


Fruit defence syndromes: the independent evolution of mechanical and chemical defences

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Received: 26 June 2017 / Accepted: 7 September 2017
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Abstract Plants are prone to attack by a great diversity of antagonists against which they deploy various defence mechanisms, of which the two principle ones are mechanical and chemical defences. These defences are hypothesized to be negatively correlated due to either functional redundancy or a trade-off, i.e., plants which rely on increased mechanical defence should downregulate their degree of chemical defence and vice versa. A competing hypothesis is that different defences perform distinct functions and draw from different pools of resources, which should result in their independent evolution. We examine these competing hypotheses using two independent datasets of fleshy fruits we collected from Madagascar and Uganda. We sampled mechanical defences, indexed by fruit puncture resistance, and defensive defences, indexed by defensive volatile organic compounds, and examined their associations using phylogenetically-controlled models. In both systems, we found no correlation between mechanical and chemical defences, thus supporting the independent evolution hypothesis. This implies that fruit defence mechanisms reflect a more complex array of selection pressures and constraints than previously perceived.

Electronic supplementary material The online version of this article (doi:[10.1007/s10682-017-9919-y](https://doi.org/10.1007/s10682-017-9919-y)) contains supplementary material, which is available to authorized users.

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Keywords Animal-plant interactions · Fleshy fruits · Mechanical defence · Chemical defence · Constraints · Trade-off

Introduction

Plants are an enormous energy source for myriad antagonists: viruses, bacteria, fungi, parasitic plants, invertebrates, and vertebrates (Hawes et al. 2000; Ellenbogen et al. 2006; Farmer 2014). All plant organs, vegetative and reproductive alike, are susceptible to attack. To mitigate the damage from these antagonists, plants employ various defences, including mechanical barriers, chemical toxins or repellents, and biotic defences via mutualistic interactions with protective animals. Most studies of plant defences have focused on leaves, which are chemically protected by both constitutive compounds, such as tannins, alkaloids, phenolics, and terpenoids (Farmer 2014), and by attack-induced green-leaf volatiles (GLVs) that function to recruit predators which then prey on the plant's attackers (Kessler and Baldwin 2000). In addition, leaves can be mechanically protected by structures such as thorns and trichomes (Westbrook et al. 2011; Farmer 2014; Kariñho-Betancourt et al. 2015). Flowers and fruits face an even greater challenge: many are self-advertising organs whose traits are often selected to attract animal mutualists (Valenta et al. 2017). This exposes them to attack which must be carefully mitigated without deterring animal mutualists.

Plant defence mechanisms have been hypothesized to form discrete defence syndromes, i.e., co-occurring defensive traits. It is predicted that within these suites of traits, mechanical and chemical defence should show a negative relationship, resulting from either redundancy or trade-off driven by the costs of maintaining multiple defences (Hermes and Mattson 1992; Agrawal and Fishbein 2006; Moles et al. 2013; Ballhorn et al. 2014; Züst and Agrawal 2017). For example, if multiple plant defences have the same outcome, one may render the other unnecessary. Similarly, if different forms of plant defence are energetically costly, investment in one defence type may drive a decrease in the other. Alternatively, these patterns of coordinated evolution of defence strategies can be evasive or absent if different defence barriers are deployed to mitigate different antagonists. For example, chemical defences may be directed at microorganisms, while mechanical defence primarily protects against animals (Agrawal and Fishbein 2006; Moles et al. 2013). In addition, defence mechanisms may be constrained by different pools of resources. For example, construction and maintenance of mechanical defence may be limited by nitrogen (Zhao et al. 2005). This may generate a trade-off with emission of N-containing VOCs, but less so with defensive carbohydrates like phenolics or terpenoids.

The ongoing debate between these two competing hypotheses has mainly focused on leaf defence and has produced supporting evidence for both. A significant negative correlation between mechanical and chemical leaf defences has been found within species (Ballhorn et al. 2013), among congeneric *Gastrolobium* species (Twigg and Socha 1996), and, independent of phylogeny, within a community of subtropical tree species (Eichenberg et al. 2015). In contrast, both a meta-analysis of studies addressing this relationship (Koricheva et al. 2004) and a large-scale phylogenetically-controlled study (Moles et al. 2013) failed to document the expected negative relationship between mechanical and chemical leaf defenses.

Fleshy fruits are prone to attack by microbial and animal antagonists, often seed predators (Herrera 1982; Zangerl and Rutledge 1996). Fruits are susceptible to attack during the maturation process and especially when fully ripe. To mitigate the risk of attack,

fruits can employ a variety of strategies such as ripening in seasons in which antagonist population densities are low, or decreasing pulp nutrient content; however, again, the two most common defence in ripe and especially in unripe fruits are mechanical, usually in the form of a thick protective exocarp, and chemical, typically through toxic or unpalatable secondary metabolites (Herrera 1982; Cipollini and Levey 1997; Cipollini 2000). A major fraction of defensive compounds are volatile organic compounds (VOCs) that are in the odours they release (Unsicker et al. 2009). At the same time, fruits often change their VOC profile upon ripeness due to the need to attract seed dispersers (Lomáscolo et al. 2010; Rodríguez et al. 2013; Nevo et al. 2016) and many of the compounds emitted by ripe fruits may, at least secondarily, function as attractants (Cipollini and Levey 1997).

Unripe fruits tend to be heavily defended. They tend to be much harder than ripe fruits, i.e. to be more mechanically defended (Lasa et al. 2017) and contain higher amounts of defensive secondary compounds (Schaefer et al. 2003). Like in leaves, expression of mechanical and chemical in unripe fruits may present a trade-off due to their metabolic costs (Herms and Mattson 1992; Ballhorn et al. 2014).

Ripe fruits, on the other hand, present an even more intriguing model system to test the defence trade-off hypothesis. Whereas in leaves and unripe fruits the costs of excess defence are likely to be metabolic, plants benefit from consumption of ripe fleshy fruits by legitimate seed dispersers and their access to the fruits can be compromised by these defences (Schaefer and Ruxton 2011; Whitehead et al. 2015). Chemical defences are especially problematic because preferences of mutualists and antagonists are often similar (Schaefer and Ruxton 2011; Whitehead et al. 2015). To minimize deterrence of seed dispersing mutualists, plants are expected to invest in the minimal effective level of fruit defence. As a result, the predicted negative relationship between mechanical and chemical defences is expected to be more pronounced in ripe fleshy fruits than it is in leave or unripe fruits.

Data on the relationship between chemical and mechanical fruit defences are scarce, and most data come from the study of seeds or of fruits whose seeds are not animal dispersed. A negative correlation between mechanical and chemical defences of fruits has been demonstrated among congeneric oak species (*Lithocarpus* spp.) (Chen et al. 2012) and among members of a liana community from the Barro Colorado island in Panama (Gipenberg et al. 2017). A comparative study of 23 species in China found a non-linear negative relationship between tannin concentration and seed-coat thickness (Zhang et al. 2016). In contrast, larger seeds in ten *Macaranga* species have been found to be more physically protected, but not necessarily less chemically protected (Tiansawat et al. 2014). However, we are unaware of published data on this topic for endozoochorously-dispersed fleshy fruits.

Here, we test the hypothesis that fruit defence strategies show distinct syndromes using two separate datasets comprised of ripe fruits originating from two communities in Madagascar (35 species) and Uganda (47 species). We test the prediction that mechanical and chemical fruit defences are negatively correlated using phylogenetically-controlled regression models. We compare the puncture resistance of ripe fruits, i.e. the amount of force required to puncture their peeling (mechanical defence), to the amount of defensive volatile organic compounds (VOCs) captured for each species using active VOC sampling and gas chromatography-mass spectrometry (GC-MS).

Materials and methods

Intact ripe fruits of 35 sympatric species (Tab. S1; Fig. S1) were collected in the tropical dry forest of Ankarafantsika National Park, north-western Madagascar (Jan–Dec 2012, Ampijoroa Field Station). Fruits of 47 (Tab. S2; Fig. S1) sympatric species were obtained Kibale National Park, Uganda near the Makerere University Biological Field Station (Chapman and Chapman 2002) (Jan 2015–Jun 2016). We considered fruit ripe when seeds were viable, fruit were fully formed and had changed their colour and were consumed by local frugivores. We identified plants to species using both published guides (Hamilton 1981; Schatz 2001) and unpublished local databases (Sato, pers. comm.). Between two and ten ripe fruits from each species were collected.

Fruit puncture resistance was used as an index of mechanical defence, as it is negatively correlated with infestation by invertebrates in domestic plant species (Lambert et al. 2004; Gonçalves et al. 2012). For example, fruit flies prefer softer fruits in later developmental stages and even species which specialize on early stages of fruit maturation to avoid competition are more likely to infest softer fruits (Follett 2017; Lasa et al. 2017; Takahara and Takahashi 2017). Puncture resistance was measured using a modified force gauge (Shimpo MF 50). The edge of the force gauge was inserted at a 90° angle into individual ripe fruits until the exocarp was punctured. We recorded the force required to puncture fruit exocarps for between five and ten ripe fruits of each species, and assign each species a puncture resistance value based on the mean for each species. The puncture resistance results represent the weight (kg/mm^2) of force required for a probe measuring 4 mm in diameter to puncture fruit exocarps (Valenta et al. 2015).

To approximate the degree of chemical defence, we calculated the sum of all VOCs (corrected for surface area) belonging to chemical classes that are known to be involved in direct and indirect leaf defence: nitrogen- and sulfur-containing compounds, phenolics, terpenoids, and green leaf volatiles (GLVs) (Farmer 2014). Different plant lineages have evolved to employ different defensive chemicals, some of which are synthesised in non-specific pathways and emitted in diverse mixtures (Fischbach and Clardy 2007) of compounds with different degrees of toxicity (Stamopoulos et al. 2007). As a result, any consideration of plants' overall reliance on chemical defence should examine all rather than only some of the chemicals. This inclusive approach is more likely to cover many potential defensive compounds used by the diverse communities than typical wet chemistry approaches examining specific major groups of compounds (i.e., alkaloids) and therefore provide the best proxy to their degree of chemical defence in a wide community of plants. At the same time, this approach also has limitations since to achieve a certain degree of toxicity, different compounds sometimes need to be emitted in different concentrations (Agrawal and Weber 2015). To address this issue, we also examined the relationship between puncture resistance and defensive VOC emission on a small subset of nine congeneric *Ficus* species in our dataset. Thus, our two community-level analyses are complemented by a similar lineage-level analysis.

Fruit VOCs of 2–10 fruits/species were sampled within 4 h of collection using a dynamic headspace adsorption technique. Fruits were enclosed in oven bags (Reynold's™ large oven bags) and air was drawn into the sampling bag at an airflow of 1 l/min for 4 h (by a pump Gilian 5000, Sensidyne™) through an inlet filtered and cleaned of atmospheric pollutants using filters filled with activated carbon (Sigma Aldrich). Air drawn out of each chamber, through a single outlet, was sampled for VOCs by a sorbent odorant probe which contained two VOC traps connected one after the other (Amberlite XAD-2™,

400–200 mg, Sigma-Aldrich). Trapped odorants were extracted in 4 ml (Madagascar) or 3 ml (Uganda) hexane (Sigma Aldrich) and manually shaken for 5 min.

VOC samples were analysed using gas chromatography coupled with mass spectrometry. Samples from Madagascar were analysed on an Agilent 7890ATM gas chromatograph equipped with a DB-5 column (Agilent; 30 m × 0.25 mm × 0.25 µm) and interfaced with an Agilent 5975 inert mass spectrometer operating in electron ionization (EI) mode. 1 µl of samples were injected splitless at an oven temperature of 50 °C with an inlet temperature of 250 °C. Carrier gas was helium at a constant flow rate of 1 ml/min. The oven program consisted of an initial hold at 50 °C for 2 min, followed by a 10 °C/min ramp to 150 °C, and then a 30 °C/min ramp to 300 °C. The transfer line temperature was held at 300 °C. We monitored analytes in full scan mode using a selected mass range of 40–300 Da.

190 µl of each sample from Uganda was mixed with 10 µl of an internal standard solution (heptadecane, 200 ng/ml) and then concentrated to 20 µl using a gentle nitrogen flow. Samples were analysed on an Agilent 7890B gas chromatograph equipped with a DB-5 column (Agilent; 30 m × 0.25 mm × 0.25 µm) and interfaced with an Agilent 5977A inert mass spectrometer operating in electron ionization (EI) mode. 2 µl of each sample were injected splitless using a cold injection system. Starting inlet temperature was 10 °C. The inlet was then heated at a rate of 12 °C/min until it reached 300 °C and then held for 4 min in this temperature. Then, the split valve was opened. Initial oven temperature was 45 °C. Carrier gas was helium at a constant flow rate of 1 ml/min. The oven program consisted of an initial hold at 45 °C for 1 min, followed by a 7 °C/min ramp to 310 °C and then a 15 min hold on this temperature. The transfer line temperature was held at 250 °C. We monitored analytes in full scan mode using a selected mass range of 40–300 Da.

We excluded VOCs suspected as contaminants based on their presence in control samples taken in identical conditions using empty bags or those present in the solvent. Odorant amounts were quantified on Amdis 2.71. VOCs were identified based on published retention indices and mass spectra (NIST 11 library). We then calculated the sum of nitrogen- and sulfur-containing compounds, phenolics, terpenoids, and GLVs (Farmer 2014). For the Madagascar samples we used the area under the TIC peaks as a measurement of VOC amounts. In the Uganda samples, for which an internal standard was available, we conducted the same calculation and then converted this measurement to an estimated absolute amount based on the internal standard. In both we then corrected the final amount to the surface area of the fruits in each sample.

Data were analysed in R 3.2.5 (R Core Team 2014) using packages Caper (Orme et al. 2012), APE (Paradis et al. 2004) and Phytools (Revell 2012). To examine the association between mechanical and chemical defence we ran phylogenetic generalized least-squares (PGLS) models using a Brownian motion model. All data were double cubic root transformed to achieve a normal distribution of all variables. We used a phylogeny by Zanne et al. (2014) extracted using Phylomatic (Webb and Donoghue 2005). We further analysed the relationship between each of the four VOC categories (GLVs, phenolics, terpenoids and N/S compounds) and puncture resistance for the Madagascar dataset. In Uganda, terpenoids constituted 84% (median 99%) of the overall defensive VOC index. As a result, all other compounds were relatively minor and their distribution was highly zero-inflated, which rendered their analysis not very informative. Therefore, in Uganda, in addition to the analysis of the full defensive VOC index we only analysed the effect of terpenoids independently. Analysis of the relationship between puncture resistance and defensive VOC within the *Ficus* family was conducted using a simple linear model since the phylogenetic relationship between the species are not well-resolved. In addition, we calculated

Blomberg's K (Blomberg et al. 2003) for puncture resistance and overall amount of defensive VOCs to estimate the degree of phylogenetic signal in both traits.

Results

Puncture resistance was similar between the systems (median values: 0.125; 0.124 kg/mm² in Madagascar and Uganda, respectively) and showed similar within-system variance (coefficient of variance: CV = 1.44 in Madagascar; CV = 1.27 in Uganda), but variance in emission of defensive compounds was higher in Uganda (Madagascar: CV = 1.21; Uganda: CV = 3.65), indicating a greater divergence in the degree of reliance on chemical defence in this system.

We identified 41 defensive VOCs in the Madagascar community and 53 in the Uganda samples (Sup. Tab. S1, S2). In both systems terpenoids were the most diverse group of defensive compounds (46% of VOCs in Madagascar and 64% in Uganda), followed by GLVs (32% and 28% in Madagascar and Uganda, respectively). (Sup. Tab. S1, S2). In Madagascar phenolics and nitrogen and sulfur compounds contributed 14% of the compounds found, whereas in Uganda VOCs of these categories were less dominant (5.6%) (Sup. Tab. S1, S2).

For both Madagascar ($F = 1.79$ (1, 33), $N = 35$, $p = 0.19$) and Uganda ($F = 0.45$ (1, 45), $N = 47$, $p = 0.51$), phylogenetically-controlled least-squares regression models found that variation in fruit puncture resistance was not a function of the variation in overall defensive VOC emission (Fig. 1). These results remained similar when examining each compound class separately. In Madagascar, a PGLS model which contained puncture resistance as a response variable and the four VOC classes as predictors found no statistically significant relationship between fruit puncture resistance and the amounts of terpenoids, phenolics, GLVs and N/S compounds ($F = 1.17$ (4, 30), $N = 35$, $p = 0.34$). In Uganda we examined only terpenoids independently (see methods) and found no correlation between terpenoid emission and puncture resistance ($F = 0.72$ (1, 45), $N = 47$, $p = 0.4$).

The complementary analysis testing this relationship among nine *Ficus* species showed similar trends: puncture resistance and defensive VOCs are not correlated ($F = 0.27$ (1, 7), $N = 9$, $p = 0.62$) (Fig. 2).

We also quantified the phylogenetic signal in puncture resistance and defensive VOC emission in the two systems. Puncture resistance in Madagascar had a significant phylogenetic signal ($K = 0.71$, n permutations = 999, $p = 0.028$), while all other variables showed no signal: (Madagascar defensive VOCs: $K = 0.33$, n permutations = 999, $p = 0.75$; Uganda puncture resistance: $K = 0.34$, n permutations = 999, $p = 0.74$; Uganda defensive VOCs: $K = 0.38$, n permutations = 999, $p = 0.53$).

Discussion

Our results support the hypothesis that different plant defences perform distinct functions or draw from different pools of resources, which results in a weak to absent relationship between mechanical and chemical defences. As such, ours is the first documentation of which we are aware that extends to fleshy fruits the trends found in the larger-scale studies of leaf defence (Koricheva et al. 2004; Moles et al. 2013), where ecological factors or

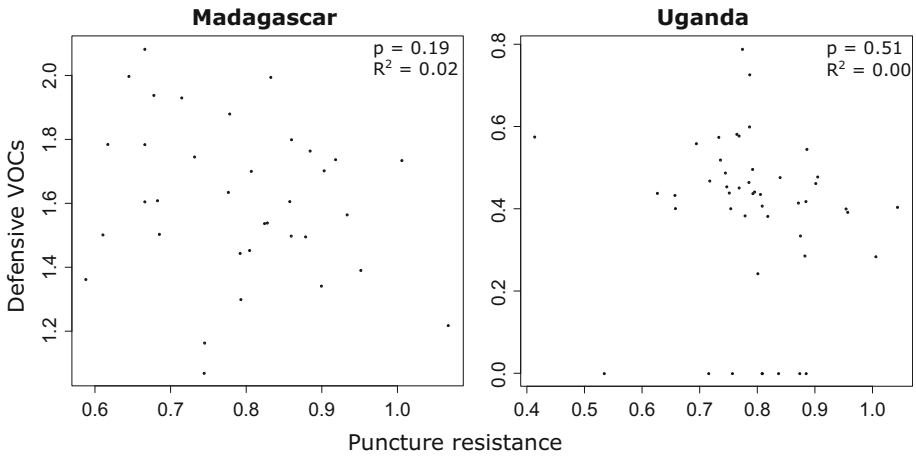
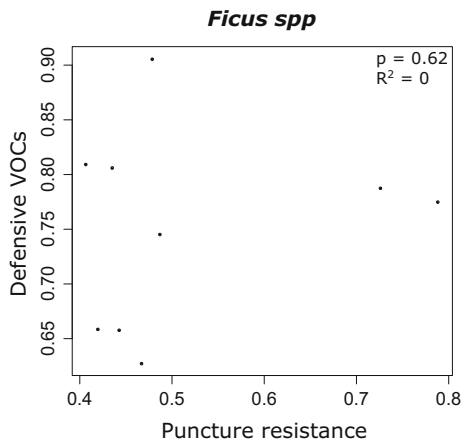


Fig. 1 The relationship between fruit puncture resistance and overall defensive VOC emission in Madagascar and Uganda. Puncture resistance is measured in kg/mm^2 . Defensive VOCs are the sum of peak area in Madagascar and estimated absolute amounts in pg in Uganda (see methods). P and R^2 values are from phylogenetic generalised least-squares models (PGLS). All values are double-cubic-root transformed to meet model assumptions

Fig. 2 The relationship between fruit puncture resistance and overall defensive VOC emission in nine *Ficus* species from Uganda. Puncture resistance is measured in kg/mm^2 . Defensive VOCs are the sum of estimated absolute amounts in pg (see methods). P and R^2 values are from a linear regression model. All values are double-cubic-root transformed to meet the assumptions of the statistical models used



constraints generate an independent evolution of the two factors. Moles et al. (2013) suggested that a lack of negative correlation between mechanical and chemical leaf defence was due to the former countering herbivory pressure, whereas the latter protects against microorganisms. This may also apply to fleshy fruits. Further, fruit puncture resistance derives mainly from exocarp thickness, which is affected by fruit size. Large fruits require a thick exocarp to support their increased pulp and seed mass (Schaefer and Ruxton 2011) and thus large fruits tend to be harder and de facto physically protected (Aguirre et al. 2003). In this situation, the coordinated evolution hypothesis predicts a strong downregulation of chemical defences because it is expected that in some plants the degree of mechanical defence will be unnecessarily high. Nonetheless, our results from both study sites show that such coordinated evolution is unlikely. The lack of correlation between the two defence systems may reflect an array of selective pressures and constraints

which is more complex than present models suggest—namely that defence systems both defend against the same antagonists and rely upon the same pool of resources. Our findings are strengthened by the fact that we conducted the analysis on species from two sites differing in abiotic characteristics, with species relying on the seed dispersal services of different vertebrates and under predation pressure by different antagonists.

Another possibility is that in absolute terms, ripe fruits are relatively lightly-defended due to the price of over-defending fruits, which may result in deterrence of seed-dispersal vectors. A decreased expression of both defence mechanisms would result in a disappearance of the trade-off if the metabolic costs become negligible relative to the advantage they provide. Support for this idea would be found if a negative relationship between mechanical and chemical defences is found in the unripe fruits of the taxa investigated here. Unfortunately, we do not have the data to examine this.

It is well documented that compounds which originally evolved as defensive compounds in flowers have been selected to be secondarily employed for other functions (Pellmyr and Thien 1986). While some authors suggested that fruit secondary metabolites are mainly the result of biochemical pleiotropy rather than adaptation (Eriksson and Ehrlén 1998), others showed that this is unlikely (Cipollini et al. 2004) and hypothesized multiple functions, including defence and attraction of animal mutualists (Cipollini and Levey 1997; Cipollini 2000). Fruit VOCs are now increasingly acknowledged to play a role in attracting frugivorous seed dispersers (Lomáscolo et al. 2010; Hodgkison et al. 2013; Rodríguez et al. 2013; Nevo et al. 2016; Valenta et al. 2017). It is therefore expected that many of the compounds considered here fulfil a dual function or even primarily serve as attractants of seed dispersers (Rodríguez et al. 2013). However, all compounds classes considered here have been documented to be involved in plant defence. Further, the most diverse group of chemicals found in both systems was the terpenoids, which tend to be toxic (Gershenzon and Dudareva 2007). Thus, it is expected that even if these VOCs fulfil other function, they also provide chemical defence and are subjected to selection pressures exerted by fruit antagonists. This conclusion is further supported by the fact that the lack of a negative relationship between mechanical and chemical defences was absent even when looking at individual compound classes separately. In addition, whereas the fruits in Madagascar are dispersed by largely-dichromatic and partially-nocturnal lemurs (Valenta et al. 2015), in Uganda the frugivore community is composed of diurnal trichromatic primates (Jacobs 2009) and tetrachromatic birds (Vorobyev et al. 1998). Levels of reliance on olfaction and hence a selection pressure to emit VOCs for animal attraction are therefore likely to be very different between the systems. The fact that a negative relationship between mechanical and chemical defences was equally weak in both systems implies that animal attraction is not likely to have strongly affected the relationship between the particular VOCs examined here and fruit puncture resistance.

Given that the approach taken here has some limitations (Agrawal and Weber 2015), we encourage future work to build upon our result and examine the relationship between fruit mechanical and chemical defences on a phylogenetically narrower model system with a large set of species whose members are more likely to use similar chemical defensive strategies. Additionally, while this and other studies focused on a single plant organ such as fruits or leaves, future studies may gain insights into the evolution of plant defence by examining them together. For example, while mechanical defence in fruits and leaves are likely functionally independent of one another, chemical defences may be shared as plants employ similar biosynthetic pathways to defend all plant structures, or retain some toxins in ripe fruits which were synthesized to defend unripe fruits or flowers. Considering such developmental constraints and opportunities which affect mechanical and chemical

defence phenotypes differently may help disentangle the complex evolution of plant defence strategies.

In conclusion, our study indicates a lack of coordinated evolution of mechanical and chemical defences in two independent communities of tropical fleshy fruits. This supports the hypothesis that defence mechanisms are complementary, and that plants do not necessarily face a trade-off when allocating resources to different defence systems. To our knowledge, this is the first time this has been documented. It joins an array of studies which examined the dynamics of the evolution of mechanical and chemical defences in leaves and non-fleshy fruits, and emphasises that in fruits, like in other plant organs, multidirectional selection pressures and constraints yield complex patterns that are difficult to explain using the straightforward trade-off model.

Acknowledgements We thank Lisa A. D'Agostino for her assistance in conducting the chemical analysis. We thank the Canada Research Chairs Program, Natural Science and Engineering Research Council of Canada, Fonds Québécois de la Recherche sur la Nature et les Technologies, the National Geographic Society for funding. ON was funded by a German Science Foundation grant (NE 2156/1-1) while working on this manuscript. We thank MICET and Madagascar National Parks, for permission to conduct this research in Madagascar. We are grateful to Paul Tsiveraza, Francette, Mamy Razafitsalama and Jean de-la-Dieu for contributions in the field.

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