Published in final edited form as:

Plant Biol (Stuttg). 2014 May ; 16(3): 659–668. doi:10.1111/plb.12081.

Adaptations for insect-trapping in brood-site pollinated *Colocasias* (Araceae)

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Abstract

The Araceae include both taxa with rewarding and deceptive trap pollination systems. Here we report on a genus in which rewarding and imprisonment of the pollinators co-occur. We studied the pollination of four species of *Colocasia* in Southwest China and investigated the morpho-anatomical adaptations of the spathe related to the attraction and capture of pollinators. All four species were pollinated by drosophilid flies of the genus *Colocasiomyia*. The flies are temporally arrested within the inflorescence and departure is only possible after pollen release. Trapping of the flies is accomplished by the closure of the spathe during anthesis. Moreover, in two species the spathe is covered with papillate epidermal cells known to form slippery surfaces in deceptive traps of Araceae. However, in *Colocasia* the papillae proved not slippery for the flies. The morpho-anatomical properties of the spathe epidermis indicate that it is an elaborate osmophore and serves for the emission of odours only. Despite its similarity to deceptive traps of other aroids, *Colocasia* and *Colocasiomyia* have a close symbiotic relationship, as the attracted flies use the inflorescence as a site for mating and breeding. The trap mechanism has presumably evolved independently in *Colocasia* and is supposed to facilitate more efficient pollen export.

Keywords

Colocasiomyia; deceptive pollination; endoplasmatic reticulum; mutualism; nursery pollination; osmophore

Introduction

Brood-site pollination, with plants providing breeding sites as reward for pollinators, occurs in various angiosperm families (Sakai 2002; Armbruster 2012). Although the relationship between the plants and the pollinators is considered mutualistic, brood-site pollination is also associated with costs for the plant because of the behaviour of the pollinators and their offspring (Pellmyr 1989; Bronstein 2001). Therefore, brood-site offering flowers such as *Ficus* and *Yucca* have evolved adaptations to reduce the detrimental impact of the insects and increase reproductive success (Huth & Pellmyr 2000; Dufay & Anstett 2003).

In the Araceae, brood-site pollination is mainly restricted to taxa of tropical Asia that are associated with drosophilid flies of the genus *Colocasiomyia* (Carson & Okada 1980; Toda & Lakim 2011). These flies have been found breeding in various aroid taxa, but the process of pollination has so far only been studied in *Alocasia*, *Furtadoa* and *Steudnera* (Mori & Okada 2001; Miyake & Yafuso 2005; Takenaka *et al.* 2006; Takenaka Takano *et al.* 2012), while their role in the pollination of *Colocasia* is unknown. *Colocasia* is estimated to comprise about 20 species distributed throughout Southeast Asia, six of which occur in China (Li *et al.* 2010). These species possess a highly synorganised inflorescence consisting of an elaborate spathe and spadix. The spathe (a modified bract) forms a basal spathe tube separated from the expanded spathe blade by a constriction. It surrounds the flower-bearing spadix with pistillate flowers in the lower part, staminate flowers in the upper part, and in some cases sterile parts below and above the staminate flowers (Fig. 1A).

Although the presence of breeding *Colocasiomyia* flies indicates a mutualistic relationship, in some species of *Colocasia* spathe movements (Cleghorn 1913) and papillate epidermal cells on the spathe (Poppinga *et al.* 2010) resembling trapping devices of brood-site mimicking aroids (Bröderbauer *et al.* 2012) have been observed. These taxa attract saprophilous insects by mimicking a brood-site such as dung or carcass without offering rewards and successful breeding is prevented (Dafni 1984; Urru *et al.* 2011). Trapping of insects is caused by papillate surfaces of the spathe epidermis that are slippery and cause the insects to glide into the floral chamber (Knoll 1926; Vogel & Martens 2000). Moreover, in some taxa, spathe movements occlude the floral chamber (Armstrong 1979). Thus, insects are prevented from escape and stay trapped until pollen is released (Vogel 1965).

The aim of the present study is to investigate the role of adaptations for brood-site pollination in inflorescences of *Colocasia* (Araceae) that resemble adaptations for trap pollination present in other taxa of Araceae. We address the following questions: (i) do the breeding *Colocasiomyia* flies act as pollinators; (ii) what is the role of spathe movements and papillate cells forming the adaxial spathe epidermis; and (iii) is the relationship between *Colocasia* and its pollinators mutualistic or has it shifted from rewarding to deceptive trap pollination?

Material and Methods

Pollination ecology

Study site—The reproductive biology of four species of *Colocasia* was studied in and around the Xishuangbanna Tropical Botanical Garden (XTBG; 21°41' N, 101°25' E, 570 m a.s.l.), Menglun, Yunnan Province, China. In this area the climate is seasonal, with most rainfall occurring between May and October. The mean annual precipitation is 1493 mm and the average temperature is 21.8 °C (Cao *et al.* 2006). The area of XTBG and its surroundings was previously covered with tropical seasonal rain forest and tropical montane rain forest, a major part of which has been converted to rubber plantations during the last decades (Zhang & Cao 1995).

Study species—Our study focuses on two species of *Colocasia*, *C. esculenta* and *C. fontanesii*. In addition, we report on two further species (*C. affinis*, *C. lihengiae*) that have

been observed in the field: (i) *C. fontanesii* Schott (synonym *C. antiquorum* Schott). Study site: XTBG. The population in the garden originates from wild collections and grows in the garden in a secondary forest within the species' natural distribution range. (ii) *C. lihengiae* C.L.Long & K.M.Liu. This species has been considered conspecific with *C. fontanesii* by Li *et al.* (2010), but differs in lacking a sterile appendix above the staminate flowers. As the examined population of *C. lihengiae* was very uniform and the specimens never produced an appendix, we consider it to represent a distinct species. Study site: XTBG. The population in the garden originates from wild collections and grows in the garden in a secondary forest within the species' natural distribution range. (iii) *C. esculenta* (L.) Schott. This species is widely cultivated in the tropics as a food crop. It probably originates from Southeast Asia (Matthews & Naing 2005), but the natural distribution range is unknown. Study site: ponds at XTBG. (iv) *C. affinis* Schott. Study site: natural population growing at the forest margin along the old road between the cities of Menglun and Jinghong northwest of XTBG.

Vouchers of the four species studied in the field (*Colocasia affinis* Yinjiantao s.n., *Colocasia esculenta* Yin jiantao1726, *Colocasia fontanesii* C310005, *Colocasia lihengiae* Yin jiantao1728) have been deposited in the herbarium of XTBG (HITBC). Two of the four species studied in the field were available in the living collections of the Botanical Garden of Vienna and were used for the morpho-anatomical analyses. Vouchers of these species have been deposited in the herbarium of the University of Vienna: *Colocasia esculenta* WU0064967, *Colocasia fontanesii* WU0064969. Fieldwork was carried out from 30 June to 19 August 2010 in and around XTBG.

The course of anthesis—The course of anthesis in *C. esculenta* (number of specimens studied = 14), *C. fontanesii* (n = 5) and *C. lihengiae* (n = 4) was recorded on different occasions from 04:00 h until 19:00 h. In *C. affinis*, several inflorescences could be observed on 2 days in August for 1 h each, from 11:00 h to 12:00 h and from 15.30 h to 16.30 h, respectively.

Thermogenesis—Thermogenesis of the spadix was recorded with a combined thermometer and data logger (Scantronik Thermofox Universal) in two inflorescences of *C. fontanesii* and three inflorescences of *C. esculenta*. Three thermocouples were inserted into the appendix, the staminate zone and the central sterile zone. A fourth thermocouple was put close to the inflorescence in order to measure the ambient temperature. Measurements were started before onset of anthesis and were stopped a couple of hours after pollen release. The temperature was recorded every 5 min.

Pollinators and visitors—Pollinators and visitors were collected from inflorescences with nets and stored in 70% ethanol. The collected insects were identified by specialists (see Acknowledgements).

Bagging experiments—Thirteen inflorescences of *C. esculenta* were covered with organza bags prior to anthesis in order to exclude pollinators. The bags were removed after the end of anthesis. Fruit set of these inflorescences was then compared to 17 open-pollinated inflorescences. In addition, fruit set was also checked for eight open-pollinated inflorescences each in *C. fontanesii* and *C. lihengiae*. For all inflorescences, the number of

fertilised and unfertilised ovaries was counted as well as the number of fertilised ovules for ten fruits per inflorescence.

Morphology and anatomy of the spathe

The morphology and anatomy of the spathe was studied from specimens of *C. esculenta*, and *C. fontanesii* cultivated in the glasshouses of the Botanical Garden of Vienna.

Spathe movements—Spathe movements in specimens of *C. esculenta* and *C. fontanesii* were recorded with a Nikon Coolpix P 5000 camera (Nikon, Tokyo, Japan), automatically taking pictures every 10 min.

Odour emission—Inflorescences were submerged in neutral red (1:10000 neutral red:tap water) and checked for staining at 1-h intervals (Vogel 1963; Dobson *et al.* 2005). The sections from the spathe tube and the spathe blade, and the pistillate, staminate and sterile parts of the spadix were enclosed in small vials and checked for odour emission by nose at intervals of 30 min (Vogel 1963).

Scanning electron microscopy (SEM)—Surface morphology of the spathe epidermis was studied with SEM. Samples used for the assessment of cell shape were dehydrated in a graduated series of ethanol and then transferred to acetone. Subsequently, samples were critical point-dried and sputter-coated with gold and investigated with a JEOL JSM6390 SEM at 10 kV. Samples used for the examination of epicuticular wax crystalloids only were air-dried before sputter-coating, as the application of ethanol and heat would alter the crystal structure of the wax (Barthlott & Wollenweber 1981).

Light microscopy (LM)—For investigation under LM, spathes were fixed in FAA for at least 7 days and transferred to ethanol 70% afterwards. Subsequently, samples were dehydrated in a graduated series of ethanol, embedded in 2-hydroxyethyl methacrylate (Kulzer's Technovit 7100, Heraclaeus Kulzer, Hanau, Germany) and cut to 6 µm with a Thermo Scientific rotary microtome (Microm HM355S, Microm, Walldorf, Germany). The sections were stained with Ruthenium red and Toluidine blue. The presence of starch and lipids in fresh spathes of *C. fontanesii* was checked through staining with iodine tincture and Sudan IV.

Transmission electron microscopy (TEM)—Pieces of the spathe from living material of *C. esculenta* and *C. fontanesii* were fixed in 3% glutaraldehyde (GA), post-fixed with 1% osmium tetroxide (OsO₄) and 0.8% potassium hexacyanoferrate (K₄Fe(CN)₆ • 3H₂O). Fixed material was dehydrated in 2,2-dimethoxypropane and then embedded in Agar's low viscosity resin (LV-Resin; Agar Scientific Limited 2004). Sections (60–90-nm thick) were cut with a diamond knife (Diatome Ultra 45°; 3.5 mm) on a Leica Ultracut EM UC6 microtome. For common contrast, the sections were stained with uranyl acetate (U: 1% methanolic solution) followed by lead citrate (Pb: 0.1% solution). The occurrence of polysaccharides was detected with the Thiéry test (Thiery 1967). Presence of lipids was investigated according to the procedure of Rowley & Dahl (1977). All sections were examined with a Zeiss EM 109 TEM at 50 kV.

Results

Pollination ecology

Course of anthesis—In all four species, the spathe is divided into a basal tube and an apical blade, which are separated by a constriction (Fig. 1B). The spathe tube forms the lower floral chamber enclosing the pistillate flowers, while the spathe blade forms the upper floral chamber, enclosing the staminate flowers and the sterile appendix. All species are protogynous, with anthesis lasting for about 24 h. In *C. esculenta*, *C. fontanesii* and *C. lihengiae* the inflorescence opened before dawn concomitantly to the emission of an intense fruity odour with a musty component (Fig. 1B). As shown in the automatic camera recordings, opening started after midnight on the first day of anthesis (Figure S1). Around the same time, the stigmas became wet. During the day, odour emission decreased and the entire spathe gradually closed again until the spathe margins overlapped completely around 17:00 h (Fig. 1C). Between 17:00 and 20:00 h, the spathe constriction closed, thereby occluding the passage between the lower and upper floral chamber. The stigmatic surface of the pistillate flowers decayed and produced large aqueous droplets. During the next morning, between 06:30 and 07:30 h, the pollen was released; at the same time, the spathe blade reopened. In *C. esculenta* the blade only opened in the lower part, while in *C. fontanesii* and *C. lihengiae* the blade reflexed and curled completely within less than 30 min (Fig. 1D). The reflexing and curling of the spathe after pollen release was also observed in *C. affinis* (Fig. 1E).

Pollinators and visitors—The insects most commonly found in inflorescences of the four *Colocasia* species studied were flies of the drosophilid genus *Colocasiomyia* (Fig. 1D). Usually ten to 30 individuals (60 in one inflorescence of *C. fontanesii*) were found per inflorescence. Three drosophilid species (*Colocasiom. alocasiae* (Okada, 1975), *Colocasiom. xenalocasiae* (Okada, 1980), *Colocasiom. sp. 3* aff. *colocasiae*) co-occurred in *C. fontanesii*, *C. lihengiae* and *C. esculenta* (Table 1). Another species, *Colocasiom. steudnerae* (Takenada & Toda, 2006), occurred only rarely in the above three taxa, but was regularly present in inflorescences of *C. affinis* (Fig. 1F). The flies arrived at the onset of anthesis, landed on the outside of the spathe blade and soon walked down into the lower floral chamber. The females oviposited mainly between the pistillate flowers (Fig. 1G). Male and female flies were frequently observed mating inside the inflorescence. Prior to closure of the spathe constriction, the flies moved upwards into the upper floral chamber and assembled on the staminate part of the spadix inside the occluded spathe blade. As the spathe margins overlapped tightly, the insects could not escape from the inflorescence at this stage of anthesis. After pollen extrusion and reopening of the spathe on the second day of anthesis, the drosophilids quickly left the inflorescence; sometimes they first aggregated on the reflexing spathe blade before departing. Fly larvae hatched within the next 24 h and developed between the pistillate and sterile flowers inside the lower floral chamber without damaging the developing fruits (Fig. 1H and I). In none of the species studied were larvae found to occur on the staminate flowers of the spadix.

Another flower visitor was *Aethina humeralis* (Grouvelle, 1890), a nitidulid beetle of the subfamily Nitidulinae (Fig. 1F); this species was, however, only rarely found. In

inflorescences of both *C. esculenta* (n = 14) and *C. lihengiae* (n = 4) only a single beetle was found. In *C. fontanesii* (n = 5) one inflorescence contained three beetles, two inflorescences a single beetle and two inflorescences none. The beetles moved around within the inflorescence, but in contrast to the drosophilid flies, they were never observed mating or laying eggs. After pollen extrusion, they fed on pollen and then left the inflorescence. Based on our short observations, beetles seem to be present more regularly in *C. affinis*, but as in the other *Colocasia* species, also only in low numbers per inflorescence.

Regular visitors of the anthetic inflorescences of all *Colocasia* species were an unidentified species of the Chrysopidae (Neuroptera; Fig. 1D) and an unidentified fly of the genus *Bactrocera* (Tephritidae; Fig. 1J). These insects were apparently attracted by the intense odour and usually arrived before dawn. They assembled on the outside of the spathe blade, but never entered the inside of the spathe and thus did not contact the flowers.

Bagging experiments—The inflorescences of *C. esculenta* contained on average 184 ± 27 pistillate flowers (number of specimens studied = 17), of which 26% produced fruits after open-pollination. Pollinated ovaries contained 2.2 ± 2.5 fertilised ovules. In bagged inflorescences (n = 13), one inflorescence was aborted as a whole, and in the remaining specimens only 0.7% of the flowers produced fruits. In open-pollinated inflorescences of *C. fontanesii* (n = 8) 169 ± 28 pistillate flowers were present; of these, 85% produced fruits that contained 16.6 ± 12.1 fertilised ovules per ovary. *Colocasia lihengiae* (n = 8) contained 162 ± 25 pistillate flowers, of which 81% produced fruits after open pollination. Pollinated ovaries contained 11.2 ± 11.8 fertilised ovules on average.

Thermogenesis—Thermogenesis in *C. esculenta* and *C. fontanesii* occurred during the pistillate and the staminate phase of anthesis (Fig. 2). The thermogenic pattern was consistent in the different specimens of the respective species studied. In both species, peaks of heat production occurred in the appendix and the staminate flowers during the first and the second morning, reaching 6–8 °C above the ambient temperature. In the second morning, heat was mainly produced by the staminate flowers. *C. esculenta* differed from *C. fontanesii* by the presence of a third but weaker phase of heat production in the afternoon of the first day of anthesis (Fig. 2A).

Morphology and anatomy of the spathe

In both *C. esculenta* and *C. fontanesii*, the whole inside of the spathe (i.e. the adaxial epidermis) stained more or less uniformly red after embedding in neutral red. The outer (abaxial) epidermis of the spathe blade showed at least a weak staining reaction.

Odour emission—Odour emission by the spathe could be detected in *C. esculenta* and *C. fontanesii* through smelling stored samples in separate glass vials. The spathe blade in particular produced a strong sweet to musty smell. In the spadix, the staminate flowers as well as the sterile appendix served as osmophores, emitting very intense odours. The appendix mainly emitted a strong musty to sweet odour, while the staminate flowers first smelled musty and in a later stage of anthesis often produced a foul smell.

Scanning electron microscopy—In both *C. esculenta* and *C. fontanesii* the adaxial (inner) epidermis of the spathe blade consisted of densely packed papillate cells (Fig. 3A) that collapsed after anthesis, while the epidermal cells of the tube were tabular to convex (Fig. 3B). The abaxial epidermal layer of the spathe blade consisted of papillate cells bearing cuticular folds, covered with wax platelets (Fig. 3C). These papillae were less densely packed than the papillae of the adaxial spathe epidermis. Moreover, they did not collapse after anthesis.

Light microscopy—After staining of fresh spathes of *C. fontanesii* with Sudan IV, lipids could be detected in the epidermis but not in the parenchyma cells. As indicated by staining with iodine tincture, starch was common in most cells of the spathe tube and very abundant in the spathe blade.

Cross-sections of embedded spathe blades showed that the papillate cells of the adaxial epidermis in *C. esculenta* and *C. fontanesii* contained dense cytoplasm. In *C. esculenta* dense cytoplasm was also present in parenchyma cells adjoining the abaxial epidermis of the spathe blade during the pistillate phase of anthesis (Fig. 3D). In both species, the mesophyll of the spathe blade contained lacunar tissue (Fig. 3D). In the spathe tube, lacunar tissue was less widespread and the lacunae were often filled with mucilage (Fig. 3E).

Transmission electron microscopy—In *C. esculenta* and *C. fontanesii*, epidermal papillae of the adaxial spathe tube contained a dense cytoplasm and high intracellular activity (Fig. 3F). Besides the presence of amyloplasts and lipids, the cells contained numerous mitochondria, dictyosomes, polyribosomes and smooth endoplasmic reticulum (sER; Fig. 3G). Moreover, vesicles were transported from the cell to the cuticle (Fig. 3H). Similar activity was also observed but to a lesser extent in epidermal cells of the abaxial spathe blade (Fig. 3I) and the adaxial spathe tube. Amyloplasts were particularly abundant in the parenchyma cells of the spathe blade (Fig. 3J). Moreover, in papillate cells of *C. fontanesii* unusual gorgon-head-shaped endoplasmic reticulum (ER), not documented so far to the best of our knowledge, was common (Fig. 3K). After anthesis, the number of organelles and the overall cytological activity within the papillae decreased significantly (Fig. 3L).

Discussion

Course of anthesis

Protogyny is a consistent feature of Araceae (Mayo *et al.* 1997) and also occurs in *Colocasia*. Moreover, as in most taxa of the large subfamily Aroideae, anthesis lasts for 2 days only (Armstrong 1979; Chouteau *et al.* 2007a; Barabé *et al.* 2008; Takenaka Takano *et al.* 2012). In three of the four *Colocasia* species studied in the field (i.e. *C. esculenta*, *C. fontanesii*, *C. lihengiae*) the attraction of pollinators and pollen extrusion occur during the early morning hours, as is also the case in other *Colocasiomyia*-pollinated Araceae, whereas in aroids pollinated by scarab beetles the major events occur at dusk (Gibernau *et al.* 2000; Maia & Schlindwein 2006; Takenaka *et al.* 2006). As *C. affinis* appears to also be pollinated by drosophilid flies, the deviating timing of anthesis – pollen release and curling of the

spathe blade take place during the afternoon - is very unusual, but the precise reason is unknown.

Thermogenesis—Thermogenesis is a common phenomenon in Araceae (Barthlott *et al.* 2009; Seymour *et al.* 2009). It enhances odour emission and serves as a heat reward for departing beetles, facilitating their warm-up before takeoff (Seymour *et al.* 2003a). In *C. esculenta* and *C. fontanesii* the peaks of heat production occur in the first and second morning of anthesis. In the first morning, when the spathe opens and a strong odour is emitted, both the appendix and the staminate flowers warm up to 6–8 °C above ambient temperatures. During the second morning, heat production is mostly from the staminate flowers. Odour emission is very weak in the staminate phase inflorescences and new insect visitors are not attracted. Thus, the second temperature peak might serve as a heat reward that attracts the insects retained within the spathe and guides them to the staminate flowers extruding pollen. Simultaneously, the heat possibly stimulates the flies to leave inflorescence after their body temperature has reached optimum. However, such a heat reward has not yet been proved in flies (Seymour *et al.* 2003b) and its occurrence in *Colocasia* remains doubtful. In *C. esculenta*, an additional but weaker – thermogenic phase takes place in the afternoon of the first day of anthesis. This intermediate peak was not observed in inflorescences of *C. esculenta* cultivated on Vanuatu (Ivancic *et al.* 2004). However, the difference could be simply a consequence of the high genetic variance in *C. esculenta*, which is especially prominent between cultivars of the Asian and Pacific regions (Kreike *et al.* 2004). Although many Araceae have two phases of heat production during anthesis, thermogenesis with more than two temperature peaks – as observed in the specimens of *C. esculenta* studied – is also known from other taxa, such as *Syngonium angustatum* Schott and *Arum* spp. (Gibernau *et al.* 2004; Chouteau *et al.* 2007a).

Spathe movements—Spathe movements are known from many aroids. In some taxa they serve as protection for developing fruits (Mayo *et al.* 1997), in others they enable the arrest of pollinators (Dakwale & Bhatnagar 1985; Bröderbauer *et al.* 2012). In the former case, the spathe constriction closes after anthesis, thereby secluding the spathe tube containing the pollinated pistillate flowers. In the latter case, *e.g.* in deceptive traps such as *Typhonium* and *Therriophorum*, the narrowing of the constriction occurs during the pistillate phase in order to arrest the insects inside the spathe tube (Armstrong 1979; Dakwale & Bhatnagar 1997). In contrast, in *Colocasia* it is not the constriction but the spathe blade that closes again during the pistillate phase of anthesis, thereby occluding the entire spadix and causing the trapping of pollinators. The constriction only closes in a later stage of anthesis. Such movements have so far only been observed in *Colocasia* and in taxa of the tribe Schismatoglottideae (Cleghorn 1913; Boyce & Wong 2007; Ulrich *et al.* 2012).

Except for *C. esculenta*, the spathe blades of the *Colocasia* species studied reflex very quickly after pollen release, similar to some schismatoglottids (Boyce & Wong 2007). The upper part of the spadix is thereby exposed within a few minutes. We hypothesise that this spathe movement serves to stimulate the pollinators to leave the inflorescence quickly, as their shelter – formed by the spathe blade – vanishes.

Pollinators and visitors

Pollinators—Flies of the genus *Colocasiomyia* were the most common insects present in each inflorescence of *Colocasia* during our study. All four species belonged to the *Colocasiom. cristata* species group. Three of the four species (i.e. *Colocasiom. alocasiae*, *Colocasiom. xenalocasiae*, *Colocasiom. sp. 3 aff. colocasiae*) visited inflorescences of all except *C. affinis*. *Colocasiom. sp. 3 aff. colocasiae* is so far known to visit *C. esculenta* in Vietnam (Toda, personal communication). *Colocasiom. alocasiae* and *Colocasiom. xenalocasiae* are known as pollinators of *Alocasia odora* and *A. cucullata* (Yafuso 1994). In our study, *Colocasiom. xenalocasiae* was the most common species and appears to be the main pollinator, at least in *C. esculenta* and *C. lihengiae*.

As far as we can conclude from our short observations, *C. affinis* was only visited by *Colocasiom. steudnerae*, which was otherwise only rarely found in *C. esculenta* and *C. fontanesii*; *Colocasiom. steudnerae* is known to be the main pollinator of *Steudnera colocasiifolia* K.Koch in XTBG (Takenaka *et al.* 2006). Contrary to the species of *Colocasia* studied in XTBG, *S. colocasiifolia* flowers from March to April. Thus, it is probable that *Colocasiom. steudnerae* switches its hosts during different times of the year (Toda, personal communication). In contrast to *Steudnera colocasiifolia* and other *Colocasia* species with pollen release occurring during the morning hours, in *C. affinis* pollen is released in the afternoon. The different timing possibly serves as a reproductive isolation mechanism from sympatric *Colocasia* species flowering at the same time of the year.

As in *A. odora*, drosophilids visiting the different species of *Colocasia* were observed to remain in the spathe tube at the beginning of anthesis, laying eggs mainly between the pistillate flowers (Miyake & Yafuso 2005). After the drosophilid flies have oviposited, larvae quickly hatched (usually within a day). The sterile flowers situated between and/or below the pistillate flowers decayed and formed a mucilaginous substrate for the larvae. In inflorescences of *C. esculenta* from which flies were excluded with organza bags, these sterile flowers remained intact, indicating that their decay is caused by activity of the larvae. The fertile pistillate flowers remained undamaged, unlike well-known examples of brood-site pollination such as in *Ficus* or *Yucca* (Armbruster 2012). The larvae did not leave the inflorescence before pupation; as in *Alocasia macrorrhizos*, they developed within the ripening infructescences (Takenaka Takano *et al.* 2012).

Unlike other aroids, oviposition of the different drosophilid species only took place in the pistillate zone of the spadix, and the larvae remained there during development. In contrast, several drosophilid species are known to form synhospitalic pairs using the same inflorescence as brood site, but one species breeds in the pistillate part of the spadix while the other uses the staminate part (Yafuso & Okada 1990). Such behaviour has also been observed in *Colocasiom. alocasiae* (staminate zone) and *Colocasiom. xenalocasiae* (pistillate zone) breeding on *A. odora* (Yafuso 1994). Despite the presence of *Colocasiom. alocasiae* we did not find eggs or larvae on the staminate part of the spadix of the *Colocasia* species studied. As *Colocasiom. alocasiae* has also been found breeding in the pistillate zone of *A. cucullata* (Miyake & Yafuso 2005), the switch to the staminate zone might be a facultative behaviour. Breeding of two synhospitalic species in the pistillate region of the spadix has

also been recorded in *A. macrorrhizos* (Takenaka Takano *et al.* 2012). Therefore, the absence of resource partitioning in our species could indicate that this is facultative behaviour, which could depend on host identity or other environmental factors.

Bagging experiments—Because of their abundance and behaviour, flies of the genus *Colocasiomyia* appear to be the most important pollinators of *Colocasia*. Our bagging experiment with *C. esculenta* proves that inflorescences do not produce fruit unless visited by *Colocasiomyia* spp. Seed set in open-pollinated *C. fontanesii* and *C. lihengiae* was even higher than in *C. esculenta*. This might be due to the fact that many inflorescences of *C. esculenta* produced only low amounts of pollen, which is probably related to selection for vegetative traits affecting reproductive traits during human cultivation.

The presence of protogyny prevents self-fertilisation in *Colocasia*. Moreover, the spathe constriction above the pistillate flowers closes before pollen extrusion, thereby preventing pollen from falling onto stigmas. In general, autogamy is uncommon in Araceae while geitonogamy has been observed in some taxa (Mayo *et al.* 1997).

Mutualism versus antagonism—*Colocasia* and *Colocasiomyia* display a very intimate pollination mutualism in which the inflorescences of *Colocasia* serve as breeding and mating sites for the flies. Despite the fact that the flies obtain rewards for their pollination services, they are also arrested in *C. esculenta*, *C. fontanesii* and *C. lihengiae*. The reason for trapping insects in *Colocasia* is not fully understood, but its resemblance to trap mechanisms in Araceae that mimic a brood site and deceive their pollinators is remarkable. According to ancestral state reconstructions, the trap mechanism has evolved independently in *Colocasia* (Bröderbauer *et al.* 2012). In general, the convergent evolution of trap pollination in different clades of Araceae has probably been facilitated by protogyny (Bröderbauer *et al.* 2012). Retention mechanisms (*i.e.* closure of the flower during anthesis) have also evolved in other protogynous lineages, mainly within the ‘basal angiosperms’ such as *Calycanthus* (Grant 1950) or *Magnolia* (Gottsberger *et al.* 2012). Trapping is necessary in protogynous species without rewards in order to retain pollinators until pollen is released in the staminate phase of anthesis. But why do rewarding protogynous species retain their pollinators? A possible explanation is that rewards may only be present for a restricted period of time. For example, in *Arum creticum* bees are rewarded with pollen during the staminate phase and can enter and leave the inflorescence unhindered while collecting pollen. However, they are arrested in the reward-free pistillate phase in order to secure fertilisation (*i.e.* female reproductive success; Diaz & Kite 2006). In contrast, in *Colocasia* the reward (*i.e.* the brood site) is only available during the pistillate phase. During this time the spathe remains open; however, later the spathe blade closes and the flies are trapped. Moreover, before the onset of the staminate phase, the spathe constriction narrows and occludes the lower floral chamber. Before this occlusion, flies must progress into the upper floral chamber containing the staminate flowers. We postulate that the trapping of the pollinators may be necessary in *Colocasia* in order to secure their presence until pollen is released. The combination of trapping and the rapid reflexing of the spathe blade increases the probability that flies will depart at the right time (*i.e.* during pollen release), whereby more efficient pollen transfer is

enabled. Consequently, adaptations for the retention and release of pollinators in *Colocasias* have probably evolved in order to increase male rather than female reproductive success.

In other taxa pollinated by drosophilids, e.g. species of *Alocasia*, the spathe does not close during anthesis (Miyake & Yafuso 2005; Takenaka Takano *et al.* 2012). Nevertheless, the drosophilids remain inside the inflorescence. Trapping is thus probably not the sole factor controlling the successful pollen export by drosophilid flies in *Colocasias* and other aroids.

Visitors—While the regularly observed *Bactrocera* flies (Tephritidae) and lacewings (Chrysopidae) can be excluded as pollinators as they never enter the inflorescences, the situation is different with the nitidulid beetle *Aethina humeralis* (subfamily Nitidulinae). Nitidulidae are known as pollinators in various plant families, including Annonaceae (Corlett 2004; Teichert *et al.* 2011), Araceae (Nunez *et al.* 2005; Fava *et al.* 2011), Magnoliaceae (Ishida 1996) and Cycadales (Kono & Tobe 2007; Proches & Johnson 2009). As in *Colocasias*, many of these taxa have flowers/inflorescences forming a pollination chamber and/or producing a fruity odour and heat. Moreover, a close relative of *A. humeralis*, *A. concolor* (Macleay, 1872), has been observed to visit *Gossypium tomentosum* on Hawaii (Burraston *et al.* 2005). In Araceae, nitidulids have been found on inflorescences of several genera (e.g. *Amorphophallus*, *Cyrtosperma*, *Typhonium* and *Urospatha*; Gibernau 2003; Punekar & Kumaran 2010) but information on their behaviour remains scarce. Chouteau *et al.* (2007b) show that the nitidulid pollinator *Colopeterus amputatus* uses inflorescences of *Monstera obliqua* as mating sites and feeds on the pollen during the staminate phase of anthesis.

Aethina humeralis behaved similarly to the drosophilids in inflorescences of *Colocasias*. It entered the inflorescence on the first morning of anthesis and departed only after pollen release on the second morning. During the staminate phase of anthesis, the beetles were observed to feed on pollen; pollen grains were also found on the beetle's body during investigations with light microscopy. Thus, the beetle might successfully transfer pollen between inflorescences. We did not find the beetle's eggs or larvae in the inflorescence, but it is possible that it might oviposit in the inflorescences and the larvae hatch there (Kirejtshuk, personal communication). Nevertheless, an important role as pollinator of *Colocasias* spp. seems unlikely because of its low abundance, at least in the season in which our observations were recorded. However, further observations are needed to examine the activities of *A. humeralis* in the inflorescences of *Colocasias*.

Morphology and anatomy of the spathe

In the two species of *Colocasias* examined for odour emission, the spathe serves as an osmophore. Odour production in Araceae is generally associated with the spadix, in later diverging clades in particular, with its sterile appendix (Vogel 1963). Nevertheless, odour production by the spathe has been recorded in several taxa (Vogel 1978; Patt *et al.* 1995; Zhu & Croat 2004). The main energy supply for odour synthesis in spathes of *Colocasias* appears to be starch, which is stored in epidermal and parenchyma cells. Lipids, known to be an important resource for odour production in other angiosperms (Hadacek & Weber 2002; Wiemer *et al.* 2009; Pansarin & Pansarin 2011), were also present but less abundant. Unlike

other osmophores (Vogel 1963), in spathes of *Colocasia* there are no specialised cell layers for the storage of starch, which is distributed in parenchyma and epidermal cells. The intense osmophore activity was most obvious in the papillate cells of the adaxial epidermis of the spathe blade in *C. esculenta* and *C. fontanesii*. These cells contain numerous mitochondria, sER, ribosomes, polyribosomes and vesicles that are transported through the cuticle. Especially in the papillate cells of *C. fontanesii* we also found unusual gorgon-head-shaped ER that appears to be associated with synthesis of odour compounds.

Papillate cells on the adaxial side of the spathe are known from several taxa of Araceae, as well as other angiosperms, where they form slippery surfaces that aid in the capture of pollinating insects (Poppinga *et al.* 2010). In contrast to these cells, papillae of *C. esculenta* and *C. fontanesii* do not point downwards. The drosophilid flies and the nitidulid beetles observed in the field were able to move along the adaxial epidermis. Therefore, we conclude that in *C. esculenta* and *C. fontanesii* the papillate cells serve only as osmophores. However, a common origin of papillate slippery surfaces and osmophoric epidermal cells in spathes of Araceae is possible, as slippery surfaces in several aroids (*e.g. Arum*, *Typhonium*) also produce odour (Bröderbauer *et al.* 2012).

The abaxial spathe blade also seemed to act as an osmophore, showing similar albeit weaker intracellular activity compared to the adaxial epidermal cells. Concordantly, osmophoric activity was only indicated as a weak staining with neutral red in both species. We conclude that the spathe emits odours in different parts, probably at varying intensity. Thus, arriving insects might be guided by an odour gradient from the outside to the inside of the inflorescence. Whether odour gradients might also be important in influencing the spatial distribution of insects within the inflorescence is unclear. It has been shown that the odour compounds produced by the spathe or specialised sterile organs can differ from those produced by the spadix (Hadacek & Weber 2002; Kakishima *et al.* 2011). These different odours might influence the behaviour and spatial distribution of pollinators and thereby cause more efficient pollen transfer. Possibly, such an odour gradient, in combination with the second thermogenic peak in the staminate flowers, stimulates flies in *Colocasia* to leave the spathe tube and move to the staminate flowers prior to the closure of the spathe constriction and pollen extrusion. Such an effect of odour on the behaviour of pollinators has already been found in the aroid *Peltandra virginica*, where flies either oviposit or feed on pollen, depending on the varying concentrations of odour compounds emitted by the spathe (Patt *et al.* 1995).

A prominent feature in the spathe blades of *C. esculenta* and *C. fontanesii* was the presence of aerenchyma-like lacunar tissue. Such tissue is known from the specialised osmophoric appendices in several members of Araceae (Vogel 1963). The intercellular spaces are thought to be important to provide oxygen for respiration during thermogenesis, thereby fuelling the odour emission (Seymour *et al.* 2009). The presence of such tissue in *Colocasia* indicates that in Araceae not only the spadix but also the spathe can be a highly elaborate osmophore.

Supporting Information

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank two anonymous reviewers for helpful comments. We also thank J.T. Yin (XTBG, China) for support during our fieldwork in XTBG; M.J. Toda (Hokkaido University, Japan), P. Sehnal (Natural History Museum, Austria), A. Kirejtshuk (Russian Academy of Sciences, Russia) and S. Hisamatsu (Louisiana State Arthropod Museum, USA) for insect identification, Y. Staedler for correction of English. The research was funded by the Austrian Science Fund (FWF): P20666-B03.

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Fig. 1.

Inflorescences and insect visitors of *Colocasia* spp. A: *C. esculenta*, spadix; a = pistillate and sterile flowers, b = intermediate sterile flowers, c = staminate flowers, d = sterile appendix. Note that parts of the spathe blade (B) and the spathe tube (T) were removed for better visibility of the spadix. B: *C. fontanesii*, inflorescence during pistillate phase of anthesis. Note that the yellow spathe blade is separated from the green spathe tube by a constriction (arrowhead). C: *C. esculenta*, spathe closure during anthesis. D: *C. lihengiae*, inflorescence during the staminate phase of anthesis visited by *Colocasiomyia* spp. (Drosophilidae) and a neuropterid species (Chloropidae). Note that the margins of the spathe blade break (arrowhead) and the blade bends back; the spadix lacks an appendix. E: *C. affinis*, inflorescence after anthesis with the spathe blade furled. F: *Colocasiomyia steudnerae* (Drosophilidae) and *Aethina humeralis* (Nitidulidae) on the spadix of *Colocasia affinis*. G: Egg (arrowhead) of *Colocasiomyia* sp. on the pistillate flowers of *Colocasia esculenta*. H: Larva of *Colocasiomyia* sp. on the pistillate flowers of *Colocasia lihengiae*. I: Pupa of *Colocasiomyia* sp. on the pistillate flowers of *Colocasia esculenta*. J: *Bactrocera* sp. (Tephritidae) and *Colocasiomyia* spp. on the inflorescence of *Colocasia fontanesii*.

Fig. 2. Heat production during anthesis in different zones of the spadix relative to the ambient temperature A: *Colocasia esculenta*. B: *Colocasia fontanesii*.

Fig. 3.

Morphology and anatomy of the spathe in *Colocasia*. A: *C. esculenta*, adaxial epidermis of the spathe blade with densely packed papillate cells. SEM. B: *C. fontanesii*, adaxial epidermis of the spathe tube with tabular to convex cells. SEM. C: *C. fontanesii*, abaxial epidermis of the spathe blade. Note that the papillate cells bear cuticular folds and are covered with wax platelets. Also note that the cells have shrunk due to drying. SEM. D: *C. esculenta*, cross-section of the spathe blade during the pistillate phase of anthesis. Note the dense cytoplasm (DC) in cells of the abaxial part of the spathe and the lacunar tissue

(asterisks) in the mesophyll. LM. E: *C. esculenta*, cross-section of the spathe tube in the pistillate phase of anthesis. Note the lacunar tissue in the mesophyll filled with mucilage (asterisks). LM. F: *C. fontanesii*, papilla of the adaxial spathe epidermis. Note the dense cytoplasm and high intracellular activity (asterisk). TEM, U + Pb. G: *C. fontanesii*, detail of a papilla of the adaxial spathe blade during the pistillate phase of anthesis. Abbreviations: lipid droplets (L), mitochondria (M), dictyosomes (D), polyribosomes (arrowhead), vesicles (arrow), and smooth endoplasmic reticulum (sER). TEM, U + Pb. H: Detail of G: vesicles (arrow) are transported from the cell to the cuticle (C). TEM, U + Pb. I: *C. fontanesii*, detail of a papilla of the abaxial spathe blade during the pistillate phase of anthesis. Vesicles are transported from the cell to the cuticle (C) via multivesicular bodies (arrow). Abbreviations: mitochondria (M), dictyosomes (D), and smooth endoplasmic reticulum (sER). J: *C. esculenta*, amyloplasts appear dark (electron dense) in the parenchyma cells of the spathe blade adjoining the adaxial epidermis. TEM, Thÿery test. K: *C. fontanesii*, irregular shaped ER (arrowhead) in the adaxial epidermis of the spathe blade during the pistillate phase of anthesis. Note the transport of cell compounds through the cell wall (asterisk) *via* plasmodesmata (arrows). TEM, Lipid test. L: *C. fontanesii*, papilla on the adaxial spathe blade after anthesis. Note the large vacuole (V) and the low intracellular activity (asterisk). TEM, U + Pb. Scale bars = 100 µm (D, E), 10 µm (A–C, J), 1 µm (F–I, K, L).

Table 1Species distribution of *Colocasiomyia* spp. in inflorescences of *Colocasia* spp.

species (n)	<i>Colocasiom. steudnerae</i> n(f)	<i>Colocasiom. alocasiae</i> n(f)	<i>Colocasiom. xenalocasiae</i> n(f)	<i>Colocasiom. sp. 3 aff. colocasiae</i> n(f)
<i>Colocasia affinis affinis</i> (3)	29 (10)	0	0	0
<i>Colocasia esculenta</i> (14)	3 (1)	20 (10)	255 (190)	17 (13)
<i>Colocasia fontanesii</i> (4)	1 (0)	6 (1)	67 (48)	36 (23)
<i>Colocasia lihengiae</i> (3)	0	2 (2)	38 (27)	3 (3)

n = number of specimens; f = number of female specimens.