REARING THE AFRICAN GRASS BLUE BUTTERFLY *Zizeeria knysna*: TOWARD THE ESTABLISHMENT OF A BIO-INDICATOR IN AFRICAN COUNTRIES

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ABSTRACT Urbanization and environmental pollution have increasingly become important issues in African countries. For environmental risk management, it is important to conduct field surveys for potential surrogate species and establish their ecotoxicological rearing experiments. A rearing system for the pale grass blue butterfly *Zizeeria maha* (Lepidoptera, Lycaenidae) has been successfully employed in Japan for the assessment of human-living environments, including rural and urban areas. A phylogenetically related species, the African grass blue butterfly *Zizeeria knysna*, lives in human residential environments widely in African countries. Thus, this species is potentially useful as a similar bioindicator in Africa. This paper reports the establishment of a simple and economical rearing system for *Zizeeria knysna* as well as some morphological traits of the immature and adult stages of this species. Females were obtained in central Kenya and confined in a meshed cage where eggs were readily obtained. Hatched larvae were reared on a natural host plant *Oxalis corniculata*. The failure rate of eclosion from pupae to adults was 6.5%, and the reared adult individuals showed normal color patterns, demonstrating the high efficiency of this rearing system. The rearing method for *Zizeeria knysna* in the present study may provide a basis for future human-living environmental assessments in Africa.

Key Words: Environmental assessment; Indicator species; Kenya; Rearing method; *Zizeeria knysna*.

INTRODUCTION

Although Africa has many unique natural environments with high biodiversity, recent rapid industrialization and urbanization have initiated serious environmental changes that affect many biological species (Clausnitzer, 2004; Arimoro, 2009; Liousse et al., 2014; Edge & Mecenero, 2015; Evan et al., 2016). Many of such environmental changes are due to chemical pollution that could cause problems also for human health; thus, management of both natural and human-living environments is a critical issue for African countries (Boadi et al., 2005; Cohen, 2006; Nweke & Sanders, 2009; Hope & Lekorwe, 2010; Simon, 2010).

In conservation biology, some surrogate species (including indicator, umbrella, keystone, and flagship species) are often employed to evaluate the quality of the

natural environment in question (Caro, 2010). In that case, species that are tightly associated with that particular natural environment are chosen as surrogates. To evaluate the quality of human-living environments, including the rural and urban residential areas and agricultural villages, an indicator species should share its living space with humans, be abundant and conspicuous for researchers to readily conduct field surveys, and be sensitive to anthropogenic and natural environmental stress. Additionally, establishment of a rearing system for the indicator species is required to reproduce and investigate the ecotoxicological aspects of environmental changes in a laboratory, so that potential causal factors of the biological impacts may be applied to this species, to obtain mortality rates and other outputs and to compare them with the field observation and survey results. For these reasons, some insects are chosen over other organisms and employed as bioindicators for human-living environments. For example, rearing systems for butterflies and beetles have been used for the risk assessment of genetically modified (GM) crops or pesticides (Olszak, 1999; Shirai & Takahashi, 2005; Wolt et al., 2005; Shirai, 2006).

Butterflies are diurnal insects with colorful wings, some of which share living spaces with humans. Without doubt, they can function as excellent bioindicators, for example, to assess the effects of climate warming (Parmesan et al., 1999), evaluate tropical biodiversity (Howard et al., 1998), and manage forest ecosystems (Maleque et al., 2009). The pale grass blue butterfly *Zizeeria maha* is a small lycaenid butterfly that can be found in human-living environments, including rural and urban residential areas and agricultural villages throughout Japan, except Hokkaido (Shirôzu, 2006). This species shares living spaces with humans, is abundant, is conspicuous for researchers, and is sensitive to environmental stress (Hiyama et al., 2013; Taira et al., 2014), making it an ideal indicator species for field surveys in Japan. Moreover, a reliable rearing system for this butterfly has been established (Hiyama et al., 2010), making it suitable for laboratory experiments that can be used in conjunction with field work. The combination of field work and laboratory experiments for this butterfly has contributed to the evaluation of the biological impacts of the Fukushima nuclear accident (Hiyama et al., 2012, 2013, 2015, 2017a, 2017b; Nohara et al., 2014a, 2014b, 2017; Taira et al., 2014, 2015a, 2015b; Otaki, 2016; Otaki & Taira, 2017). Similarly, this species has been used to investigate the potentially harmful effects of GM corn pollen on nontarget species (Shirai & Takahashi, 2005; Wolt et al., 2005).

The author's ultimate objective is to establish an evaluation method for human-living environments using grass blue butterflies or related species in many countries worldwide. The pale grass blue butterfly *Zizeeria maha* is widely distributed in Asia from Japan through India to the Middle East, but not in Europe, Africa, Australia, and America. However, other species of grass blue butterflies are distributed in temperate to tropical regions where the pale grass blue butterfly is not found (Kawazoé & Wakabayashi, 1976; Fiedler & Hagemann, 1992; Wiemers, 1995; Braby, 2000; Daniels, 2004). These facts suggest that it may be possible to use the other grass blue butterfly species, such as *Zizeeria knysna* (the African grass blue) and *Zizina otis* (the common grass blue), as bioindicators over a wide

range of the globe. Coincidentally, the idea that *Zizeeria knysna* serves as a bioindicator for excessive use of agrichemicals in Africa has been briefly mentioned in Vieira (2008).

As the first step to evaluate the grass blue butterflies as a bioindicator, it is necessary to establish rearing methods for various grass blue butterflies comparable to that for *Zizeeria maha*. The simplest way is to examine whether the standard rearing method for *Zizeeria maha* is applicable to other related species. In a previous study, the authors reared six subspecies of grass blue butterflies found in the Pacific rim regions, *Zizeeria maha argia*, *Zizeeria maha okinawana*, *Zizeeria karsandra karsandra*, *Zizina emelina emelina*, *Zizina otis labradus*, and *Zizina otis riukuensis*, and confirmed that minor modifications in the standard rearing method were sufficient to rear these subspecies efficiently (Gurung et al., 2016). Moreover, the immature stages of these subspecies were compared morphologically with one another to allow researchers to clearly identify species and examine their morphological status (Gurung et al., 2016). However, in that study, the authors did not directly observe another important member of the grass blue butterflies, *Zizeeria knysna*, distributed in Africa and southern Europe (Larsen, 1991; Fiedler & Hagemann, 1992; Wiemers, 1995; Daniels, 2004), which will be the subject for this paper.

In the present study, the authors demonstrate the high efficiency of a simple and economical rearing method for *Zizeeria knysna* and report some morphological traits of the immature and adult stages of this species. This species is found all year round in many African countries (Clark & Dickson, 1971) and shares living spaces with humans (Woodhall, 2005). It is one of the most abundant butterfly species in Kenya (Larsen, 1991). Considering the similarities between *Zizeeria knysna* and *Zizeeria maha* in terms of natural history and morphology, the authors believe that *Zizeeria knysna* is useful as a bioindicator for human-living environments in Africa. More concretely, this species may be employed in place of *Zizeeria maha* in Japan to examine the potential biological effects of agrichemicals and test GM crop safety in light of GM crop cultivation in the African countries such as Kenya and South Africa (James, 2015).

METHODS

I. Butterflies

The African grass blue butterfly *Zizeeria knysna* (Trimen, 1862) (Lepidoptera, Lycaenidae) is common in Kenya. According to Larsen (1991), this species is distributed throughout tropical Africa. The adult butterfly can be found all year round in Africa, although the best period is from October to April in South Africa (Clark & Dickson, 1971). This species can be found in various habitats with humans, including parks, gardens, and villages from flatlands to mountains (Woodhall, 2005). The known host plants of this species to date include *Tribulus terrestris*, *Amaranthus deflexus*, *Medicago sativa*, and *Oxalis corniculata* (Clark & Dickson, 1971).

Zizeeria knysna has two closely related species, *Zizeeria karsandra* and *Zizeeria maha*. In some studies, *Zizeeria maha* is categorized into the genus *Pseudozizeeria* (Kawazoé & Wakabayashi, 1976; Fukuda et al., 1984; Yago et al., 2008). However, the authors treated the pale grass blue butterfly as *Zizeeria maha*, following Shirôzu & Hara (1962), Shirôzu (2006), and Gurung et al. (2016). Importantly, *Zizeeria maha* has been accepted as a formal name of this species in the current checklist of Japanese butterflies (Inomata et al., 2010).

In addition to samples of *Zizeeria knysna* reared in this study, the authors used the samples of *Zizeeria maha argia* from Hiyama et al. (2012) and *Zizeeria maha okinawana* from Hiyama et al. (2010) for cross-species comparison in the pupal period and the failure rate (FR). The offspring individuals of *Zizeeria maha argia* and *Zizeeria maha okinawana* were reared exclusively with their natural host plants *O. corniculata* in a laboratory at the University of the Ryukyus, Okinawa, Japan. Their rearing conditions were 16L:8D at 25–27°C. The parents of the offspring of *Zizeeria maha argia* and *Zizeeria maha okinawana* were collected in Tsukuba (Ibaraki prefecture) and Nishihara (Okinawa-jima Island, Okinawa prefecture), Japan, respectively.

II. Establishment of a Rearing System

To establish an efficient rearing system for *Zizeeria knysna*, six males and three females were caught around the housing complex for researchers and employees of the Mpala Research Centre in Laikipia District, central Kenya (0°17'N, 36°53'E), in the middle of September 2013 (Fig. 1a). The Mpala Research Centre is located in a highland savanna environment. Mpala is a working cattle ranch and wildlife conservancy with a nearly intact mammalian community. As shown in the climate graph of this region (Fig. 1b), September is in the dry season. The annual rainfall in 2013 was 761.3 mm, and the monthly rainfall in September was 27.8 mm for this year (Caylor et al., 2017). The collected butterflies were confined in a meshed cage (300 × 300 × 300 mm) placed outside one of the houses of the Mpala Research Centre to harvest eggs. Also placed inside the cage were two potted creeping woodsorrel *O. corniculata*, one of the natural host plants for this species, some wild Asteraceae flowers in a glass bottle, and two artificial flowers with yellow petals made with plastic tape to supply sugar water as artificial nectar. These plants were obtained locally nearby. Among the three females in the cage, only one appeared to have deposited eggs. The ovipositioning period was 6 days after the confirmation of the first deposited egg.

After hatching, bundled cut leaves were placed on the potted plant with larvae, and then the bundled leaves with larvae were transferred to a plastic container (150 × 150 × 60 mm). The container was placed indoors at one of the houses of the Mpala Research Centre to avoid potential wild animal attacks, but with sufficient indirect sunlight. These larvae were exclusively fed *O. corniculata* from the end of September to the middle of October. After pupation, two pupae were each transferred to small Styrofoam cups (110 mm in top diameter, 60 mm in bottom diameter, 30 mm in height) that allowed their wings to expand success-

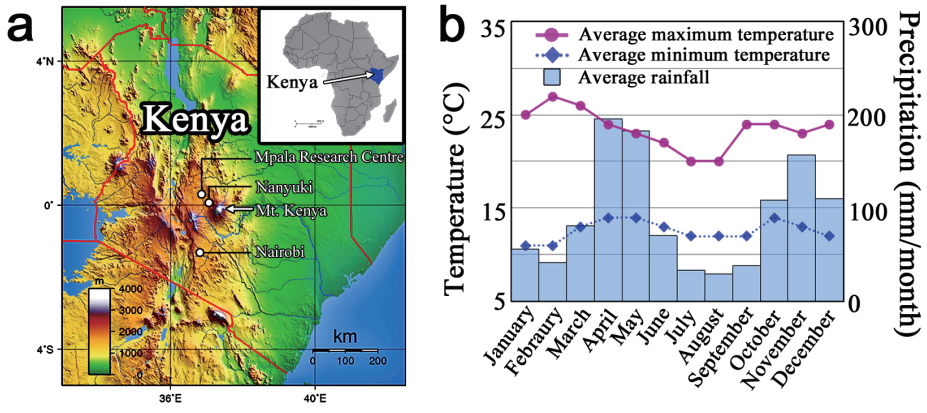

Fig. 1. Collection Locality and Its Climate
(a) Map of Kenya. The Mpala Research Centre is located near Nanyuki and Mt. Kenya. Inset indicates the location of Kenya on the map of Africa. Original public domain maps were obtained from web sites free map viewer (http://www.freemapviewer.com/en/map/Map-Kenya_467.html) and Wikimedia Commons (https://commons.wikimedia.org/wiki/File:Blank_Map-Africa.svg) and modified as necessary.
(b) Temperature and rainfall data of Nanyuki near the Mpala Research Centre: Average monthly temperature with maximum and minimum values and average monthly rainfall records of Nanyuki (2000–2012). The original data were obtained from world weather online (http://www.worldweatheronline.com/), accessed in October 2015, and used with permission. Temperature and rainfall graphs were constructed using Microsoft Excel.

fully after eclosion but covered with a net for confinement. The room temperature was maintained at 25 ± 3°C without the use of an air conditioner.

To evaluate this rearing system, the authors calculated the FR for eclosion: FR = [{(number of pupae that were obtained) – (number of pupae that successfully eclosed)}/(number of pupae that were obtained)] × 100. That is, the FR indicates the percentage of dead pupae combined with individuals that eclosed with severe wing deformations to the total number of individuals that pupated successfully.

The authors measured the forewing size of the reared individuals of *Zizeeria knysna* (n = 4 in male; n = 3 in female) from the wing base to the apical end, following Hiyama et al. (2010), using ImageJ v. 1.48 of the National Institute of Health, Bethesda, MD, USA (Schneider et al., 2012). Only a small number of *Zizeeria knysna* individuals were used for measurements because most samples were damaged severely by animals such as mice and ants. The forewing size was expressed as the mean ± standard deviation (SD).

III. Statistical Analyses

Numerical data were analyzed with R version 3.2.1 (The R Foundation for Statistical Computing, Vienna, Austria). For comparisons between each species, the Wilcoxon rank sum test (a nonparametric test) with Holm adjustment (multiplication of original P-values not to change the conventional significance levels)

was performed to compare the pupal period of *Zizeeria maha argia* and *Zizeeria maha okinawana* with that of *Zizeeria knysna*. To compare the FR of eclosion between species, Fisher's exact test for count data was performed with Holm adjustment.

RESULTS

I. The Rearing System and Morphological Traits of the Immature Stages

Adult *Zizeeria knysna* butterflies mated within 30 minutes after confinement in the cage. One of the three females started laying light blue-green eggs, approximately 0.5 mm in diameter, on the surface of *Oxalis corniculata* leaves one day after confinement (Fig. 2a–d). The authors confirmed that at least 32 eggs (but 46 pupae were obtained) were deposited mainly on the host plant leaves and fruits, and even on non-host plant leaves and nearby objects, which had not been recorded in previous literature where eggs were laid on flower buds and leaves of host plants (Clark & Dickson, 1971; Daniels, 2004).

Eggs were light blue-green immediately after oviposition (Fig. 2d), and the color gradually changed to white by the hatching time (Fig. 2e). Similar color changes are known to occur in other species of the genus *Zizeeria* and its related genus *Zizina* (Gurung et al., 2016). The size of the outline ridge (or notch) of an egg from the top-down view (Fig. 2e) was relatively large in *Zizeeria knysna* among the *Zizeeria* and *Zizina* species, as shown in Downey & Allyn (1981, 1984), Munguira et al. (2015), and Gurung et al. (2016). The first larvae hatched 8 days after the first oviposition. Egg shells were opened circularly at the top without damage to the lateral wall (Fig. 2f).

Hatched larvae transferred to the bundled cut leaves by their own locomotive activity (Fig. 3a). The entire leaves with the larvae were then transferred to a plastic container (Fig. 3b). First instar larvae of *Zizeeria knysna* were indistinguishable from those of other species of the genera *Zizeeria* and *Zizina* (Fig. 3c). Larvae of *Zizeeria knysna* at the third instar stage had dorsal white lines (Fig. 3d), as in the illustrations in Clark & Dickson (1971), but larvae at the fourth (last) instar stage did not have clear dorsal and lateral white lines in the present study (Fig. 3d), which is not consistent with the illustrations in Clark & Dickson (1971).

The first individual that pupated was observed 21 days after the first hatch. Pupae had various levels of pigmentation on the lateral and dorsal surfaces (Fig. 3e, f). The majority of pupae appeared to be the blot type (*sensu* Gurung et al., 2016), which has a pair of arrays of extensive pigmentation parallel to arrays of spiracles, as shown in Clark & Dickson (1971), but fine-type pupae (*sensu* Gurung et al., 2016), which have low levels of pigmentation arrays, were also found in the present study (Fig. 3f).

The first individual that eclosed was observed 8 days after the first pupation, and the pupal period of this species was 8.8 ± 1.0 days (n = 45), significantly

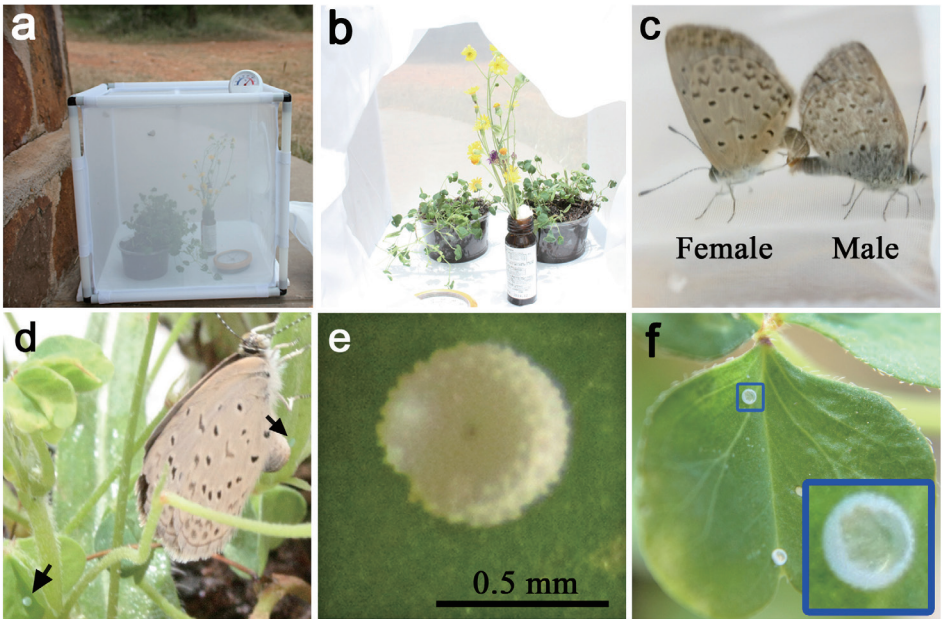

Fig. 2. Rearing Method for *Zizeeria knysna* Adults in Kenya
(a) The cage. (b) Inside the cage. (c) Copulation in the cage. (d) Oviposition in the cage. Arrows indicate eggs, which are light blue green. (e) An egg that turned white. A complex chorionic structure is observed. (f) Egg shells after hatch. Inset is an enlargement of the small boxed area.

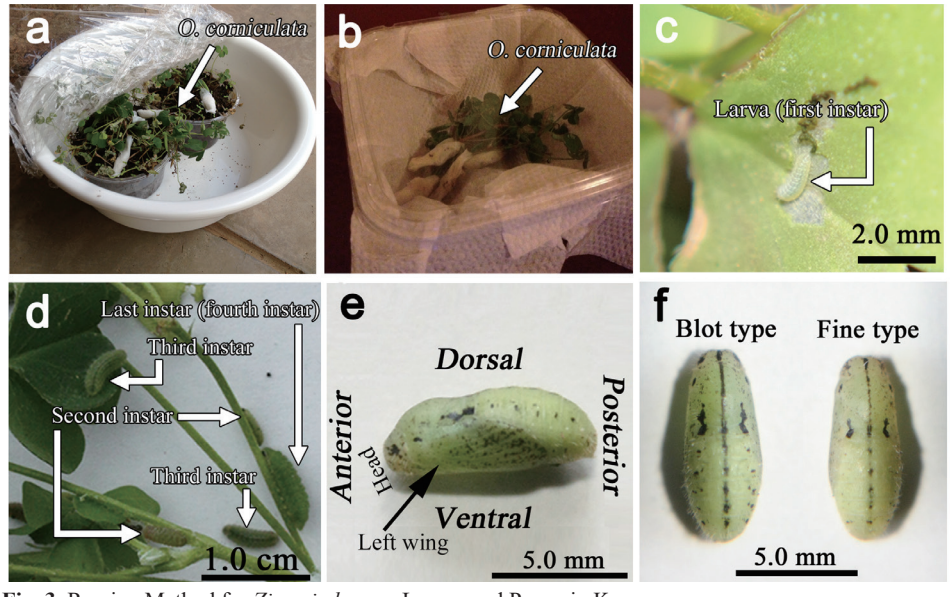

Fig. 3. Rearing Method for *Zizeeria knysna* Larvae and Pupae in Kenya
(a) Transfer of the first instar larvae to bundles of cut plants in a plastic container. The container is loosely covered with plastic wrap to prevent the plants from drying quickly. (b) Rearing larvae in a small plastic container. (c) A first instar larva. (d) Larvae of various instars. (e) Lateral view of a pupa. (f) Top-down view of two pupae with different degrees of pigmentation. The blot type has more pigmentation than the fine type along the arrays of spiracles.

longer than those of two subspecies of the pale grass blue butterflies: 6.9 ± 0.7 days (n = 30) for *Zizeeria maha okinawana* (W = 1272, P < 0.0001) (Hiyama et al., 2010) and 6.4 ± 0.5 days (n = 161) for *Zizeeria maha argia* (W = 7073, P < 0.0001) (Hiyama et al., 2012). The total period between the day when the first egg was laid and the day when the first adult emerged was 37 days.

II. Failure Rate of Eclosion

The failure rate (FR) of eclosion for the present rearing experiment was 6.5% (n = 46). This value was low enough for rearing system and not significantly different from those of the previous studies on *Zizeeria maha argia* (FR = 18.5%, n = 194; P = 0.095) and *Zizeeria maha okinawana* (FR = 0.0%, n = 30; P = 0.27).

III. Wing Color Patterns of the Adult Offspring

The wing color patterns of the adult offspring of *Zizeeria knysna* that were obtained in this rearing system showed normal color patterns in comparison with those of their parents and with those described in the literature (Clark & Dickson, 1971; Larsen, 1991; Woodhall, 2005). The male dorsal side of the wings showed metallic violet blue with black borders, whereas the female dorsal side showed various degrees of metallic blue scales in basal area surrounded by a relatively large black background (Fig. 4). On the ventral side of the wings, both sexes exhibited many black spots that composed several arrays against the grayish white background (Fig. 4). This spot pattern was essentially indistinguishable from that of other *Zizeeria* species.

The average male forewing size of the reared individuals of *Zizeeria knysna* was 11.3 ± 0.2 mm (n = 4), and the female forewing size was 12.0 ± 0.3 mm (n = 3).

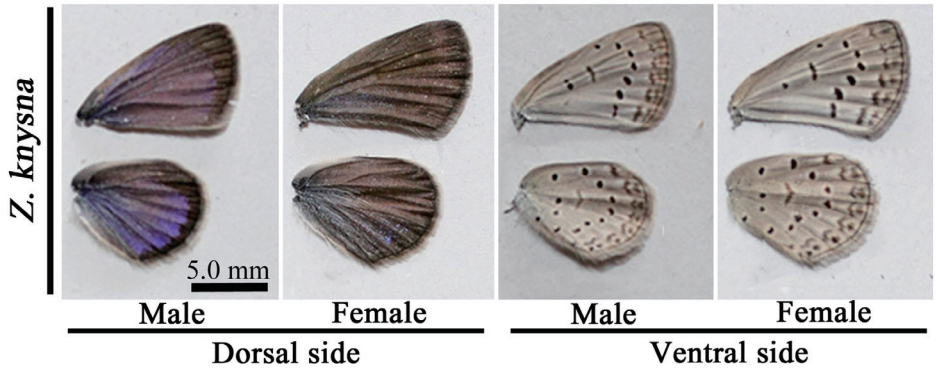

Fig. 4 Wings of *Zizeeria knysna*
All wings are from the offspring individuals that were obtained using the present rearing method. The scale bar is applicable to all panels in this figure.

DISCUSSION

In this study, the authors reared *Zizeeria knysna* for an entire life cycle in Kenya using a simple and economical rearing method modified from the standard method for *Zizeeria maha* (Hiyama et al., 2010). Previously, Fiedler & Hagemann (1992) and Daniels (2004) reported on successfully rearing *Zizeeria knysna*. In these studies, *Zizeeria knysna* larvae were reared with *Medicago sativa* for experiments on larval myrmecophilous function using advanced laboratory equipment. However, to the authors' knowledge, precise rearing procedures from eggs to adults, and the efficiency of rearing *Zizeeria knysna* with *Oxalis corniculata*, have not been reported. This study used minimal laboratory equipment and did not depend on an electrical supply. The technical simplicity could be important in rearing this butterfly in African countries, although confirming reproducibility and further refinement of the rearing method should be further explored.

The present results on the morphological traits and periods of each life stage of *Zizeeria knysna* are mostly consistent with Clark & Dickson (1971), indicating that the authors' rearing system was sufficient for the species to complete a normal life cycle. The system also demonstrated that *O. corniculata* was a suitable host plant for egg deposition and larval consumption, although a variety of plants are known to be host plants (Clark & Dickson, 1971; Larsen, 1991; Fiedler & Hagemann, 1992). Most importantly, the simple rearing method resulted in a low FR, that is, mostly normal adult individuals with non-deformed wings and normal color patterns were gained, demonstrating the high efficiency and quality of the system. A less adequate rearing method would have resulted in adult individuals with deformed wings and aberrant color patterns, e.g., disappearance of black spots on the wings (Hiyama et al., 2010; Iwata et al., 2013).

The authors obtained 46 offspring individuals (pupae) in this study from three females, although only one of the females appeared to have deposited most, if not all, of the eggs. This is not unusual because the degree of oviposition likely varies among individuals, depending on the mating and ovipositioning experience at the time of being captured, as has been reported for *Zizeeria maha* (Hiyama et al., 2010). The authors did not make an effort to increase the number of eggs to be obtained in the present study, but more eggs would be practical for future ecotoxicological studies, by increasing both the number of females and the length of the ovipositioning period in the cage.

The pupal period of *Zizeeria knysna* was significantly longer than that of *Zizeeria maha* in the present study. However, the authors believe that this is unlikely to reflect species differences. Instead, it may reflect a methodological difference. *Zizeeria maha* was reared in Japan under the controlled conditions of a laboratory, but not *Zizeeria knysna*, which was reared in a Kenyan environment that lacked equivalent laboratory equipment. Nonetheless, it is important to stress that the economical rearing method presented in this study did not harm the final morphology and survival of the butterflies, as demonstrated by the low FR and the normal color patterns.

The observed morphological traits of the immature stages of *Zizeeria knysna*

were mostly consistent with the previous records (Clark & Dickson, 1971; Daniels, 2004). However, some inconsistencies were discovered, including a lack of dorsal and lateral white lines in the fourth-instar larvae and less pigmentation in pupae. These are likely variations of phenotypes not well recorded in the literature. Such phenotypic variations are not surprising, considering that *Zizeeria knysna* is distributed widely in Africa.

The forewing size of *Zizeeria knysna* is presented for reference, because of the small sample size. However, the forewing size information may be important because it could reflect environmental stress, as has been shown for *Zizeeria maha* (Hiyama et al., 2012, 2013). Based on the forewing size measurements, it may be possible to identify potential factors such as pesticides that may negatively impact the environment. In the case of *Zizeeria maha*, the forewing size of the field-collected adults from Fukushima immediately after the Fukushima nuclear accident was shown to be smaller than those from other localities (Hiyama et al., 2012, 2013). Furthermore, similar reduction in the forewing size was observed in the external and internal exposure experiments (Hiyama et al., 2012, 2013). Similar future measurements in field-caught and experimental samples may be performed using *Zizeeria knysna* in African countries. Geographical and seasonal size changes may also be expected in *Zizeeria knysna*, as has been shown for *Zizeeria maha* (Taira et al., 2015b).

Because the *Zizeeria maha* rearing system has contributed to the assessment of the environmental and biological impacts of anthropogenic disturbances in Japan (Shirai & Takahashi, 2005; Wolt et al., 2005; Hiyama et al., 2012, 2013; Nohara et al., 2014a, 2014b, 2017; Taira et al., 2015a, 2015b), the *Zizeeria knysna* rearing system reported here may also be employed for similar environmental assessments in African countries experiencing rapid urbanization and environmental pollution. Moreover, the rearing system may serve as a basis for economically and efficiently conducting genetic, behavioral, and physiological studies of this butterfly in the future.

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