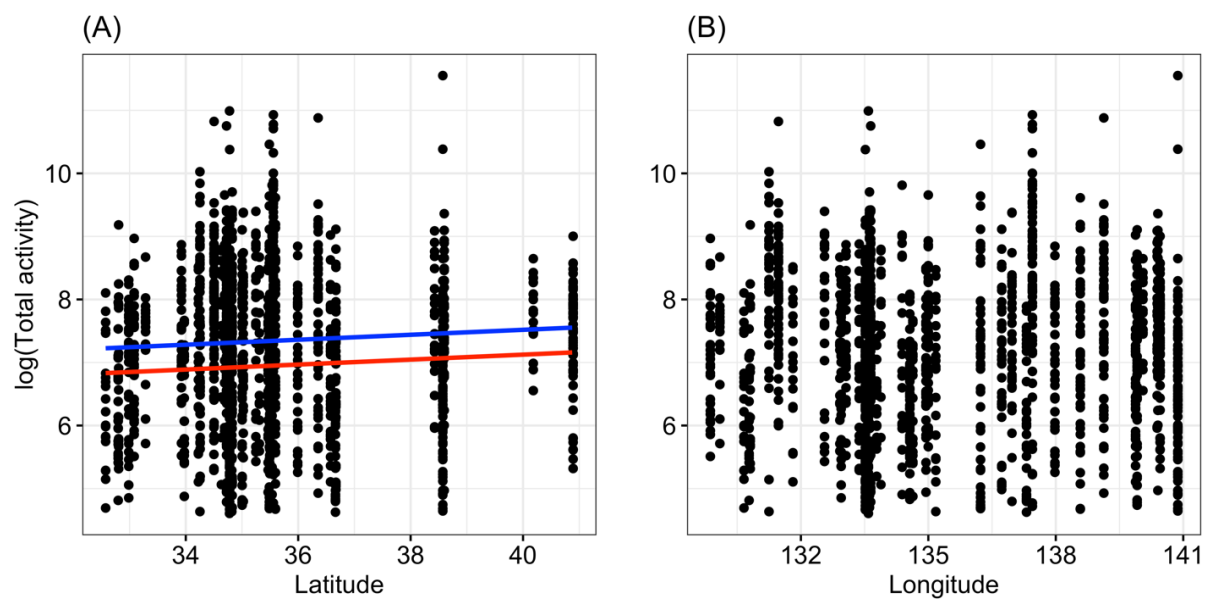
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443 Figure 3

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446 Figure 4

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Table S1. Collection site of *T. castaneum* populations with latitude and longitude.

Collection site	Latitude	Longitude	Sample size
Aomori	40.8909	140.4552	68
Akita	40.1825	140.0518	20
Yamagata-B	38.5981	140.4029	39
Miyagi	38.5726	140.8723	72
Yamagata-A	38.4264	140.3856	20
Ishikawa	36.6707	136.7276	36
Tochigi	36.5747	139.8776	35
Gunma	36.3546	139.1299	55
Nagano	35.9904	137.9795	36
Yamanashi	35.5952	138.5768	47
Gifu	35.5583	137.4489	62
Tottori	35.5357	134.3827	24
Hyogo-A	35.4942	134.5642	61
Shiga	35.4862	136.2289	49
Hyogo-B	35.4649	134.9447	35
Shimane-A	35.3094	132.9177	17
Shimane-B	35.2467	132.5565	24
Aichi	35.0232	136.9688	35
Chiba	35.0029	139.9148	42
Okayama (Kinoyama)	34.8251	133.6153	66
Hyogo-E	34.8183	135.1759	20
Okayam (Nariwa)	34.7806	133.5178	128
Okayam (Ochiai)	34.7779	133.5887	65
Hyogo-D	34.7263	134.9985	20
Okayama (Minagi)	34.7247	133.6436	77
Hyogo-C	34.6903	134.9988	23
Yamaguchi-B	34.5033	131.472	36
Hiroshima	34.5006	133.3735	28
Kagawa-A	34.2553	133.7846	23
Yamaguchi-A	34.224	131.8132	18
Tokushima-B	33.9734	134.6358	23
Ehime	33.9224	133.0625	32
Saga	33.2845	130.0928	17
Nagasaki	33.0817	129.8691	33
Kochi-B	32.9847	132.9482	31
Kumamoto-B	32.8051	130.8083	18
Kumamoto-A	32.7974	130.7815	22
Kumamoto-C	32.5741 ³¹	130.6605	20