DAVIS EXPEDITION FUND

REPORT ON EXPEDITION / PROJECT

Expedition/Project Title:	Floral colour change and the attraction of insect flower visitors in the invasive herb *Ageratum conyzoides* in Amani Nature Reserve, Tanzania
Travel Dates:	23 June 2017 – 4 August 2017
Location:	Amani Nature Reserve, Tanzania
Group Members:	Galina M Jönsson
Aims:	(1) Test whether insect pollinators show a preference towards blue or white flowers of *A. conyzoides* and (2) test whether floral colour change is driven by pollination or removal of stamens.

Outcome (not less than 300 words):-

Floral colour change and the attraction of insect flower visitors in the invasive herb *Ageratum conyzoides* in Amani Nature Reserve, Tanzania

INTRODUCTION

Increased human activities are contributing to nonindigenous species invading new geographical areas at unprecedented rates, which is posing a serious threat to biodiversity and human welfare worldwide (Mooney *et al*., 2005; Pimentel *at al*., 2005). Plant invasions in particular have been linked to the disruption of ecosystem structure and function (Hobbs and Humbhries, 1995; Dukes and Mooney, 2004; D'Antonio and Hobbie, 2005), ultimately making them an important component of global environmental change (Theoharides and Dukes, 2007). Pollination is fundamental for the sexual reproduction of many angiosperm species and therefore plays a major ecological role. This plant-animal mutualism has been suggested as a key reason for the observed diversity and success of angiosperms (Pellmyr, 1992); however, since pollinators can also aid in the reproductive success of invasive plants, it has also been proposed to act as a facilitator for invasive nonindigenous plant species (INIPS). Brown *et al.* (2002) found that the INIPS *Lythrum salicaria* reduces the pollinator visitations of its native sister species *L. alatum* while Bartomeus *et al.* (2008) showed that INIPSs with large conspicuous flowers can both facilitate and compete with native species for pollinators. These studies demonstrate that pollinator responses to INIPSs are unpredictable, and further, underline the importance of studying the pollinator ecology of INIPSs in their new ranges to allow for informed conservation decisions.

Floral colours act as visual signals that allow plants to evolve adaptive strategies to increase their attractiveness to pollinators by advertising the quantity and quality of floral rewards (Melendez-Ackerman *et al*., 1997; Aragón and Ackerman, 2004). Colours do not only differ between species of flowering plants or between and within populations, but floral colour change (FCC; a sequential change in the colouration of fully turgid flowers during the flower life, disregarding darkening or fading of floral colouration) has been observed in more than 450 species from 78 families (Weiss, 1995; Weiss and Lamont, 1997; Willmer *et al.*, 2009). Pollinators act as agents of directional selection on floral colouration and multiple non-mutually exclusive hypotheses have been proposed to explain the functional significance of FCC (summarised by Yan *et al*., 2016). Three hypotheses include, firstly, since pre-change colours are usually associated with higher floral

rewards, FCC involving the retention of older flowers increases distant pollinator attraction while at a close range, it provides an honest visual signal for pollinators to avoid flowers that have already been pollinated and therefore carry less floral rewards (Weiss, 1995; Larson, and Barrett, 1999; Oberrath, 1999), secondly, that different pollinators are attracted to different floral colour stages (Yan et al., 2016), and thirdly, that pollen transfer among flowers of the same plant is reduced by discouraging pollinators to stay on plants where flowers have been pollinated and thus changed to a less attractive colour (Jones and Cruzan, 1999; Ida and Kudo, 2003).

Ageratum conyzoides L. is an annual herb that shows FCC from blue to white (Kaur *et al*., 2012). However, to my knowledge, no work has identified drivers of this colour change, nor whether flower visitors (FVs) show a preference for either colour in the species. *A. conyzoides* is native to Central America and the Caribbean (Xuan *et al*., 2004) but occurs as an invasive species in large parts of the world, including Tanzania where it is considered a serious INIPS of tropical forests (Hewood, 1993). This study took place in the Amani Nature Reserve in the East Usambara Mountains (Tanzania), which are part of the Eastern Arc Mountains. The Eastern Arc Mountains is one of the most species-rich areas for its size globally, and has the highest proportion of endemic species in East Africa (Newmark, 2002). However, due to human activities such as deforestation and the introduction of invasive species, 90% of bird species and 52% of tree species in the Eastern Arc tropical forests were considered critically endangered, endangered or vulnerable as of 1996 (IUCN).

This study aimed to (1) test whether insect pollinators show a preference towards either blue or white flowers of *A. conyzoides* by comparing flower visitor (FV) abundance and taxonomic richness, and to (2) test whether floral colour change is driven by pollination or removal of stamens. I expect insect pollinator diversity and abundance to be higher at blue flowers as this is in line with previous work suggesting that the initial flower colour should be most attractive to pollinators.

METHODS

Study species

Ageratum conyzoides L., is an annual herb, native to Central America and the Caribbean (Xuan *et al*., 2004 (Bhatt)) but it occurs as an invasive species in the Hawaiian islands, South America, South East Asia, Australia, West-, South- and East Africa (Hewood, 1993; Kong *et al*., 2004; Sankaran, 2007). *A conyzoides* has great invasive potential, partly due to fast propagation, strong competitive ability, shade tolerance, allelopathic properties, and its ability to grow in a wide range of soils and soil pH (Rodriguez and Capero, 1984; Kong *et al.*, 1999; Batish *et al*., 2009a, b). Its great invasive potential contributes to why it is considered a serious weed of cultivated lands, grasslands, tropical forests and wetlands in most of its invasive range, causing substantial economical and ecological costs (summarised by Kaur *et al*., 2012). As a member of the Asteraceae family, *A conyzoides* has inflorescences composed of florets, and the inflorescences together form corymbs (Fig 1). The colour of the florets of one inflorescence is all blue, all white or all brown (seed produced). Inflorescence colouration can vary within individuals such that completely blue, completely white or mixed corymbs exist.

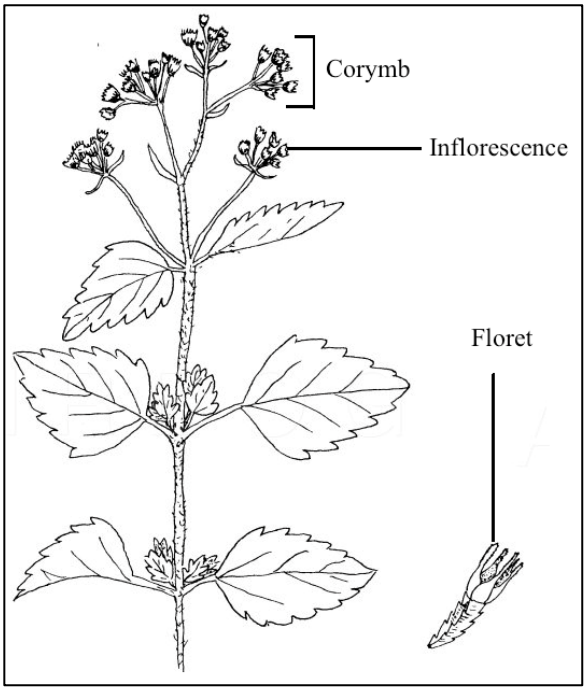

Fig 1: Annotated diagram of *Ageratum conyzoides* showing the position of an inflorescence, a corymb and a separate diagram of one floret. Adapted from Sauerborn and Sauerborn, 1985.

Sample areas

The present study was conducted in the Amani Nature Reserve in the East Usambara Mountains, which are part of the Eastern Arc Mountains, between 16 and 22 July 2017. The experiment was conducted in two habitats, one wetland area (Swamp; N0458854, E9436706) and one garden used to grow cinnamon (*Cinnamomum* sp.) in low densities (Garden; N0458745, E9435983). In each habitat, focal plant individuals with different corymb colourations (blue, white and mixed inflorescences) were selected (see table 1 for sample sizes).

Table 1: Sample sizes (number of individual plants) of blue, mixed and white plants in the two habitat types: garden and swamp. Numbers in brackets indicate the number of observations per plant colour and habitat.

	Plant colour		
	Blue	Mixed	White
Garden	4 (53)	6 (52)	4 (53)
Swamp	6 (45)	4 (45)	4 (44)

Drivers of inflorescence colour change

Three corymbs were randomly chosen on each focal plant and ascribed to one of the treatments: artificially pollinated, stamens removed, or control. I artificially pollinated corymbs by transferring pollen from separate individuals to each inflorescence using a paintbrush, stamens were removed by using forceps and control corymbs were not manipulated. Florets of each treatment were counted and their initial colour recorded before I covered the corymbs with mosquito mesh to prevent FVs from accessing the inflorescences. The number and colour of inflorescences in all treatments were counted after having been covered for six days to detect any change in colouration. The data was entered such that an inflorescence colour change after six days was distinguished from no change in colour for each of the three inflorescence colours blue, white and brown for each treatment of each study plant.

Pollination ecology

Pollinator visitations are normally affected by factors including temperature, humidity and time of day (Heinrich and Raven, 1972). Therefore, I observed FVs on randomly chosen plants for 6 minutes each between 9 AM and 5 PM for two days. I found the hours with the highest average abundance and diversity of FVs to be between 10 AM and 2 PM. Focal individuals were observed between these times for 4 following days. During one hour, six minutes long observations of 6

different and randomly chosen focal plants were performed. Insect individuals landing on and remaining on flowers for longer than 3 seconds were considered FVs. Humidity and temperature was recorded hourly throughout all observations.

Statistical analysis

The data on inflorescence colour change was analysed using a mixed generalised linear model with a binomial error distribution (the "lme4" package V. 1.1-12; Bates *et al.*, 2015) in R (V. 3.3.2; R Core Team, 2015), because the response variable is binary. I initially constructed maximal models with, firstly, treatment, habitat, corymb colour and inflorescence colour as fixed factors, secondly, plant ID as a random factor nested within habitat since the two are not independent and each plant ID can only be found in one habitat and finally, two-, three- and four-way interactions between all fixed factors. Further, I estimated the effects of fixed factors and interactions using type III Wald Chi-square tests (the "car" package V. 2.1-4; Fox and Weisberg, 2011), and the effect of the random factor using likelihood ratio tests by running one Chi-square test between two nested models: the full model and the same model excluding the random factor. The model was subsequently simplified by removing interactions that were not significant. The final model included no interactions since no interactions were found to be significant.

For the data on abundance and taxonomic richness, neither untransformed or transformed data (log-, square root and box-cox) met assumptions of normality and homogeneity of variance. I therefore analysed the data in two separate generalised nested mixed models with Poisson distributions (the "lme4" package V. 1.1-12; Bates *et al.*, 2015) in R (V. 3.3.2; R Core Team, 2015). For both models, I initially constructed maximal models with, firstly, habitat and corymb colour as fixed factors, secondly, an interaction term between habitat and corymb colour, thirdly, humidity, temperature, observation date and time of observation as random factors and finally, plant ID as a random factor nested in habitat since the two are not independent and each plant ID can only be found in one habitat. The models were subsequently simplified until all remaining random factors and interactions were statistically significant. For taxonomic richness, the final minimal model included the two fixed factors habitat and corymb colour, and the sole random factor temperature (see Appendix A for test statistics and p-values). For abundance, only the interaction term between habitat and corymb colour was removed from the maximal model (see

Appendix A for test statistics and p-values). I estimated the effects of all random factors in both models using likelihood ratio tests by running one Chi-square test for each random factor between two nested models: the full model and the same model excluding the random factor. Further, I estimated the effects of fixed factors and interactions using type III Wald Chi-square tests (the "car" package V. 2.1-4; Fox and Weisberg, 2011). For both models, I performed post hoc pairwise comparisons of levels using Tukey contrasts (the "multcomp" package V. 1.4-6; Hothorn *et al.*, 2008).

RESULTS

Drivers of inflorescence colour change

Floral colour change was not affected by treatment (Type III Wald chi-square test: df=2, χ^2=5.55, p-value=0.0624) or by habitat type (Type III Wald chi-square test: df=1, χ^2=2.43, p-value=0.119).

Pollinator ecology - Flower visitor taxonomic richness

Both habitat and corymb colour affect the FV taxonomic richness at *A. conyzoides* (Table 2; Fig 2). In both habitats, corymb colour affects FV taxonomic richness such that it is higher for blue corymbs compared to white corymbs, but there is no difference in FV taxonomic richness between mixed and blue corymbs or mixed and white corymbs (Table 3; Fig. 2). FV taxonomic richness is higher in the swamp habitat than in the garden habitat (Table 2; Fig. 2). Among the random factors, only temperature explains variation in FV taxonomic richness (Table 2).

Table 2: The effects of temperature, corymb colour and habitat on FV taxonomic richness. See methods for further details on data analyses. See Table 1 for sample sizes. The test statistic given is (χ^2), degrees of freedom (*df*) and associated p-value is given for each factor.

Factor	F/R[1]	*df*	χ^2	p-value
Temperature	R	1	5.8246	0.0158
Corymb Colour	F	2	15.6268	0.0004043
Habitat	F	1	4.1806	0.0408888

[1]F: Fixed; R: Random

Table 3: Post hoc pairwise comparisons of the three levels of corymb colour (blue, mixed and white) for the effects of corymb colour on FV taxonomic richness using Tukey contrasts. See methods for further details on data analyses. See Table 1 for sample sizes. The test statistic Z and the p-value are given for each pairwise comparison.

Levels compared	z	p-value

Blue - Mixed	2.274	0.0591
Blue – White	3.833	0.000126
Mixed - White	1.631	0.2314

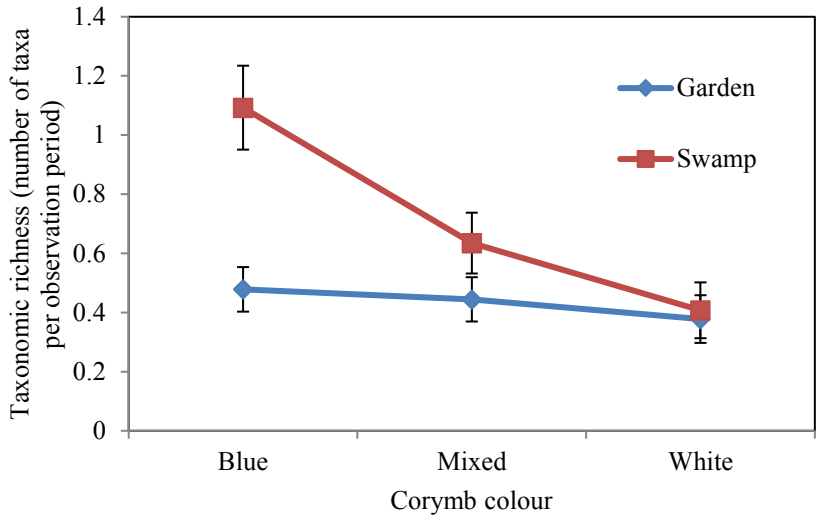

Fig. 2: Average taxonomic richness (number of taxa observed per 6 minute observations) for blue, mixed and white corymbs in the two habitats garden (blue line with diamond markers) and swamp (red line with square markers). Error bars indicate standard errors of the mean. See table 1 for sample sizes.

Pollinator ecology - Flower visitor abundance

Both corymb colour and habitat affect FV abundance such that FV abundance is higher in the garden compared to the swamp (Table 4; Fig 3). Blue corymbs show higher FV abundance than white corymbs and mixed corymbs have higher FV abundance than white ones; however, no difference in abundance was detected between mixed and white corymbs (Table 5; Fig 3).

Table 4: The effects of plant ID, time of day, date, humidity, temperature, corymb colour and habitat on FV abundance. See methods for further details on data analyses. See Table 1 for sample sizes. The test statistic given is (χ^2), degrees of freedom (*df*) and associated p-value is given for each factor.

Factor	F/R[1]	Df	χ^2	p-value
Plant ID	R	1	46.568	8.851e-12
Time of day	R	1	9.8533	0.001695
Date	R	1	21.186	4.168e-06
Humidity	R	1	13.02	0.0003083
Temperature	R	1	30.749	2.937e-08
Corymb Colour	F	2	16.0605	0.0003255
Habitat	F	1	4.0263	0.0447958

[1]F: Fixed; R: Random

Table 5: Post hoc pairwise comparisons of the three levels of corymb colour (blue, mixed and white) for the effects of corymb colour on FV abundance using Tukey contrasts. See methods for further details on data analyses. See Table 1 for sample sizes. The test statistic Z and the p-value are given for each pairwise comparison.

Levels compared	z	p-value
Blue - Mixed	1.427	0.3262
Blue – White	3.992	<0.001
Mixed - White	2.654	0.0216

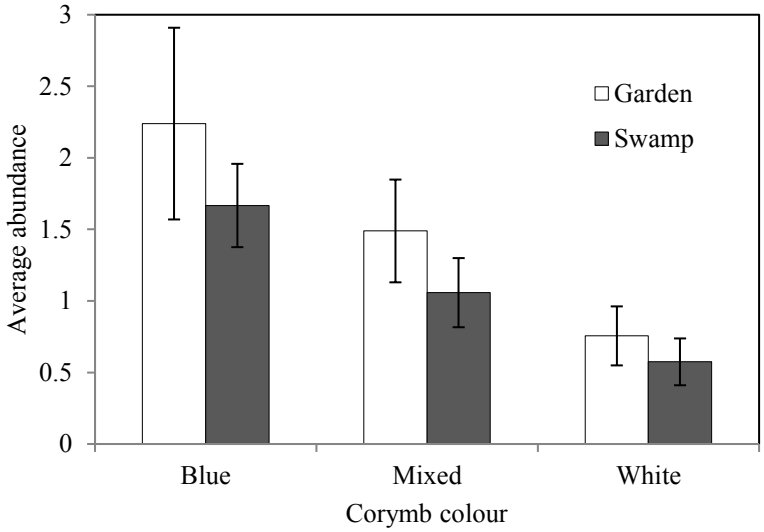

Fig 3. Average abundance (number of individuals observed per 6 minute observations) for blue, mixed and white plants in the two habitats garden (white bars) and swamp (grey bars). Error bars indicate standard errors of the mean. See table 1 for sample sizes.

DISCUSSION

Drivers of inflorescence colour change were not determined by this study since treatment type had no effect on floral colour change. Possibly, colour change does not depend on the activity of pollinators and is solely time dependent, such that a colour change occurs after a specific time. However, it is possible that the analysis did not have enough power to detect any treatment effect due to small sample sizes. Further, it is unclear whether the mesh used to hinder pollinators from accessing study corymbs were effective against small pollinators and whether the artificial pollination treatment actually did transfer pollen.

Both the taxonomic richness (Fig. 2) and the abundance (Fig. 3) of FVs were higher in blue corymbs compared to white corymbs, indicating that blue flowers of *A. conyzoides* are preferred

by FVs in both studied habitats. This observation is consistent with my hypothesis that the initial blue floral colour is preferred by pollinatiors. This has been demonstrated in other angiosperm species with colour change where the flower colour that is less attractive is believed to function to increase the floral display size and contribute to long-distance signaling in order to increase visitation rates (Lamont, 1985; Weiss, 1991; Larsson and Barrett, 1999). Nonetheless, I still observed FVs on white flowers (Fig. 2-3). This could be explained by either that *A. conyzoides* is an invasive species and colour preference by native FVs may not match those found in *A. conyzoides*, or that some native FVs show preferences toward white flowers (Willmer, 1953; Yan *et al*., 2016). Further studies should quantify whether FCC in *A. conyzoides* occurs together with changes in other factors such as floral rewards and scent, which has been observed in many other angiosperms (Yan *et al*., 2016). If white inflorescences are found to have lower floral rewards, their role in long-distance signaling is supported. However, if floral rewards are similar between inflorescence colours, different floral colour changes may be retained to attract pollinators with different colour preferences (Yan *et al*., 2016).

FVs were found to be more taxonomically diverse in the swamp habitat (Fig. 2), while more abundant in the garden habitat (Fig. 3). Human disturbance was less evident and other species of plants were more abundant in the swamp habitat. *A. conyzoides* is an invasive species that does not have specialised pollinators in Africa. It therefore has to rely on pollinators that are mostly generalists, which in turn are expected to be more abundant in areas with higher plant diversity. Further studies should attempt to identify FVs to species-level and test whether *A. conyzoides* relies on generalist pollinators in its non-native range.

REFERENCES

Aragón, S. J. and Ackerman, D. 2004. Does flower color variation matter in deception pollinated Psychilis monensis (Orchidaceae)? Oecologia **138**: 405-413.

Bartomeus, I., Vila, M. and Santamaria, L. 2008. Contrasting effects of invasive plants in plant-pollinator networks. *Oecologica* **155**: 761-770.

Bates, D., Maechler, M., Bolker, B. and Walker, S. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* **67(1)**: 1-48.

Batish, D. R., Kaur, S., Singh, H. P. and Kohli, R. K. 2009a. Role of root-mediated interactions in phytotoxic interference of *Ageratum conyzoides* with rice (*Oryza sativa*). *Flora* **204**: 388-395.

Batish, D. R., Kaur, S., Singh, H. P. and Kohli, R. K. 2009b. Nature of interference potential of leaf debris of *Ageratum conyzoides. Plant Growth Regulations* **57**: 137-144.

Brown, B. J., Mitchell, R. J. and Graham, S.A. 2002. Competition for Pollination Between An Invasive Species (Purple Loosestrife) and A Native Congener. *Ecology* **83**(8): 2328-2336.

D'Antonio, C. M. and Hobbie, S. E. 2005. Plant species effects on ecosystem processes. In: SaxDF, StachowiczJJ, GainesSD, eds. *Species invasions: insights from ecology, evolution and biogeography*. Sinauer Associates: Sunderland, MA, USA. pp. 65–84.

Dukes, J. S. and Mooney, H. A. 2004. Disruption of ecosystem processes in western North America by invasive species. *Revista Chilena de Historia Natural* **77**: 411-437.

Fox, J. and Weisberg, S. 2011. *An {R} Companion to Applied Regression*, Second Edition. Thousand Oaks CA: Sage. Accessed April 15, 2017 [http://socserv.socsci.mcmaster.ca/jfox/Books/Companion].

Heinrich, B. and Raven, P. H. 1972. Energetics and Pollination Ecology. *Science* **176**: 597-602.

Hewood, V. H. 1993. The flowering plants of the world. BT Bast Ford Ltd, London.

Hobbs, R. J. and Humphries, S.E. 1995. An integrated approach to the ecology and management of plant invasions. *Conservation Biology* **9**: 761–770.

Hothorn, T., Bretz, F. and Westfall, P. 2008. Simultaneous Inference in General Parametric Models. *Biometrical Journal* **50**(3): 346-363.

Ida, T. Y. and Kudo, G. 2003. Floral color change in *Weigela middendorffiana* (Caprifoliaceae): reduction of geitonogamous pollination by bumble bees. *American Journal of Botany* **90**: 1751-1757.

Jones, C. E. and Cruzan, M. B. 1999. Floral morphological changes and reproductive success in deer weed (*Lotus scoparius*, Fabaceae). *American Journal of Botany*, **86**: 273-277.

Kaur, S., Batish, D. R., Kohli, R. K. and Singh, H. P. 2012. *Ageratum conyzoides: and alien invasive weed in India*. In: *Invasive alien plants: an ecological appraisal for the Indian subcontinent*. Eds: Bhatt, J. R., Singh, J. S., Singh, S. P., Tripathi, R. S and Kohli, R. K. Cabi: New Delhi, India. pp. 57-76.

Kong, C., Hu, F., Xu, T. and Lu, Y. 1999. Allelopathic potential and chemical constituents of volatile oil from *Ageratum conyzoides. Journal of Chemical Ecology* **25**: 2347-2356.

Kong, C., Liang, W. Hu, F., Xu, X, Wang, P., Jiang, Y *et al.* 2004. Allelochemicals and their transformations in the *Ageratum conyzoides* intercropped citrus orchard soils. *Plant and Soil* **264**: 149-157.

Larsson, B. M. H. and Barrett, S. C. H. 1999. The pollination ecology of buzz-pollinated *Rhexia virginica* (Melastomataceae). *American Journal of Botany* **86**: 502-511.

Melendez-Ackerman, E., Cambell, D. R. and Waser, N. M. 1997. Hummingbird behavior and mechanics of selection on flower color in *Ipomopsis*. *Ecology* **78**: 2532-2541.

Mooney, H. A., Mack, R.N., McNeely, J.A., Neville, L.E., Schei, P.J. and Waage, J.K. 2005. *Invasive Alien Species: A New Synthesis*. Island Press, Washington, DC.

Newmark, W. D. 2002. *Conserving Biodiversity in East African Forests: A study of the Eastern Arc Mountains*. Springer: Verlag Berlin Heidelberg.

Oberrath, R. and Bögning-Gaese, K. 1999. Floral color change and the attraction of insect pollinators in

lungwort (*Pulmonaria collina*). *Oecologa* **121**: 383-391.

Pellmyr, O. 1992. Evolution of Insect Pollination and Angiosperm Diversification. *Tree* **7**(2): 46-49.

Pimentel, D., Zuniga, R. and Morrison, D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics.* **52**: 273-288.

R Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [https://www.R-project.org/].

Rodriguez, G. S. and Capero, G. S. 1984. Number of seeds produced by some weed species. *Centro Agricola* **11**: 45-50.

Sankaran, K. V. 2007. Billy-goat weed (*Ageratum conyzoides*). *Newsletter of the Asia-Pacific Forest Invasive Species Network* **13:** 2.

Sauerborn E., Sauerborn J. 1985. *Wichtige Unkrautarten der Tropen und Subtropen*. Verlag Josef Margraf, Weikersheim, Germany.

The World Bank. 1998. Tanzania – Eastern Arc Forests Conservation and Management Project.

Theoharides, K.A. and Dukes, J.S. 2007. Plant invasion across space and time: factors affecting nonindigenous species success during four stages of invasion. *New Phytologist* **176**: 256-273.

Theoharides, K. A. and Dukes, J. S. Plant invasion across space and time: factor affecting nonindigenous species success during four stages of invasion. *New Phytologist Journal* **176(2)**: 256-273

Weiss, M. R. 1995. Floral color change: a widespread functional convergence. *American Journal of Botany* **86**: 167-185.

Weiss, M. R. and Lamont, B. 1997. Floral color change and insect pollination: a dynamic relationship. *Israel Journal of Plant Sciences* **45**: 185-199.

Willmer, P., Stanley, D. A., Steijven, K., Matthews, J. M. and Nuttman, C. V. 2009. Bidirectional flower color and shape changes allow a second opportunity for pollination. *Current. Biology.* **19**: 919-923.

Yan, J., Wang, G., Wang, M. and Zhang, L. 2016. Pollinator response to floral colour change, nectar, and scent promote reproductive fitness in *Quisqualis indica* (Combretaceae). *Scientific Reports* **6**: 24408.

Xuan, D., Shinkichi,T., Hong, N. H., Khanh, T. D. and Min, C. 2004. Assessment of phytotoxic action of *Ageratum conyzoides* L. (billy goat weed) on weeds. *Crop Protection* **23**: 915-92.

Appendix A

Table A1: Output from chi square likelihood ratios for each factor used in the maximal model to explain FV taxonomic richness. The chi square test statistic and associated p-value is given for each factor. See methods for details on the model and Table 1 for sample sizes.

Factor	F/R[1]	*df*	χ^2	p-value
Plant ID	R	1	0.0201	0.8874
Humidity	R	1	2	0.1041
Temperature	R	1	5.8246	0.0158
Date	R	1	0	0.9997
Time	R	1	0	0.9999
Habitat*Plant Colour	F	2	3.7492	0.1534
Plant Colour	F	2	0.6879	0.708948
Habitat	F	1	7.2081	0.007257

Table A2: Output from chi square likelihood ratios for each factor used in the maximal model to explain FV abundance. The chi square test statistic and associated p-value is given for each factor. See methods for details on the model and Table 1 for sample sizes.

Factor	F/R[1]	Df	χ^2	p-value
Plant ID	R	1	46.568	8.851e-12
Time of day	R	1	9.8533	0.001695
Date	R	1	21.186	4.168e-06
Humidity	R	1	13.02	0.0003083
Temperature	R	1	30.749	2.937e-08
Habitat*Plant Colour	F	2	0.0719	0.96469
Plant Colour	F	2	16.0605	0.0003255
Habitat	F	1	4.0263	0.0447958