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Original Paper

Simultaneous movement of style and stamen set during anthesis in *Etlingera elatior* (Zingiberaceae)

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Abstract

Etlingera is a genus of the family Zingiberaceae that is native to tropical Asia. Many species are economically important as a source of food, spices, medicines, and ornamental plants. This study aims to elucidate the floral morphology of *E. elatior* and understand the behavior of the set of stamen and style during floral anthesis, seeking to identify the mechanisms that promote xenogamy in the species. Flower morphology, time of anthesis and movement of the stamen and style during this period, pollen viability, nectar volume, floral visitors, and the reproductive system were evaluated. The flowers of *E. elatior* exhibited synchronous movement of the style and stamen, however, this movement was not considered as flexistyly, since the species does not present dichogamy. The synchronous movement of the stamen and pistil, by itself, was not enough to promote self-pollination. Fruiting depends on pollinators. The stigma fluid is an important means whereby pollen adheres to the mouthpart of the pollinator. Hummingbirds and bees were considered the most effective pollinators of *E. elatior* in the area studied.

Key words: flexistyly, ginger, ornamental plant, reproductive biology, Zingiberaceae.

Resumo

Etlingera é um gênero da família Zingiberaceae nativo da Ásia tropical. Muitas espécies são economicamente importantes como fonte de alimentos, especiarias, medicamentos e plantas ornamentais. Este estudo tem como objetivo elucidar a morfologia floral de *E. elatior* e compreender o comportamento do estame e do estilete durante a antese floral, buscando identificar os mecanismos que promovem a xenogamia na espécie. Foram avaliados morfologia floral, tempo de antese e movimentação do estame e estilete neste período, viabilidade polínica, volume de néctar, visitantes florais e sistema reprodutivo. As flores de *E. elatior* exibiram movimento sincrônico do estilete e do estame, porém, esse movimento não foi considerado como flexistilia, uma vez que a espécie não apresenta dicogamia. O movimento sincrônico do estame e estilete, por si só, não foi suficiente para promover a autopolinização na espécie. A frutificação depende da ação de polinizadores. O fluido do estigma é um meio importante pelo qual o pólen adere ao aparelho bucal do polinizador. Beija-flores e abelhas foram considerados os polinizadores mais eficazes de *E. elatior* na área estudada.

Palavras-chave: flexistilia, gengibre, planta ornamental, biologia reprodutiva, Zingiberaceae.

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Introduction

Self-fertilization is a type of sexual reproduction where the gametes involved are produced by the same individual. This process serves as a reproductive guarantee, but it can cause inbreeding depression and reduction of genetic variability in the species. Therefore, numerous systems evolved as mechanisms to reduce self-fertilization and promote cross breeding between individuals of different plants (Barrett 2002; Cardoso *et al*. 2018).

Among the floral mechanisms that prevent autogamy is the flexistyly (Li *et al*. 2001). Flexistyly is characterized by a sexual polymorphism combining herkogamy and dichogamy, in which populations exhibit two floral morphs that vary in the temporal patterns of style growth and orientation (Barrett 2002; Cardoso *et al*. 2018).This floral system was described in *Alpinia blepharocalyx*, in which Zhang *et al.* (2003) observed two floral morphs, the cataflexistylous, in which the receptive stigma remains erect above the dehiscent anther in the morning and curves over the indehiscent anther in the afternoon, and the anaflexistylous, where the receptive stigma bends over the indehiscent anther in the morning and becomes erect, in a superior reflex position in the afternoon, when the anther begins to release pollen. This mechanism has been documented in 24 Zingiberaceae species (Cui *et al.* 1996; Li *et al.* 2001; Zhang *et al.* 2003; Jia *et al.* 2015), including the genus *Etlingera* (*E. yunnanensis*). However, Cui *et al.* (1996) did not characterize in detail flexistyly in *E. yunnanensis*.

The genus *Etlingera* Giseke (Zingiberaceae) is distributed throughout southeastern Asia, New Guinea, the Bismarck Archipelago, the Philippines, Australia (Queensland), and Polynesia with centers of species diversity in Borneo and New Guinea. The cone-shaped inflorescence gives the genus the common name of torch ginger and is composed of bracts and flowers sustained on a peduncle of variable lengths (Yeats 2013). Some inflorescences are partially subterranean, for example in *E. megalocheilos*. Territorial spider-hunter birds (*Arachnothera longirostra*) and long-tongued bees have been reported as pollinators of *Etlingera* species (Poulsen 2006; Jia *et al.* 2015).

Etlingera elatior is a plant with aromatic properties. Essential oils are extracted from its rhizomes, leaves, and inflorescence. The plant also has pharmacological and phytochemical properties (Abdelwahab *et al.* 2010; Abdelmageed *et al.* 2011) and is used for the control of fungal diseases (Punnawich *et al.* 2009) and in the cosmetics industry (Voon *et al.* 2012). In addition, torch ginger is used in ornamental and landscape projects for gardens or urban areas. The extravagant and showy inflorescence with bright colors is suitable for use as a cut flower. Torch ginger has been used in floral arrangements in Australia, Brazil, Hong Kong, Thailand, and the United States (Mohamad *et al.* 2005; Lachumy *et al.* 2010; Jackie *et al.* 2011).

The reproductive biology of *E. elatior* was studied by Aswani *et al*. (2013) who concluded that the species is xenogamous, pollinated by birds or insects, with the occurrence of fruiting through manual self-pollination and cross-pollination. The authors did not go into detail about the floral morphology of the species and the mechanisms that favor cross-pollination.

In the present study, we seek to elucidate the floral morphology of *E. elatior* and understand the behavior of stamen and style during floral anthesis, seeking to identify the mechanisms that promote xenogamy in the species. The floral biology of *E. elatior* will be compared with that observed in other species of Zingiberaceae that present flexistyly.

Material and Methods

Plant material

The experiment was conducted at the active germplasm bank of tropical flowers of the State University of Mato Grosso, Tangará da Serra Campus, MT, Brazil (14º39’2.5’’S, 57º25’53.8’’W), from May 2018 to April 2019. Specimens of *E. elatior*, were planted in 2015, for the purpose of studies. The region is characterized by a tropical climate with a dry season from May to September and a rainy season from October to April, average annual rainfall of 1830 mm, and altitude of 446.6 m (Dallacort *et al.* 2011).

Flower morphology

The flower morphology of *Etlingera elatior* was analyzed in detail by visual observation and under a Leica DMLB stereomicroscope equipped with a Zeiss Axio Cam digital camera (Aswani *et al*. 2013). The inflorescence, flower and floral structures, were illustrated.

Flower lifespan

For the determination of flower lifespan, 20 inflorescences from five different individuals were bagged using organza bags. In each inflorescence, 40 flower buds were labeled and observed at two-hour intervals between 6:00 and 20:00 h for five days. The times of opening of the flower buds (anthesis) and wilting (senescence) were recorded. At each assessment interval, five flowers were taken to the laboratory and the occurrence of anther opening and the presence of stigma fluid were evaluated under a light stereomicroscope Carl Zeiss Stemi DV4.

Movement of stamen and style during anthesis

Every two hours, five flowers, from five different individuals, were collected and excised at the basal portion of the labellum and the angle between the stamen and style, which form a set because of their proximity, was measured in relation to the labellum. The removal of flowers did not impact the remaining parts of the inflorescence.

Movement of the stamen and style was determined under laboratory conditions. The main movements of the set (stamen and style) were recorded during the following key processes: 1) during pre-anthesis at 20:00 h of the previous day; 2) onset of anthesis (6:00–10:00 h); 3) mid-anthesis (12:00–14:00 h); 4) end of anthesis (18:00–20:00 h). The angle for determination of stamen/style movement was recorded with a protractor.

Pollen viability and stigma receptivity

Pollen viability was determined in the pre-anthesis and anthesis stages (6:00 to 20:00 h) of *E. elatior* flowers at intervals of 2 hours, totaling eight assessment intervals. At each interval, slides were prepared for five anthers from different flowers and different individuals. The anthers were removed and crushed on glass slides and then stained with 3% acetic carmine (Kearns & Inouye 1993). Pollen viability was evaluated by counting up to 200 pollen grains. Pollen showing stained protoplasm was classified as viable and transparent pollen was classified as non-viable.

Stigma receptivity was evaluated at the same intervals and with the same flowers as used for the assessment of pollen viability. Receptivity was determined by reaction of the peroxidase enzyme present in the stigma with hydrogen peroxide (3%) (Kearns & Inouye 1993).

Nectar volume and nectar sugar concentration

The nectar volume and sugar concentration were measured at intervals of two hours from 6:00 to 20:00 h. At each interval, the mean nectar volume was obtained with a graded micropipette (0.5 to 10 µL). The mean sugar concentration was determined with a Vodex® pocket refractometer in five flowers collected from different inflorescences.

Reproductive system

The pollinator dependence for seed production was tested using two treatments: spontaneous self-pollination and open pollination (control). For each test, five fully developed inflorescences divided across eight different plants were used. Spontaneous self-pollination was tested by bagging young inflorescences using an organza bag in order to prevent the flowers from being pollinated by pollen from other inflorescences. For determination of the occurrence of open pollination, the inflorescences were labeled and left open to natural pollination. The labeled inflorescences were monitored until senescence when the presence or absence of fruiting was evaluated.

Floral visitors

Observations were made during two periods of the day totaling 20 hours of observation. Visitors were monitored for a period of 5 non-consecutive days, with 4 hours of observation/day (2 hours in the morning 7:00–9:00 h and 2 hours in the afternoon 15:00–17:00 h). For assessment, photographs were obtained and possible visitors were collected. Insects or birds that, when visiting the flowers, made contact with the reproductive organs were classified as potential pollinators. The identification of bees and hummingbirds was carried out with the help of a specialist. The nomenclature of hummingbirds followed the guidelines of the Brazilian Committee of Ornithological Records -CBRO (Pacheco *et al.* 2021).

Results

Flower morphology

The inflorescences of *Etlingera elatior* are terminal and densely congested, with involucral sterile and fertile bracts (Figs.1-2). Each fertile bract contains a single flower (Figs. 1a; 2a). The flowers are bright red with a yellow border (Fig. 1a-b) and have a gamosepalous (Fig. 2b-c) calyx with serrated lobes (Fig. 2b). The bracteoles (minor bracts) are tubular and persistent with two to three indentations, as presented in the Figures 2d and 2e. The floral tube is long (approximately 4 cm, Fig. 2f) and is composed of two short lateral staminodes (Fig. 2g) and an up to 3 cm long labellum (Fig. 1c), resulting from the fusion of other staminodes (Fig. 2h). The bisexual flower typical of Zingiberaceae produces a single fertile stamen (Fig. 1d) with two thecae (Figs. 1e; 2i). The thin style has an average length of 3 mm and passes between the thecae of the stamen (Fig. 2i). The stigma is dark red and has a round shape, with a transverse ostiole facing downward or forward, and is located just above the anther apex (Figs. 1e-f; 2i). The stigma fluid is released on the receptive surface. The ovary is inferior, densely pubescent (Fig. 2j), tricarpellary and trilocular, with axial placentation (Fig. 2k), and two nectareous glands are present at the base of the style (Fig. 2j).

Anthesis of flowers

Etlingera elatior bloomed throughout the year, with a peak bloom from March to September. Flower opening occurred from bottom to top, taking 90 to 120 days for all flowers of the inflorescence to open. The beginning of anthesis was observed between 6:00 and 6:30 h and was characterized by opening of the labellum and horizontal positioning of the flowers, in related to the axis of the inflorescence (Fig. 1a-b). Senescence of the flowers occurred at about 20:00 h of the same day when the flowers exhibited an opaque and dark color and the labellum closed on the reproductive organs. In general, the senescent flowers remained adhered to the inflorescence.

Anther dehiscence was observed at about 7:00 h. Pollen presentation occurred concomitantly with the release of stigma fluid. The stigma fluid

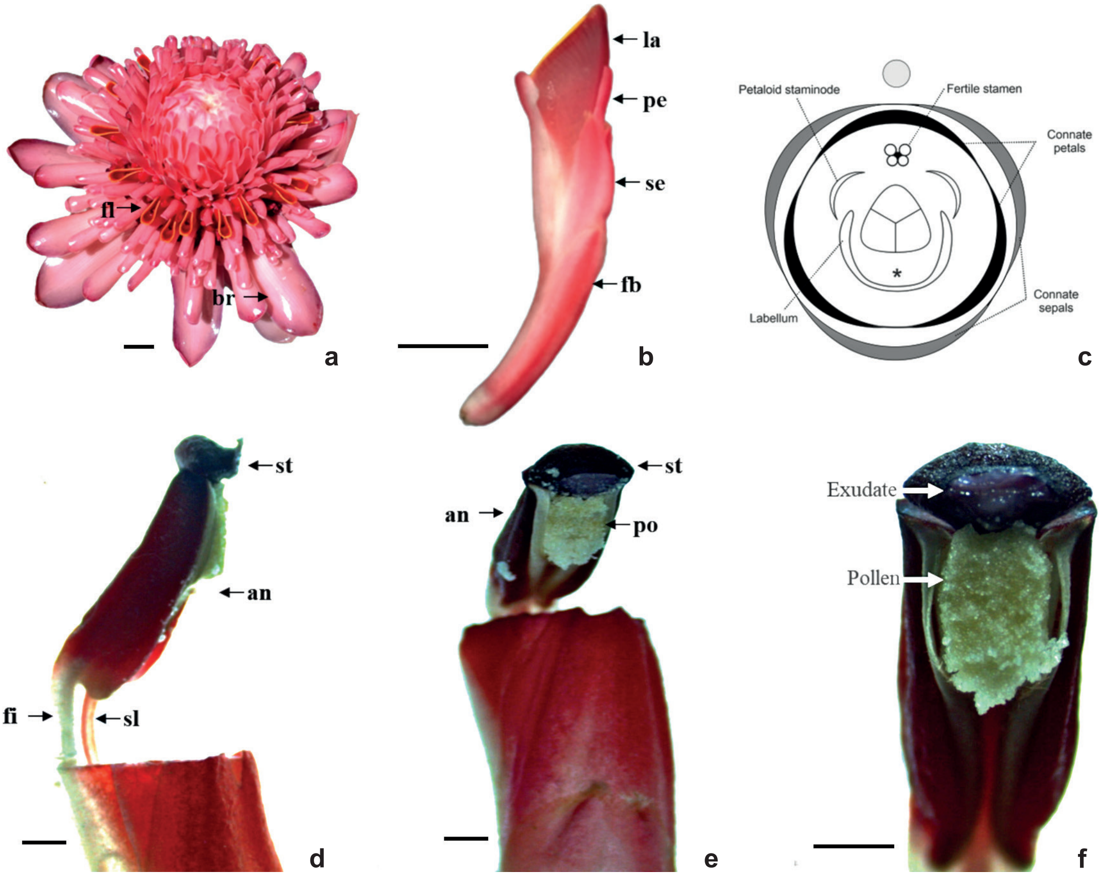

Figure 1 – a-f. Inflorescence and flower details of *Etlingera elatior* – a. flower (fl), and bract (br); b. lateral view of the flower with fertile bracts (fb), sepal (se), petal (pe), and labellum (la); c. internal side view of flower with corolla removed; d. lateral view of the anther, arrows show stigma (st), anther (an), style (sl), and filament (fi); e. frontal view of the anther (an), stigma (st) with exudate (ex), and pollen (po); f. presence of pollen and exudate (arrows). Bars: a-b = 10 mm; d-f = 1 mm.

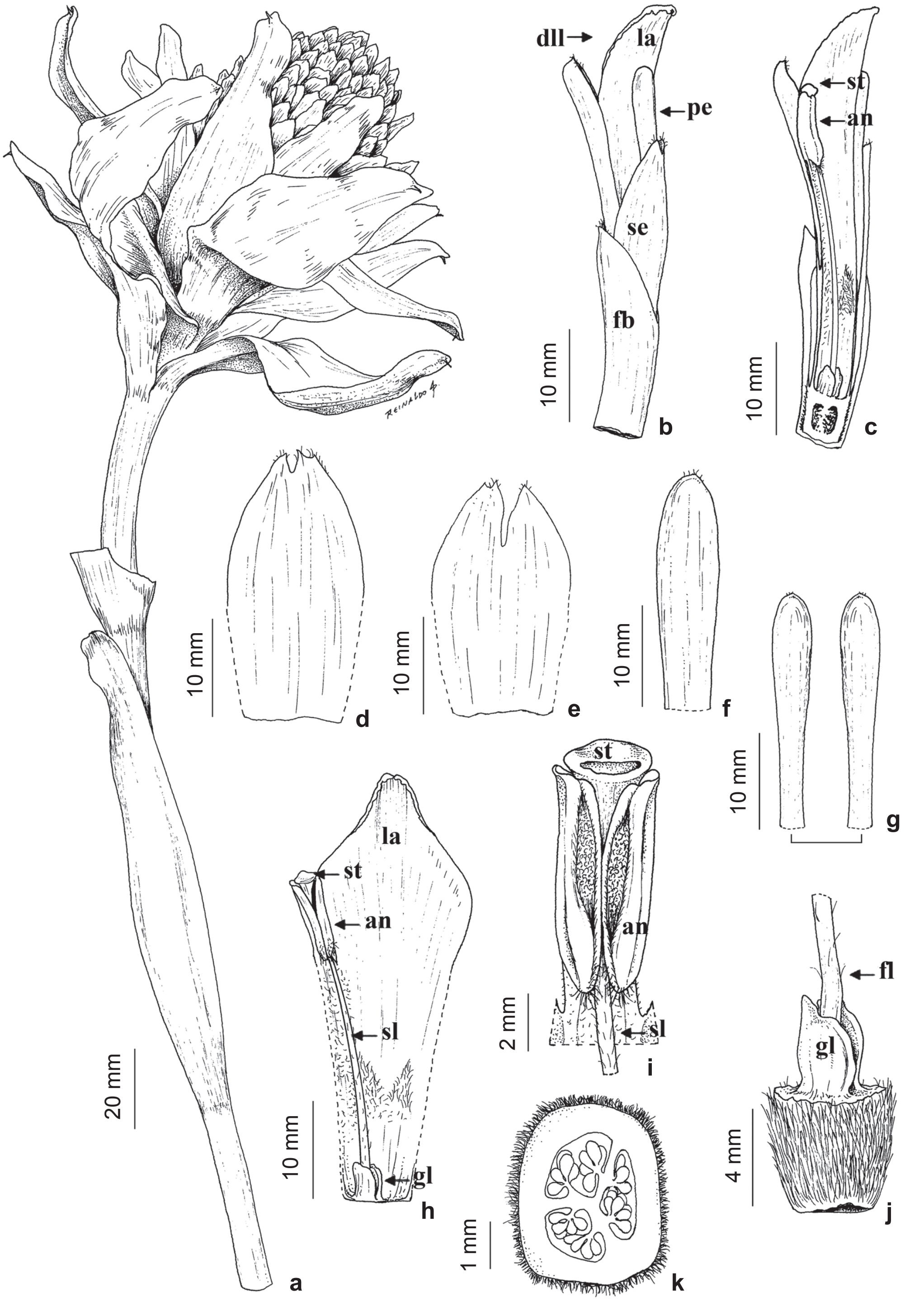

Figure 2 – a-k. Floral structures – a. inflorescence of *Etlingera elatior*; b. lateral view of the *E. elatior* flower with fertile bracts (fb), sepal (se), petal (pe), labellum (la), and dorsal labellum lobe (dll); c. side view of the of the flower showing the internal structures, arrows show stigma (st), and anther (an); d. structure of calyx, fused sepals; e. detail of the calyx: gamosepalous; f. detail of the corolla: central petal; g. detail of the corolla: lateral petals; h. detail of the corolla: labellum (la), gland (gl), and reproductive structures stigma (st), style (sl), and anther (an); i. detail of the reproductive structures stigma (st) above the anther (an) composed of two thecae, and style (sl); j. inferior ovary with nectareous glands (gl) at the base of the filament (fi); k. detail of the tricarpellary ovary. Drawing: Reinaldo Pinto, 2019.

accumulated in the ostiole of the stigma, had a milky color, and small amounts could be perceived even before dehiscence of the anthers, around 10 am (Fig. 3a-b). The period in which the stigma is receptive started at 14 h. During floral anthesis, the fluid formed a mixture with the pollen grains, resulting in a sticky and moist mass (Fig. 3c-e). At the end of anthesis, the amount of stigma fluid decreased and the fluid became translucent close to the ostiole (Fig. 3f).

Movement of stamen and style during anthesis

The movements of the stamen and style occur simultaneously in *E. elatior*. About 10 hours before anthesis, the flowers exhibited an erect position, forming an angle of almost 90° in relation to the basal portion of the labellum (Fig. 4a); the stamen and style set moved in the direction of the basal portion of the labellum over time (Fig. 4b-c). A reduction in the angle formed between the stamen/style set and the basal portion of the labellum was observed to 10:00 h (Fig. 4c). Movement away from the base of the labellum occurred between 14:00 and 18:00 h of the same day (Fig. 4d-e). At the end of anthesis (20:00 h), the angle was 40° (Fig. 4f).

Pollen viability and stigma receptivity

The slides examined exhibited pollen viability higher than 70% at all anthesis times evaluated. Peak viability was observed at 12:00 h (mean 92.7 ± 1.8%) and the lowest percentage of viable pollen was recorded at 16:00 h (mean 71.6 ± 10.8%). In newly opened flowers, the onset of bubble formation due to the reaction with H_2O_2 was observed in 80% of the flowers analyzed. After 14:00 h, 100% of the stigmas were receptive and this receptivity persisted until the end of anthesis.

Nectar volume and sugar concentration

Nectar production began at 8:00 h and the highest volumes were recorded starting at 14:00 h (Fig. 5). The mean volume of nectar produced throughout the period of anthesis was 18.8 ± 0.42 µL. Peak nectar production (40.2 µL) was observed at 14:00 h (Fig. 5). The mean sugar concentration was 7.95 ± 0.55%.

Reproductive system and floral visitors

The mean number of flowers/inflorescence in *E. elatior* ranged from 161–208 ± 8.3 (N = 5 inflorescences). Fruiting was observed only in open pollination tests, with the formation of 4

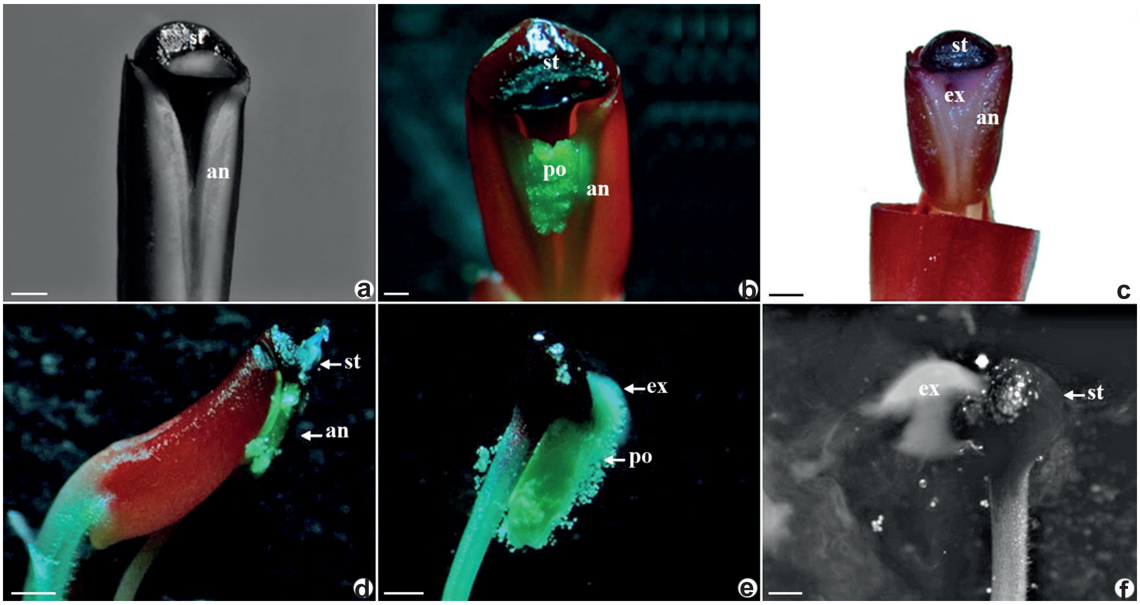

Figure 3 – a. Frontal view of the anther (an) and stigma (st) during pre-anthesis. b. Frontal view of the dehiscent anther (an) and non-receptive stigma (st). c. Frontal view of the anther containing pollen mixed with stigma (st) exudate (ex). d. Lateral view of the anther (an) and stigma (st). e. Lateral view of pollen (po) adhered to the exudate (ex) (anthers were removed). f. Reaction of the stigma fluid with H2O2. Bars: a-f = 1 mm.

to 19 fruits per inflorescence. The percentage of fruiting ranged from 2.56 to 10.2%.

The pollination process in *E. elatior* occurs as follows: when inserting the mouthpart in the search of nectar stored at the base of the corolla tube, the pollinator comes in contact with the pollen mass mixed with stigma fluid that adheres to the mouthpart. During retraction of the mouthpart, the hairs present in the basal region of the anther (Fig. 2i) assist in the cleaning of the mouthpart.

During the observations, bees, butterflies and hummingbirds were recorded visiting the *E. elatior* flowers. The hummingbirds *Thalurania furcata* (male), *Amazilia versicolor* and *Phaethornis pretrei*, bumblebees (*Xylocopa* sp.) and butterflies were the most frequent visitors, which together accounted for 74% of all visits. Bees accounted for 35% of the visits and hummingbirds and butterflies for 27.5% and 11.25%, respectively. Both hummingbirds and bees visited several flowers of the same inflorescence.

Discussion

Morphology and movement of stamen and style

The morphology of congeneric *Etlingera* species is quite similar in terms of vegetative features such as leaf blade color, leaf margin, height of the aerial part of the leaf (8 to 10 m), and striate or reticulate leaf sheath (Chongkraijak *et al.* 2013). On the other hand, the flower morphology of these species is highly variable, including features such as size, shape and color of the inflorescence and infrutescence, labellum length and width, and length, width and angle of stamen opening (Trimanto & Hapsari 2018).

Etlingera elatior has predominantly asexual propagation via rhizomes. The plant develops in the form of clumps that spread over varied distances, its flowering occurs throughout the year, the inflorescences during of 3 to 4 months, the anthesis occur between 5:00 and 6:30 h, and the lifespan of a single flower is of one day. These observations are similar to those reported by Aswani *et al.* (2013)

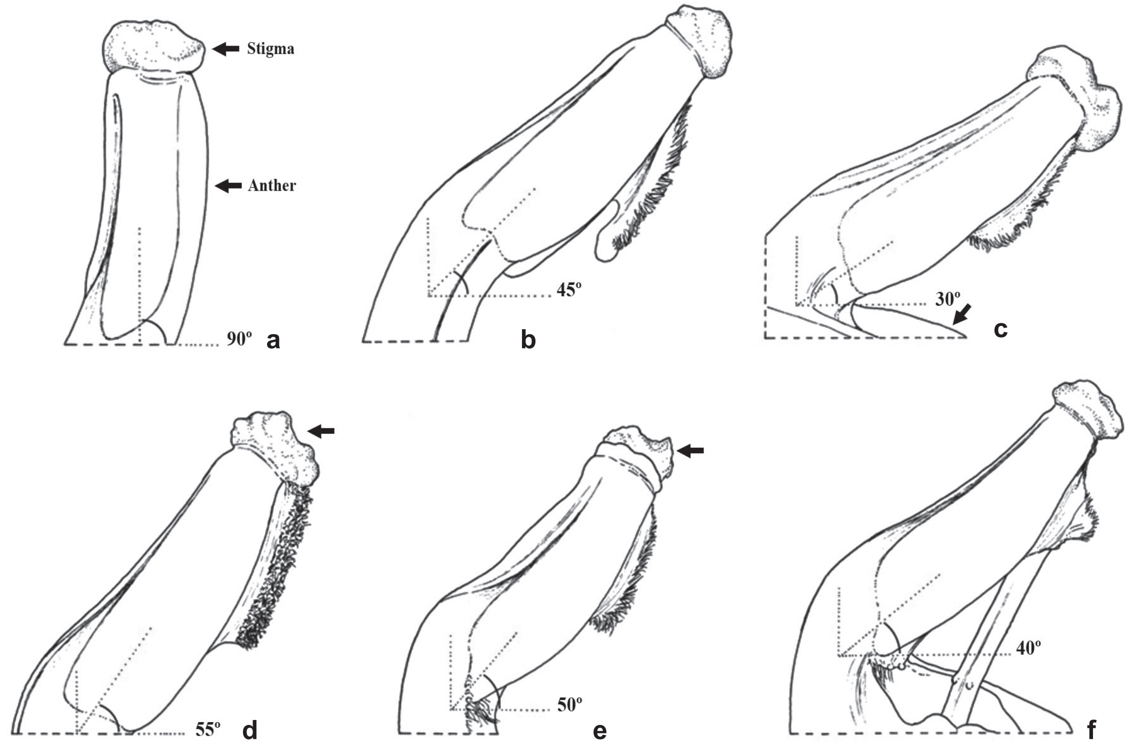

Figure 4 – a-f. Patterns of stamen/style movement in *Etlingera elatior* – a. position of the organs during pre-anthesis (20:00 h of the previous day); b. pre-anthesis and start of stamen/style movement (6:00 h); c. anthesis (10:00 h) and movement in relation to the base of the labellum (arrow); d. anthesis (14:00 h) with initiation of stigmatic fluid formation (arrow); e. anthesis (16:00 h) and stigmatic fluid in the stigma (arrow); f. end of anthesis (20:00 h).

studying the reproductive biology of *E. elatior* and similar to that described for other species of Zingiberaceae (Poulsen 2006; Poulsen *et al.* 2012).

The simultaneous movement of stamen and style during floral anthesis of *E. elatior* cannot be considered a type of flexistyly since it does not exhibit dichogamy. The simultaneous movement of the reproductive organs can influence the transfer of pollen to the pollinator's body, according to the place of deposition and the size of the pollinator (Fan & Li 2012). In botany, the stamen filament has always been used as a taxonomic characteristic that, among other, has contributed to the differentiation of Zingiberaceae species such as *Etlingera littoralis* (Chongkraijak *et al.* 2013), *Etlingera megalocheilos* (Trimanto & Hapsari 2018) and *Etlingera poulsenii* (Leong -Škorničková *et al.* 2016).

The stigma fluid is an important means whereby pollen adheres to the mouthpart of the pollinator. The presence of accessory adhesive substances of the flowers becomes important in situations when the pollen must adhere to smooth surfaces (Moyano *et al.* 2003). Although pollinators such as bees, hummingbirds and butterflies have mouthparts of varying sizes, in our observations it was not possible to state that pollen *plus* stigma fluid were adhering effectively to one possible pollinator as to another.

Histochemical and biochemical analyses of the stigma exudate in Angiosperms have demonstrated a heterogenous composition, including lipids, polysaccharides, and proteins (Wetzstein & Law 2012). The presence of exudate in the region of the stigma has been reported for other Zingiberaceae species such as *Caulokaempferia coenobialis* (Wang *et al.* 2005) and *Roscoea debilis* (Fan & Li 2012) and is considered to facilitate pollen dispersal.

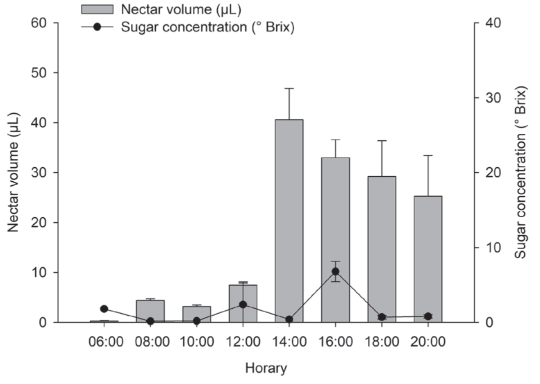

Figure 5 – Nectar volume and sugar concentration during floral anthesis of *Etlingera elatior*.

In *Etlingera elatior*, the nectar produced is stored in a narrow corolla tube, but does not reach the stigma. Our field observations during peak bloom of *E. elatior* in Brazil showed that, if not collected by visitors, the nectar accumulates in the corolla tube and is mixed with pollen and stigma fluid, resulting in two antagonistic pathways: 1) pollen adherence and possible facilitation of their transport, or 2) occlusion of the stigma surface by self-deposition of pollen.

Fructification and pollinators

The fruiting rate in the open pollination treatment of *E. elatior* observed in the present study (1.8–10.2%) was similar to the rate (8%) reported for hand self-pollination treatment of the same species in India and about 70% lower than the rate reported for *Caulokaempferia coenobialis* (Zingiberaceae) (Wang *et al.* 2005).

The absence of spontaneous autogamy of *E. elatior* flowers observed in India was confirmed in the present study conducted in Brazil. Xenogamy seems to be the predominant reproductive system among *Etlingera* sp. According to Kittipanangkul & Ngamriabsakul (2006) and Aswani *et al*. (2013), cross-pollination is the main mechanism required for fruiting in *Etlingera* species. Aswani *et al.* (2013) found no fruit formation by spontaneous self-pollination or apomixis.

The horizontal position of *Etlingera elatior* flowers, their red and orange colors, the tubular perianth, and the abundant and low sugar concentrated nectar are characteristics commonly associated with pollination by birds (Stiles 1981; Johnson & Nicolson 2008). These characteristics are similar to the findings for species of other families typically pollinated by birds, such as species of the family Maranthaceae including *Calatheia timothei*, *C. lutea* and *C. platystachya* (Kennedy 2000; Claßen-Bockhoff & Heller 2008). The vertical arrangement of the bracts of *E. elatior* also serves as a reservoir of rainwater, possibly rendering the inflorescences an important water source for birds.

Nectarinia asiatica, *N. zeylonica*, *Tetragonula irridipennis*, and *Certina* sp. were the most effective pollinators of *E. elatior* in Thailand (Kittipanangkul & Ngamriabsakul 2006; Aswani *et al*. 2013). The pollinators of *Etlingera brevilabris* are *Arachnothera* spp. (Nectariniidae). Spider-hunter birds were found to be pollinators of *E. elatior* in an area of the Khao Nan National Parque. These birds were also reported to be pollinators of three *Etlingera* species, *E.* aff. *metriocheilos*, *E. punicea* and *E.* aff. *brevilaris*, in Borneo (Sakai *et al.* 1999).

Our results indicate movement of the stamen and style during anthesis; however, this movement cannot be characterized as typically flexistyly, since was not observed protogynous or protandrous phase (dichogamy). The synchronous movement of the stamen and style, by itself, was not enough to promote autogamy in the species. The flowers of *E. elatior* visited by bees and birds that promoted pollination and thus ensured fruiting. A more in-depth study is needed to draw conclusions about probable and possible pollinators of this plant.

Acknowledgments

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