SCIENTIFIC REPORTS

OPEN

The biological impacts of the Fukushima nuclear accident on the pale grass blue butterfly

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The collapse of the Fukushima Dai-ichi Nuclear Power Plant caused a massive release of radioactive materials to the environment. A prompt and reliable system for evaluating the biological impacts of this accident on animals has not been available. Here we show that the accident caused physiological and genetic damage to the pale grass blue *Zizeeria maha*, a common lycaenid butterfly in Japan. We collected the first-voltine adults in the Fukushima area in May 2011, some of which showed relatively mild abnormalities. The F_1 offspring from the first-voltine females showed more severe abnormalities, which were inherited by the F_2 generation. Adult butterflies collected in September 2011 showed more severe abnormalities than those collected in May. Similar abnormalities were experimentally reproduced in individuals from a non-contaminated area by external and internal low-dose exposures. We conclude that artificial radionuclides from the Fukushima Nuclear Power Plant caused physiological and genetic damage to this species.

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Massive amount of radioactive materials were released from the Fukushima Dai-ichi Nuclear Power Plant (NPP) to environment due to the Great East Japan Earthquake[1–6]. However, precise information on exactly what occurred and on what is still ongoing is yet to be established[7,8]. This lack of information raises serious concerns about biological influences on living organisms that could ultimately produce long-term destruction of ecosystems and cause chronic diseases. Prompt and reliable evaluation of the biological influences of the artificial radionuclides from the Fukushima Dai-ichi NPP is lacking, and only a few studies have been performed to date[9,10]. In the case of the Chernobyl accident, changes in species composition and phenotypic aberration in animals[11–17] and an increase in the incidence of thyroid and lymph cancers in humans[18] have been reported. Similarly, an increase in the incidence of cancers has been reported for atomic bomb survivors in Hiroshima and Nagasaki, Japan[19]. However, the effects of low-dose radiation exposure on animals, including humans, are still a matter of debate[20–22] despite the relatively rigorous documentation of physiological damage to animals from external high-dose radiation exposure. Moreover, one of the greatest concerns is the possible inheritance of the adverse effects of exposure by the offspring of the exposed individuals. However, experimental demonstration of genetic mutations in the germ-line cells that are inherited by the offspring of radiation-exposed parents has been scarce, although the germ-line damage was shown in barn swallows[23].

We address these important issues in connection with the recent Fukushima Dai-ich NPP accident. We use the pale grass blue butterfly *Zizeeria maha* (Lepidoptera, Lycaenidae) as an indicator species to evaluate the environmental conditions. A reliable rearing method has been established for this species[24]. Butterflies are generally considered useful environmental indicators[10,15,25,26], and this species is particularly suitable for this purpose because it is widespread in Japan, including the Fukushima area, and because its wing colour patterns are sensitive to environmental changes[24,27]. In the past, this species was used to evaluate the ecological risk associated with transgenic maize pollen[28,29].

The meltdown and explosion of the Fukushima Dai-ichi NPP occurred on 12 March 2011, when *Z. maha* was overwintering as larvae. On that date and thereafter, these larvae were exposed to artificial radiation not only externally but also internally from ingested food. In this study, we examine possible physiological and genetic changes in this butterfly in the Fukushima area resulting from damage by artificial radionuclides from the Fukushima Dai-ich NPP. We also evaluate the risk of external exposure and that of internal exposure to ingested radionuclides.
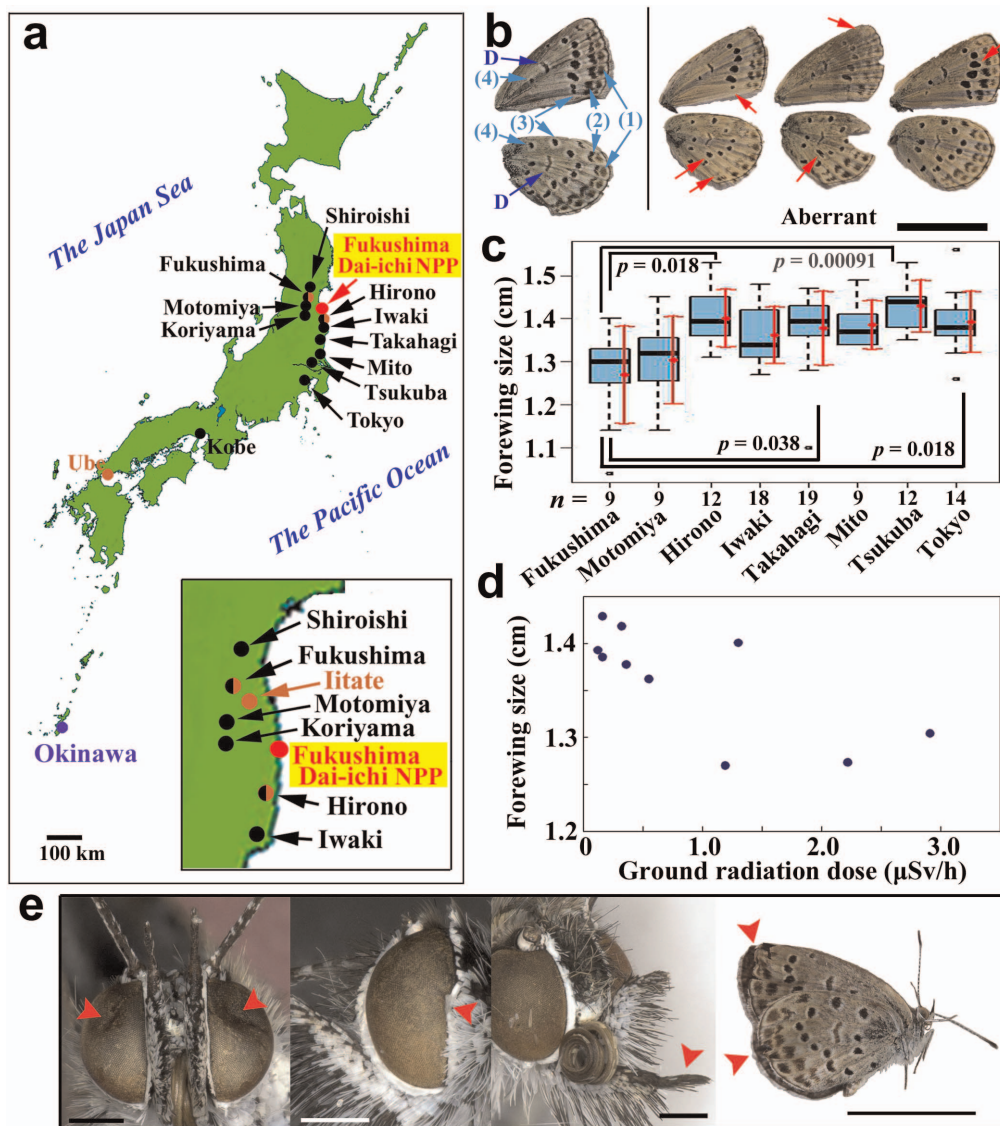
Figure 1 | First-voltine collection and abnormalities. (a) Collection localities. A red dot indicates the location of the Fukushima Dai-ichi NPP. Black dots and black half dots indicate the cities from which the first-voltine adults were collected. Brown dots and brown half dots indicate cities from which the host plant leaves were collected for the internal exposure experiment. All experiments were performed in Okinawa, marked by a blue dot. Inset shows the collection localities around the NPP. (b) Representative wings with normal (leftmost) and aberrant colour patterns. Numbers 1, 2, 3, and 4 indicate the first, second, third, and fourth spot arrays, respectively, and "D" indicates the discal spot. Red arrows indicate loss, dislocation, and weak expression of spots (left individual), weak expression and dislocation of spots (middle individual), and enlargement of spots (right individual). These samples were caught in Mito except for the leftmost aberrant specimen, which was caught in Iwaki. Scale bar, 1.0 cm. (c) Male forewing sizes from various localities. The first quartile and third quartile were indicated by horizontal bars at the bottom and top of the box, respectively. Median is indicated as the centre line inside the box. Outliers were indicated by dots. A red dot indicates the mean value and a red bar the standard deviation (SD). Holm-corrected p-values are shown, which were obtained for pairwise comparisons among 8 localities using t tests with pooled SD. Only male samples were used here because when the female samples were used to obtain eggs, broken wings resulted from the egg collection procedure. Samples from Shiroishi ($n = 5$) and Koriyama ($n = 3$) were excluded because of small sample sizes. (d) Scatter plot of the male forewing size and ground radiation dose at each collection locality. Pearson correlation coefficient $r = -0.74$ (Holm-corrected $p = 0.029$). (e) Representative morphological abnormalities. From left to right, dented eyes (Shiroishi), deformed left eye (Iwaki), deformed right palpus (Takahagi), and deformed wing shape (Fukushima). Arrowheads indicate deformation. Scale bars, 0.50 mm with the exception of the rightmost bar, which is 1.0 cm.

Results

Abnormalities in field-caught individuals and their F_1 offspring.
We collected 144 first-voltine adults (111 males and 33 females) from 10 localities (Shiroishi, Fukushima, Motomiya, Koriyama, Hirono, Iwaki, Takahagi, Mito, Tsukuba, and Tokyo) approximately 2 months after the accident on 13–18 May 2011 (Fig. 1a; Supplementary Table 1). Most of the collected adults appeared morphologically and behaviorally normal, but mild morphological abnormalities were detected in some individuals upon close

inspections (Fig. 1b–e; Supplementary Table 2). The overall abnormality rate for 7 localities (excluding Shiroishi, Koriyama, and Tokyo to allow comparisons with the second phase of field work in September) was 12.4% (Table 1). The male forewing size (from the base to the apical end) was different among populations (ANOVA, df = 7, $F = 4.0$, $p = 0.00093$); it was significantly reduced in the Fukushima population in comparison with the Tsukuba population (t test with pooled SD, $p = 0.00091$, Holm-corrected by 28 combinatorial pairs, excluding Shiroishi and Koriyama due to
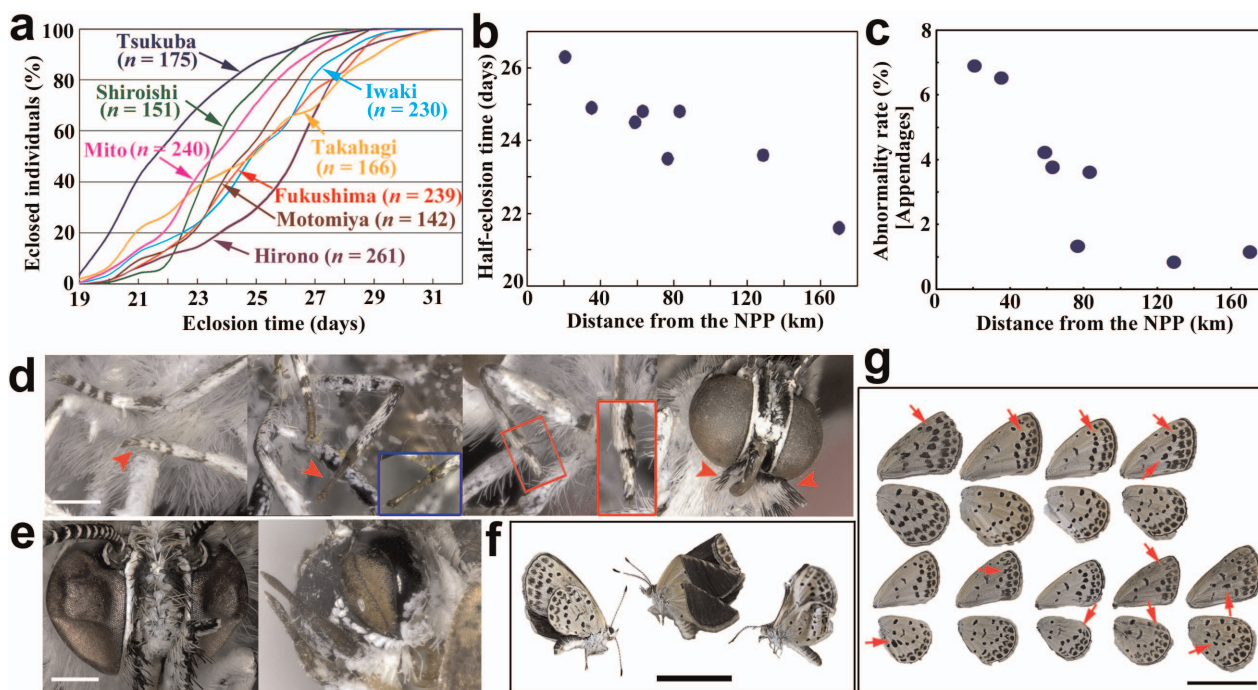
Table 1 | Overall abnormality rate (OAR) of adults

| Adult samples	OAR (%)
Field-caught (P generation) in May (7 localities)*	12.4
Field-caught (P generation) in May (10 localities)	13.2
F_1 from the May samples (8 localities)	18.3
F_2 from the May samples (6 localities, 9 strains)	33.5
Field-caught (P generation) in September (7 localities)*	28.1
F_1 from the September samples (6 localities including Kobe)	51.9
F_1 from the September samples (5 localities excluding Kobe)	60.2
External radiation exposure	31.7
Control (no exposure)	16.7
Internal radiation exposure (excluding the Ube samples)	39.6
Internal radiation exposure (including the Ube samples)	24.5

*can be compared directly.

Based on the established rearing method[24] (Supplementary Fig. 1), we obtained F_1 offspring from the female parents caught in the Fukushima area. This and all the following experiments were performed in Okinawa, located 1,750 km from the Fukushima Dai-ichi NPP (see Fig. 1a), where artificial radiation can scarcely be detected. Some of these field-caught parents had mild abnormalities (Supplementary Table 2), but those from Motomiya showed no detectable abnormalities and appeared to be morphologically and behaviorally healthy. In the F_1 generation (Supplementary Tables 3, 4), the mortality rates of larvae, prepupae, and pupae and the abnormality rate of adults were high for Iwaki, Hirono, Motomiya, and Fukushima, and overall abnormality rate of the F_1 adults was 18.3% (Table 1), 1.5 times the overall abnormality rate of the parent generation. The eclosion-time dynamics (Fig. 2a) as well as the pupation-time dynamics (Supplementary Fig. 2a) varied among the F_1 populations from different localities. The eclosion curves of all of the local populations examined differed significantly from the eclosion curve of the Tsukuba population (generalized Wilcoxon test, $p < 0.00001$ in all population groups, Holm-corrected by 28 pairs). Essentially identical results were obtained in the pupation curve (Supplementary Figure 2a). The half-eclosion time was negatively correlated with the distances of the collection localities from the Fukushima Dai-ichi NPP ($r = -0.91$, df = 6, $p = 0.045$, Holm-corrected by 30 pairs [{ground radiation dose and distance from the NPP} versus {abnormality rates of four stages (called "total"),

small sample sizes) and with the Hirono, Tokyo, and Takahagi populations (t test with pooled SD, $p = 0.018$, 0.018, and 0.038, respectively, Holm-corrected by 28 pairs as above) (Fig. 1c). The male forewing size was negatively correlated with the ground radiation dose at the collection localities (Pearson correlation coefficient $r = -0.74$, df = 8, $p = 0.029$, Holm-corrected by 2 pairs [ground radiation dose and distance from the NPP]) (Fig. 1d).

Figure 2 | F_1 abnormalities. (a) Eclosion-time dynamics. Cumulative percentages of eclosed individuals were plotted against eclosion day. All local populations differ significantly from the Tsukuba population (generalized Wilcoxon test, Holm-corrected $p < 0.00001$). (b) Scatter plot of half-eclosion time and distances of the collection localities from the NPP. Half-eclosion time was derived from the eclosion-time dynamics shown in (a) as the time when 50% of the pupae eclosed. Pearson correlation coefficient $r = -0.91$ for half-eclosion time (Holm-corrected $p = 0.045$). (c) Scatter plot of abnormality rate of appendages and distances from the NPP. Pearson correlation coefficient $r = -0.86$ (Holm-corrected $p = 0.18$). (d) Representative morphological abnormalities of appendages. Miniaturized left foreleg tarsus (Fukushima F_1, leftmost), undeveloped left middle leg tarsus (Fukushima F_1 and Hirono F_1, second and third from the left, respectively), and undeveloped palpi (Takahagi F_1, rightmost) were structurally abnormal, reminiscent of *Drosophila Distal-less* mutants. Arrowheads indicate abnormal structures. Insets show enlargements of boxed areas. Scale bar, 0.50 mm. (e) Representative morphological abnormalities of eyes. Both compound eyes were dented (Fukushima F_1, left), and left compound eye was bar-like in shape (Hirono F_1, right), reminiscent of *Drosophila Bar* mutants. Scale bar, 0.50 mm. (f) Representative wing size and shape deformation. Right hindwing was much smaller than the left hindwing of the same individual (Fukushima F_1, left), wings were folded (Takahagi F_1, middle), and wings were rumpled (Iwaki F_1, right). Scale bar, 1.0 cm. (g) Representative wing colour-pattern modifications. The top left three individuals are F_1 individuals from an Iwaki parent, and the top rightmost individual is a Hirono F_1. The bottom samples, from left to right, are F_1 individuals from Hirono, Mito, Shiroishi, Motomiya, and Motomiya. Arrows indicate modified spots. Scale bar, 1.0 cm.
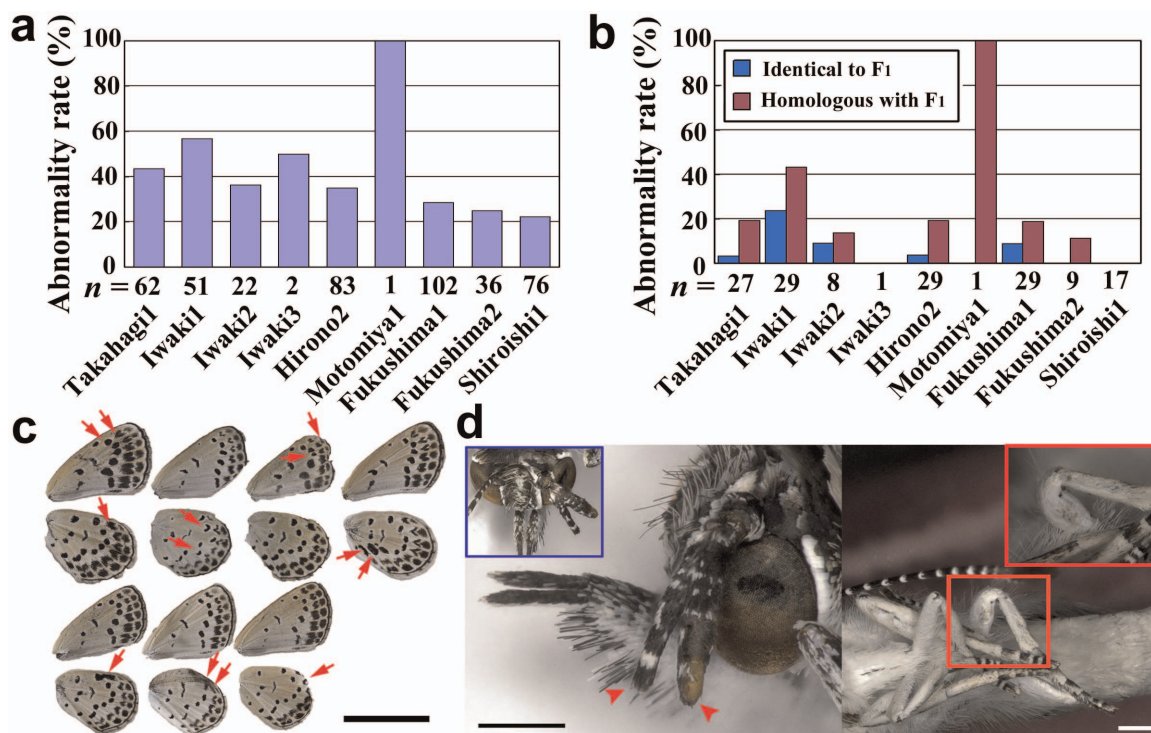
Figure 3 | F$_2$ abnormalities. (a) Abnormality rate for the F$_2$ generation. The *x*-axis shows strain names that indicate the local origin of their P generation. The total number of individuals (corresponding to 100%) was indicated for each strain. See also Supplementary Table 3. (b) Identical and homologous abnormality rates. The number of individuals that show abnormal traits identical to the F$_1$ parents was divided by the total number of individuals obtained and expressed as a percentage. Similarly, the number of individuals that show abnormal traits in organs, such as wings and appendages, homologous with those in their F$_1$ parents was divided by the total number of individuals obtained and expressed as a percentage. The total number of abnormal individuals (corresponding to 100%) was indicated for each strain. (c) Representative wing colour-pattern aberrations. Arrows indicate modified spots and wing parts. The top leftmost wings are the wing-wide spot elongation type of the Iwaki F$_2$, a phenotype similar to that of its F$_1$ parent shown in Fig. 2g. The top four samples, from left to right, are Iwaki F$_2$, Takahagi F$_2$, Iwaki F$_2$, and Fukushima F$_2$ individuals. All of the samples at the bottom are Fukushima F$_2$ individuals. The bottom middle and rightmost wings show a deformation of the hindwing shape, which were obtained from the offspring of the Fukushima F$_1$ parent that had the small hindwing shown in Fig 2f. Scale bar, 1.0 cm. (d) Antenna and leg malformations. The left panel shows a Takahagi F$_2$ individual with a malformation of left antenna, which is short and forked (arrowheads). The right panel shows a Takahagi F$_2$ individual with a deformation of the left hindleg femur. Insets show pictures taken from different angles. Scale bars, 0.50 mm.

adults, wings, colour patterns, appendages, and others; mortality rates of pupae, prepupae, and larvae; periods of prepupae and pupae; peak days of eclosion and pupation; half days of eclosion and pupation}]) (Fig. 2b). Similarly, half-pupation time was negatively correlated, but not significant statistically (Supplementary Fig. 2b).

We also observed a negative correlation between the F$_1$ abnormality rate of appendages and the distances from the NPP, although not significant statistically ($r = -0.86$, df $= 6$, $p = 0.18$, Holm-corrected as above) (Fig. 2c). We detected morphological malformations in various parts (Supplementary Table 4) including legs, antennae, palpi, eyes, abdomen, and wings (Fig. 2d–g). In addition to dented compound eyes (Fig. 2e, left), the entire eye structure was deformed in a pattern similar to that of *Drosophila Bar* mutants (Fig. 2e, right). Wing aberrations, including broken or wrinkled wings, were found in many individuals (Fig. 2f). Asymmetric hindwing size reduction was observed in a few individuals (Fig. 2f, left). Colour-pattern changes were relatively frequent (Fig. 2g). In one individual, the third spot array was located closer to the second array. In another individual, spots were deleted or added, which was occasionally found only in a right or left wing. In another individual, spots were fused together. Additionally, wing-wide spot enlargement was relatively common, especially in individuals from Iwaki. This spot enlargement pattern differs from those observed at the northern range margins and from those induced by temperature shocks[24,27]. It also differs from those seen in sibling inbreeding[24].

It is noteworthy that we obtained relatively high abnormality rates for the F$_1$ populations from Motomiya, the parents of which showed no detectable abnormal phenotype. Other parents used for egg collection were also comparably normal and vigorous. The abnormal F$_1$ individuals obtained from healthy parents suggest that genes that are important in morphological development were damaged by radiation at the stage of germ-line cell development in the parents.

Inheritance of abnormalities by the F$_2$ generation. We tested the fertility of these abnormal F$_1$ individuals and the inheritance of their abnormal traits. We chose 10 F$_1$ females with abnormal traits (except one female from Shiroishi, which did not have any detectable abnormal trait) and crossed them with non-abnormal F$_1$ individuals from Tsukuba. Of the collection localities from which the F$_1$ offspring were obtained, Tsukuba was the farthest from the Fukushima Dai-ichi NPP (Supplementary Table 4) and therefore chosen as the source of the non-abnormal F$_1$ adults. To avoid failure due to the unexpected infertility of the males, we put 3 normal virgin males for 1 virgin female in a single cage. Although our mating system is nearly always successful and yields more than 100 offspring per female if both males and females are fertile[24], 3 females out of 10 produced only a limited number of offspring, i.e., less than 2 adult offspring (Supplementary Table 5). Nonetheless, we were able to obtain a reasonable number of eggs from other females and successfully reared these offspring to the adult stage.
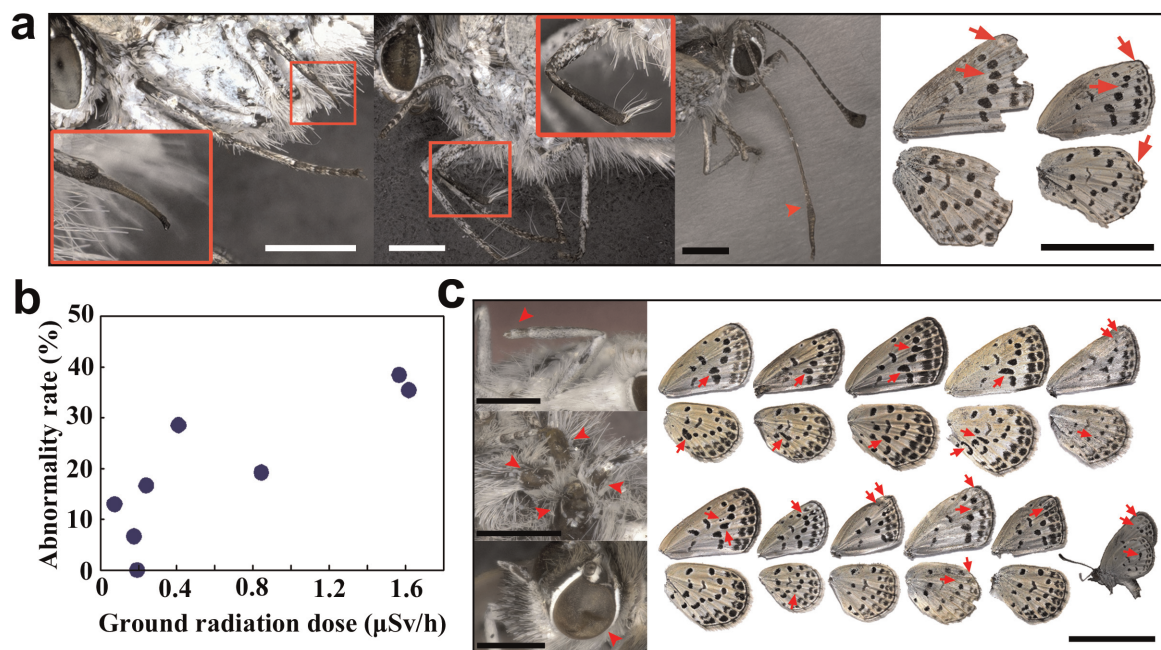
Figure 4 | **Abnormalities in the adult samples collected in September 2011 and in their F₁ offspring.** (a) Representative morphological abnormalities of the field-caught individuals. Insets are enlargement of the boxed areas. The tarsus of the left hindleg was structurally abnormal (Hirono, left), the tarsus of the right foreleg was not developed at all (Fukushima, second from left), the right antenna (an arrowhead) was elongated with abnormal structure and colouration (Motomiya, second from right), and the wing colour-patterns and wing shape were modified as indicated by arrows (Iwaki and Fukushima, right). All scale bars indicate 1.0 mm with the exception of the rightmost bar, which is 1.0 cm. (b) Scatter plot of ground radiation dose and abnormality rate of the field-caught adults. Pearson correlation coefficient $r = 0.84$ (Holm-corrected $p = 0.13$). (c) Representative abnormalities in the F₁ generation. The left three panels indicate malformations of left foreleg tarsus (an arrowhead) (Takahagi F₁, top), tumor-like solid protuberance (arrowheads) in the ventral side of the thorax (Takahagi F₁, middle), and dented eyes (Fukushima F₁, bottom). Scale bars in the left three panels all indicate 1.0 mm. Wing colour-pattern modifications (arrows) of the F₁ samples were shown on the right: from left to right, Iwaki, Iwaki, Motomiya, Hirono, and Takahagi (top), and Takahagi, Motomiya, Motomiya, Fukushima, Motomiya, and Motomiya (bottom). Scale bar in the wing panel indicates 1.0 cm.

The F₂ generation showed a relatively high abnormality rate (Fig. 3a; Supplementary Table 6). The overall abnormality rate in the F₂ adults was 33.5% (Table 1). An important finding was that certain traits observed in the F₁ generation were inherited by the F₂ generation (Fig. 3b; Supplementary Table 6). Colour-pattern modifications were relatively frequent (Fig. 3c). Wing-wide spot enlargement was evident especially in the Iwaki F₂ generation as in the Iwaki F₁ generation discussed above. In particular, 52.4% of the Iwaki F₂ females in the strain "Iwaki1" inherited this trait; this inheritance was biased towards females (Supplementary Table 6). Abnormalities of appendages were also relatively frequent (Fig 3d). A striking antenna malformation, or a forked antenna, was observed in a F₂ individual from Takahagi (Fig 3d). This abnormality had never been seen in the F₁ and other individuals that were reared in our laboratory. These results demonstrated that the abnormal traits observed in the F₁ generation were inherited by the F₂ generation and that it is highly probable that these characteristics are caused by genetic damage introduced to the parent germ-line cells, possibly due to the Fukushima Dai-ichi NPP accident.

More severe abnormalities 6 months after the accident. To assess the possible genetic and ecological impacts of the Fukushima nuclear accident on the populations of *Z. maha*, we asked if any abnormalities similar to those found in the F₁ and F₂ generations in the laboratory from the first-voltine females were observed in the field 6 months after the accident. We again collected *Z. maha* adults from the 7 localities (i.e., Fukushima, Motomiya, Hirono, Iwaki, Takahagi, Mito, and Tsukuba; 18–21 September 2011) and from Kobe (3–4 October 2011) (see Fig. 1a). They were probably the fourth- or fifth-voltine individuals. We collected a total of 238 individuals (168 males and 70 females) from these localities

(Supplementary Table 1). We observed frequent malformations of legs and antennae as well as wing colour-pattern aberrations (Fig. 4a; Supplementary Table 7). The overall abnormality rate for the 7 localities (excluding Kobe to allow comparisons with the first phase of field work in May) was 28.1%, more than double that observed in the field-collected first-voltine adults in May (Table 1). The total abnormality rate of the field-collected adults in September 2011 was correlated with the ground radiation dose at the collection localities ($r = 0.84$, df = 6, $p = 0.13$, Holm-corrected by 14 pairs [{ground radiation dose and distance from the NPP} versus {abnormality rates of adults, wings, colour patterns, appendages, and others, and wing sizes of males and females}]) (Fig. 4b).

In the F₁ generation from the September samples, the mortality rate and the abnormality rate were relatively high (Supplementary Tables 3, 4), and abnormalities similar to those observed in the field-collected September samples were observed (Fig. 4c). The overall abnormality rate of the F₁ adults was 59.1% (Table 1). These results demonstrated that the September populations in the Fukushima area deteriorated in comparison with the May populations, possibly due to genetic damage caused by radiation from the Fukushima Dai-ichi NPP, as predicted from the results of our previous breeding experiments using the first-voltine adults.

Effects of external and internal exposures. To experimentally reproduce the abnormal phenotypes obtained from the field and in the breeding experiments, we artificially exposed larvae and pupae that were obtained from females caught in Okinawa to radiation from ^{137}Cs, one of the major radionuclides released from the Fukushima Dai-ichi NPP, up to 55 mSv (0.20 mSv/h) or 125 mSv (0.32 mSv/h). In both exposure levels, we observed abnormal traits (Fig. 5a) and forewing size reduction in both sexes in comparison
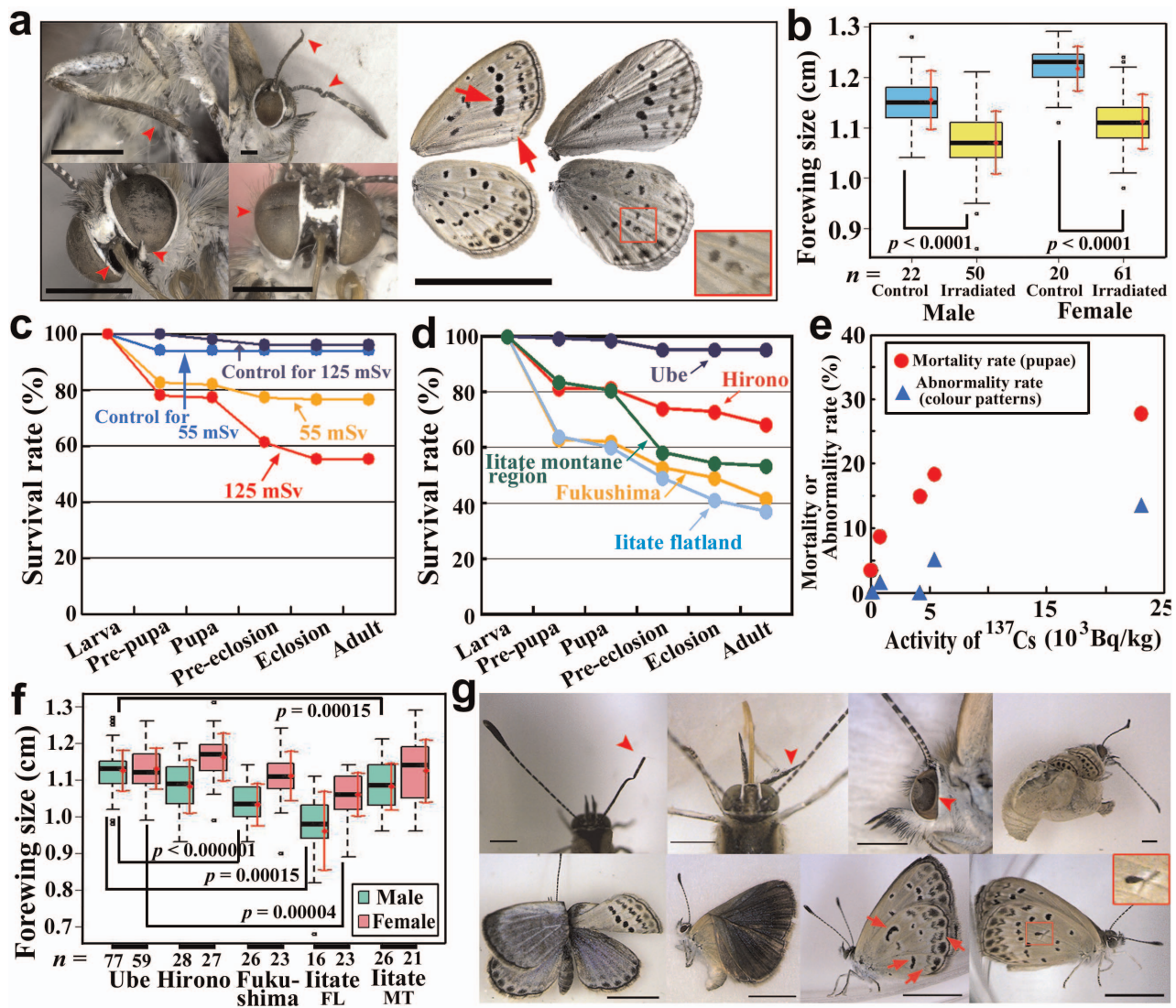
Figure 5 | **External and internal exposures.** (a) Representative abnormalities obtained by external exposure. Left hindleg tibia and tarsus, antennae, palpi, and an eye showed abnormal structures (All exposed at 125 mSv with the exception of the left bottom individual, which was exposed at 55 mSv. All scale bars, 1.0 mm). Aberrant wing colour patterns are indicated by arrows and boxes (Left wings exposed at 55 mSv and right wings at 125 mSv. Scale bar, 1.0 cm). Inset shows the enlarged boxed area. (b) Forewing size reduction in the externally exposed individuals at 55 mSv (t test). (c) Survival curves of individuals that were exposed externally. Differences between the exposed at 55 mSv and its control (Holm-corrected $p = 0.018$), between the exposed at 125 mSv and its control (Holm-corrected $p = 0.0000026$), and between the exposed at 55 mSv and at 125 mSv (Holm-corrected $p = 0.0040$) were statistically significant (generalized Wilcoxon test). (d) Survival curves of individuals that ingested contaminated leaves from different localities. The host plant collection localities are shown. All curves differed from the non-contaminated Ube curve (generalized Wilcoxon test, Holm-corrected $p < 0.000001$). The Hirono curve was different from the Fukushima curve (Holm-corrected $p = 0.0017$) and from the Iitate flatland curve (Holm-corrected $p = 0.00035$) (generalized Wilcoxon test). (e) Scatter plot of the ^{137}Cs activity of the host plant and pupal mortality rate ($r = 0.91$) and colour-pattern abnormality rate ($r = 0.96$).
(f) Forewing size reduction in the internally exposed individuals (t test). (g) Representative abnormalities of individuals that ingested contaminated leaves. From the top left to the right bottom, the panels show right antenna malformation (Iitate montane region), right palpus abnormality (Fukushima), dented left compound eye (Iitate flatland), eclosion failure (Fukushima), bent wings (Fukushima), additional bent wings (Hirono), aberrant wing colour patterns (Fukushima), and an ectopic black spot beside the discal spot (Iitate flatland; enlargement in the inset). Arrowheads indicate abnormal parts, and arrows indicate deformed wing spots. Scale bars for the top four panels indicate 1.0 mm, and those for the bottom four panels indicate 5.0 mm.

with non-irradiated controls (t test, $p < 0.00001$ in both sexes) (Fig. 5b). The survival curves indicated dose-dependence; the 55-mSv and 125-mSv curves differed significantly from each other (generalized Wilcoxon test, $p = 0.0040$, Holm-corrected by 6 pairs). We also observed significant differences between the 55-mSv curve and its control (generalized Wilcoxon test, $p = 0.018$, Holm-corrected as above) and between 125 mSv curve and its control (generalized Wilcoxon test, $p = 0.0000026$, Holm-corrected as above). The survival curves also indicated that external exposure caused frequent deaths at the prepupal stage and

that the higher dose mainly affected the pre-eclosion and eclosion stages (Fig. 5c).

To evaluate the effects of internal exposure caused by ingested food, we fed host plant leaves collected from the Fukushima and other areas (i.e., Fukushima, Iitate montane region, Iitate flatland, Hirono, and Ube; see Fig. 1; Supplementary Table 8) to Okinawa larvae, which had never been exposed to artificial radionuclides. We confirmed that these leaves indeed contained high activities of ^{134}Cs and ^{137}Cs (Supplementary Table 8). Almost all individuals that consumed leaves from the non-contaminated locality (i.e., Ube)

survived, whereas many individuals that consumed leaves from the contaminated localities could not survive well (Fig. 5d). Survival curves in the 4 groups fed leaves from the contaminated localities differed significantly from that in the Ube group (generalized Wilcoxon test, $p < 0.000001$ in all groups, Holm-corrected by 10 pairs). A dose-dependent trend was indicated by the significant differences between the Hirono and Fukushima curves ($p = 0.0017$, Holm-corrected as above) and between the Hirono and Iitate flatland curves ($p = 0.00035$, Holm-corrected as above). The pupal mortality rate ($r = 0.91$, df $= 3$, $p = 1.0$, Holm-corrected by 32 pairs [{ground radiation dose, ground β-ray dose, activity of ^{137}Cs in host plant, and activity of ^{134}Cs in host plant} versus {abnormality rates of four stages (total), adults, wings, colour patterns, appendages, and others, and mortality rates of pupae and larvae}]) and colour-pattern abnormality rate ($r = 0.96$, df $= 3$, $p = 0.34$, Holm-corrected as above) showed high r values with the ^{137}Cs activity of the collected leaves, although not significant statistically (Fig. 5e). Forewing size difference was observed among males (ANOVA, df $= 4$, $F = 25$, $p < 0.0000001$) and among females (ANOVA, df $= 4$, $F = 8.0$, $p = 0.0000073$) (Fig. 5f). In comparison with the Ube samples, forewing size reduction in males was detected in the Fukushima population (Student t test, $p < 0.000001$, Holm-corrected by 10 pairs), in the Iitate flatland population (Welch t test, $p = 0.00015$, Holm-corrected as above), and in the Iitate montane region males (Welch t-test, $p = 0.00015$, Holm-corrected as above) (Fig. 5f). Similarly, forewing size reduction in females was observed between the Ube and Iitate flatland samples (Student t test, $p = 0.000041$, Holm-corrected as above). Morphological abnormalities, including colour-pattern aberrations (Fig. 5g), were detected in the adults that ingested contaminated leaves during the larval period.

These results demonstrated that both external and internal irradiation processes led to similar adverse physiological effects. An important result was that the abnormal traits of the irradiated individuals were similar to those observed in the field-collected adults from the Fukushima area and were also similar to those of their F_1 and F_2 generations.

Discussion

We demonstrated that the *Z. maha* population in the Fukushima area is deteriorating physiologically and genetically. Most likely, this deterioration is due to artificial radionuclides from the Fukushima Dai-ichi NPP, as suggested by our field work and laboratory experiments. We observed abnormalities of the F_1 generation that were not necessarily seen in the first-voltine parents, and these F_1 abnormalities were inherited by the F_2 generation, suggesting that genetic damage was introduced in germ-line cells of this butterfly. We also observed a higher abnormality rate in the field-collected samples in September than in May. Moreover, we successfully reproduced the results of the field work and breeding experiments with artificial external and internal exposures. All of these results suggest that radiation caused adverse effects at the physiological and genetic levels. It is probable that germ-line cells of the first-voltine individuals were genetically damaged by the massive external exposure from iodine and other artificial radionuclides that were released at one burst immediately after the accident from the Fukushima Dai-ichi NPP on 12 March, 2011[1–6]. The subsequent damage may result primarily from low-level internal exposure caused by ingested leaves. The cumulative effects of the external and internal exposures could have resulted in the deterioration at the population level.

In the F_1 generation from the May samples, a significant delay of eclosion and pupation was evident. In addition to various morphological abnormalities of the somatic cells, infertility was observed in a few females. Somewhat unexpectedly, the half-eclosion time and half-pupation time were correlated with the distance from the NPP but not with the ground radiation dose (data data not shown). The reason for this outcome is not apparent, but it may be that distance

values are more resistant to measurement deviations. Dose measurements in a given collection site could sometimes vary more than one order of magnitude within a several-meter range due to uneven distribution of radionuclides in the field. Probably because of this variability, data set for the ground radiation dose was less normally distributed (although it was within the normal range), as indicated by normality tests (data not shown), which likely made the dose-abnormality correlation ambiguous. We also admit that our field estimates of absolute doses did not consider time-dependent changes, mainly caused by the radioactive decay of short half-life radionuclides.

The overall abnormality rate (OAR) was higher in the September samples than in the May samples. Longer exposure or higher doses of exposure per individual cannot explain this finding, because the exposed doses per individual is certainly much higher in the May samples than in the September samples due to the massive release of short half-life radionuclides such as ^{131}I immediately after the meltdown. Also note that an individual of the pale grass blue butterfly does not live long: its life cycle completes in about one month. Considering other experiments performed in this study, the OAR difference may be explained by mutation accumulation caused by continuous low-dose exposure through generations.

It appears difficult to distinguish physiological and genetic adverse effects on the basis of morphological examination. Malformations of appendages and wing colour-pattern aberrations were observed in the field-collected first-voltine adults and in the irradiated adults. Thus, these abnormalities are due to physiological effects of radiation. However, similar abnormalities were also found in the F_1 and F_2 generations from the non-abnormal first-voltine adults. These findings suggest that the abnormalities result from genetic effects. Our irradiation experiments demonstrated that forewing size reduction, colour-pattern changes, and certain malformations of appendages and eyes can be induced as physiological effects. These characteristics were also observed in the first-voltine adults collected in May 2011. However, certain types of abnormalities were found only in the F_1 and F_2 generations. These abnormalities included deformed compound eyes similar to the eyes of *Drosophila Bar* mutants, asymmetric hindwing size reduction, wing-wide spot enlargement, and a forked antenna. Most likely, these aberrations are due to genetic damage, although more experiments are necessary to distinguish between physiological and genetic effects.

Throughout this study, many abnormal traits were asymmetric, and abnormal traits detected in offspring were not necessarily identical to those of their parents. It is probable that these results were obtained because the expressivity of mutant phenotypes was not high. In addition, it is possible that a group of pleiotropic genes involved in the development of both appendages and wing colour patterns in butterflies, such as *Distal-less*[30–35], may be mutated because abnormal traits in appendages and colour patterns were frequently observed.

It has been reported that the sterilising dose of external radiation to pupae of the melon fly had no influence on eclosion rate and adult longevity[36]. Similarly, silk moth pupae have been reported to be highly resistant to external radiation exposure[37]. However, closer examination of the adult morphology of various insects after irradiation has demonstrated morphological damage to testes, antennae, and wings, and frequent pupal and larval death due to molting failure[38–40]. We showed that *Z. maha* is sensitive to external radiation at the level of 55 mSv from ^{137}Cs. It is probable that this level of radiation is similar to the field exposure level at least in some areas around the Fukushima Dai-ichi NPP. Although exposure levels of *Z. maha* in this study are relatively low, the abnormalities observed in this butterfly were similar to those previously reported in other insects[38–40]. Furthermore, our results are consistent with the previous field studies that showed that butterfly populations are highly sensitive to artificial radionuclide contamination in Chernobyl and

Fukushima[10,15,17]. Together, the present study indicates that the pale grass blue butterfly is probably one of the best indicator species for radionuclide contamination in Japan.

Although epigenetic effects cannot be entirely excluded[41], it is most likely that the abnormal phenotypes observed are produced by random mutations caused by the exposure to radiation. This outbreak of abnormal phenotypes in the Fukushima area is very different from the outbreak of wing colour-pattern changes previously observed at the northern range margins of this species, i.e., the Fukaura area[27], which is located approximately 400 km northwest of the Fukushima Dai-ichi NPP. The Fukaura populations at the time of the outbreak were composed of temperature-shock types that exhibit distinct wing colour-pattern modifications but no other wing modifications or aberrations. Moreover, to the best of our knowledge, no malformations of appendages and other parts have been detected[27,41–43]. In contrast, the outbreak in the Fukushima area includes various unexpected abnormal phenotypes. These abnormalities cannot be expressed within the range of phenotypic plasticity exhibited by normal populations. This information and the experimental data obtained in this study allow us to conclude that the present outbreak of abnormal individuals in the Fukushima area was caused by random genetic mutations in addition to physiological effects due to the artificial radionuclides from the Fukushima Dai-ich NPP.

Our demonstration of heritable germ-line genetic damage caused by low-dose exposure due to radioactive contamination in a species of butterfly has invaluable implications for the possible future effects of radiation on animals. In view of the detection of plutonium released from the Fukushima Dai-ichi NPP[44], the possible risk of internal exposure from ingestion should be investigated more accurately in the near future.

Methods

Butterflies. The pale grass blue butterfly *Z. maha* (*Yamato Shijimi* in Japanese, meaning the Japanese lycaenid) occurs commonly throughout Japan, as its Japanese name implies, including Okinawa (the Ryukyu Archipelago) but excluding Hokkaido[45,46]. This species is monophagous. It eats the wood sorrel *Oxalis corniculata* (*Katabami* in Japanese), a short plant whose leaves are close to the ground. The plant *O. corniculata* and the butterfly *Z. maha* thrive in cities and villages, where human activities influence the environment. This species is multivoltine, and one generation requires approximately 1 month. The pale grass blue overwinters in the larval stage on the surface of the ground. Thus, it may be sensitive to accumulated radioactive materials on the ground, and it is suitable as an indicator species for monitoring the biological effects of environmental pollutants in human habitats. At the time of the accident, the populations of this species were overwintering as larvae and were externally exposed to artificial radiation. It is possible that they ate contaminated leaves during the spring and were thus also exposed to internal radiation.

Field work. We collected first-voltine *Z. maha* adults from 10 localities (Fig. 1a; Supplementary Table 1) on 13–18 May 2011. Since the meltdown and explosion of the Fukushima Dai-ichi NPP on 12 March 2011, the overwintering larvae had been exposed to radiation for approximately 2 months as larvae, pupae, and adults. We also collected fourth- or fifth-voltine adults on 18–21 September 2011 from 7 localities and on 3–4 October 2011 from one locality (Kobe) (Fig. 1a; Supplementary Table 1). These September localities are almost identical to those examined during the first phase of field work in May 2011.

Collection sites were mostly artificial open fields such as parks and rice paddies. A collection locality, defined by administrative boundaries, could include a few different sites. At each collection site, at least one 20-min collection attempt was made by four people simultaneously by foot using insect nets around a single physical point. Collectors were expected to catch *Z. maha* adult individuals as many as possible in this 20-min attempt, although females were preferred. After the first collection attempt, decision was made as to whether the collection at that site was to be continued. If decided to continue, later attempts were made preferably around highly populated places. Although we cannot exclude the possibility of sampling bias, one locality included a few different collection sites, which reduces the possible sampling bias. Collected live adults were stored in a cooled box and transferred to our laboratory. We measured radiation doses on the surface of the ground at 0 cm, 30 cm, and 100 cm high at the location of the host plant nearest to the place where butterflies were caught using a Hitachi Aloka Medical TCS-161 scintillation survey meter (Tokyo, Japan). Similarly, we also measured count rate per min using Hitachi Aloka TGS-133 GM survey meter (Tokyo, Japan) (data not shown). Measurements were made at each collection sites, and thus the ground dose of a given locality was expressed by their mean value. When only one collection site was visited for a locality,

dose measurements were made twice in two different places within a given site (see Supplementary Table 1).

Mating and rearing. Mating, egg collection from females, and rearing of larvae were performed as in a previous study[24] except that we used a glass tank of 30 (H)×30 (W)×30 (D) cm for mating and egg collection at 26 ± 1°C. For the collection of adults in May 2011, we selected healthier and vigorous males and females from specified collection localities. The adults from each locality were confined in a single tank (2 males and 1 female for Shiroishi, 3 and 5 for Fukushima, 3 and 3 for Motomiya, 3 and 4 for Hirono, 3 and 6 for Iwaki, 3 and 4 for Takahagi, 4 and 5 for Mito, and 3 and 5 for Tsukuba). Although some of these individuals showed mild abnormalities, the males and females from Motomiya and the females from Iwaki, Takahagi, and Tsukuba showed no detectable abnormalities (Supplementary Table 2) and appear to be morphologically and behaviorally healthy. The females were allowed to lay eggs for 9 or 10 days. For the adults collected in September and October 2011, we selected both healthier (non-abnormal) and abnormal males and females from a collection locality and confined them in a single tank (2 males and 5 females for Kobe, 2 and 4 for Takahagi, 2 and 4 for Iwaki, 2 and 3 for Hirono, 2 and 5 for Motomiya, and 4 and 5 for Fukushima). Among these individuals, 7 adults from Kobe were all non-abnormal, but the adults from other localities included both non-abnormal and abnormal individuals. The females were allowed to lay eggs for 7 or 8 days.

The larvae were reared on host plant leaves collected around the Nishihara Campus of University of the Ryukyus unless otherwise indicated. For the mating between F₁ individuals from the first-voltine females collected in May 2011, a relatively aberrant virgin female was selected (Supplementary Table 5) and was housed together with 3 virgin males from Tsukuba in a tank, producing 10 strains from 6 localities. The F₂ generation was obtained and reared in a manner similar to that used for the F₁ generation. For constructing pupation curves and eclosion curves, pupated and eclosed individuals were recorded every day. Days at the pupation and eclosion were counted from the very first day of setting egg collection for each locality group.

Abnormality identification and scoring. A conventional anatomical microscope was used for abnormality identification. An external morphological examination of the entire specimen was performed. All abnormalities were searched for by two persons, A.H. and C.N., to avoid possible inter-personal discrepancies in the assessment of morphological abnormalities. Disagreement often arose from overlooking apparent abnormalities. Thus, when two persons disagreed, they discussed discrepancies together and reached agreement on those issues. Abnormal traits of adults were classified into the following 4 categories based on the organ to which an abnormal trait belong: wing morphology (except colour patterns), wing colour patterns, appendages (including legs, antennae, palpi, and proboscis), and other abnormalities (including eyes, thoraces, and abdomens). An abnormality rate was calculated for each category at each collection locality. The adult abnormality rate included all 4 categories at a given collection locality. The total abnormality rate (the abnormality rate of four stages) included the adult abnormality rate plus mortality and abnormality rates of larvae, prepupae, and pupae. The overall abnormality rate was defined as the total number of abnormal adults divided by the total number of inspected adults at all (or most) localities at a given sampling or breeding trial, and it was expressed as a percentage. Pictures of aberrant wings were transposed horizontally if necessary using Adobe Photoshop to compare colour patterns among wings. The forewing size was measured from the wing base to the apical end with an SKM-S30A-PC digital microscope and its associated software called SK measure (Saitoh Kougaku, Yokohama, Japan).

External radiation exposure. An irradiation experiment with ¹³⁷Cs radiation from a CsCl source (14.3 MBq at the time of the experiment; Japan Radioisotope Association, Tokyo, Japan) was performed twice with different radiation levels. In each case, 200 larvae were collected from a pool of females, and randomly selected 150 larvae out of 200 were placed in a plastic container and irradiated for 180–280 hours or for 177–387 hours, resulting in a cumulative radiation exposure of 3–55 mSv or 57–125 mSv, respectively. In each case, 50 larvae were not irradiated and were reared as a control.

Internal radiation exposure. For the internal exposure experiment, we collected wild *O. corniculata* leaves from 5 localities (Fig. 1a; Supplementary Table 1). For all localities except Ube, leaves were collected on 17–19 July 2011 and 2 August 2011. For Ube, leaves were collected on 15, 17 and 28 July 2011. The collected leaves were stored in refrigerators until they were used for feeding. Eggs were deposited on the potted Okinawa leaves by the field-caught Okinawa females. Leaves from a given locality were provided to the late first-instar larvae or second-instar larvae. The β-ray radiation doses associated with the plant samples were measured with a low-background 2π gas-flow counter (Hitachi Aloka Medical LBC-4211-R2, Tokyo, Japan). The cpm values were measured twice in 60 min on 1.60 g ash from the leaves (see below). The mean value minus the background value was used for the final output, together with the ashing rate. The ashing rate for the collected wild *O. corniculata* plants, which depends on water content or dryness of the initial materials, was determined as 2.07% (mean, $n = 3$) on the sample from Tokyo that was collected a few days before processing.

To quantify the activities of artificial radionuclides in the leaves, the following ashing process was performed. First, the leaves were completely air-dried and then baked on a silicon-coated hot plate without catching on fire until the sample turned completely gray. The ash was spread evenly in a plastic container (5.0 cm in diameter,

3.0 cm high) and placed in a germanium semiconductor radiation detector (Canberra GCW-4023, Meriden, CT, USA) with a heavy shield of lead. Measurements were performed until the error rate scored below 5% of the measured values. The activities of cesium were calculated using the following values: the detection efficiency values of ^{134}Cs (605 keV) and ^{137}Cs (662 keV) were 3.81% and 3.54%, respectively, and their branching ratios were 97.6% and 85.1%, respectively. An ashing rate 2.07% was used, as indicated above.

Statistics. The numerical data were subjected to statistical analyses using R version 2.14.2 (The R Foundation for Statistical Computing, Vienna, Austria). For forewing-size differences of field-collected samples, two-tailed paired t test (with pooled SD) for pairwise comparisons was performed, due to the small number of samples, with Holm correction (sequential correction) method using pairwise.t.test(X,Y, p.adj="holm"), as a post-hoc test of ANOVA with summary(aov(X~Y)). For forewing-size differences of external exposure experiment, Student t test was performed using t.test(X,Y, var.equal=T). For forewing-size differences of internal exposure experiment, either Student or Welch t test was performed using either t.test(X,Y, var.equal=T) or t.test(X,Y, var.equal=F), depending on equality of variance, as a post-hoc test of ANOVA, and p-values were corrected by Holm method using p.adjust(p, method="holm"). Before performing t test, both normality and equality of variance of samples were checked.

For correlation analysis, mean values were used as representative values for localities. Pearson correlation coefficient r was determined for scatter plots using cor.test(X,Y, method="pearson") after checking normality of samples, and p-values were adjusted by Holm method using p.adjust(p, method="holm"). For survival curves, generalized Wilcoxon test was performed using survdiff(formula=Surv(X)~Y, rho=1), and p-values were adjusted by Holm method using p.adjust(p, method="holm").

The normal distribution of data sets for t test and correlation analysis was checked by linearity plot using qqnorm(X) and by Shapiro-Wilk test using Shapiro.test(X). If null hypothesis of random sampling from a normal population was not rejected at the significance level of 1%, we considered it normally distributed. Equality of variance was checked by F test using var.test(X,Y).

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Acknowledgements

Author contributions

Additional information