Toxin mediated plant choice among Danainae butterflies from the districts of West Bengal, India

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Abstract: Relationship between herbivore insects and their respective food plants constitute the central theme of any investigation on community ecology. Heterogeneous environment with fluctuating floristic composition and structure, influence the distribution of butterflies in a non-random manner based on the pattern of larval and adult resource distribution. Therefore the present study was designed to identify toxin (predominantly glycosides and alkaloids) based plant species preferred by butterflies. Subsequently their polyphagy index and niche breadth was determined to highlight their utilization of larval resource. Exploitation of damaged plant substrates by adult species was also recognised. 16 Danainae butterflies as larvae were observed feeding on glycoside rich plant species while damaged parts of pyrrolizidine alkaloid rich plant were recognised by adults. *Euploea core* with highest niche breadth and polyphagy index was indicative of their utilization of widest repertoire of larval food plants. Feeding guild of adult butterflies was represented by regression analysis. Hierarchial Clustering was employed to create a dendrogram for identification of probable clusters formed among danainae species based upon their similarity in larval resource utilization. Highest Shannon - Wiener Function values for *Ageratum conyzoides* revealed the maximum information content generated by them in turn attracting the largest assemblages of adult butterflies. Thus phylogenetic conservatism in adaptation to different phytochemical profile of their host plants probably forms the basis of such preference among lepidopterans.

Keywords: Alkaloids; butterflies; Cardiac glycosides; danainae; polyphagy

I. INTRODUCTION:

Butterfly communities are influenced by its surrounding vegetation [1,2,3] which is said to be governed by the vegetation matrix [4,5,6,7,8]. Heterogeneous environment with fluctuating floristic composition and structure, influence the distribution of butterflies in a non-random manner based on the pattern of larval and adult resource distribution. Larval diet predominantly derived from plants is responsible for a large part of adult's reproductive potential [9,10]. Based on pioneering efforts of [11] a general correlation exists between host plant diversity and herbivore species richness associated with sequential events of adaptive radiation and enhanced diversification. The generalised pattern of lepidopteron host plant associations is the occurrence of host plant specialization [12,13,14] along with a propensity of clades to remain associated with the same genus or family of host plants [15,16,17]. Although most insect herbivores possess a specialised diet breadth, they are known to utilize a small proportion of the total host plants actually available to them [18,19,20,21,22]. Herbivore diet breadth provides insight into the niche breadth of such insects [23]. Additionally, the influence of niche breadth on body size [24] range size and abundance [25] of herbivore is also worth mentioning.

Importantly, the real mystery of Danainae larvae lies in their ability to sequester high concentration of cardiac glycosides (CGs) in their bodies as compared to other species [26]. Thus sequestered CGs confers protection from predators [27] and parasitoids [28]. However, adult insects exhibiting pharmacophagy are known to gather 1,2-dehydropyrrolizidine ester alkaloid (PA) [29] usually from dried / withered / damaged plant substrates or injured living matter of plant species.

Therefore the present study was designed to identify toxin (predominantly glycosides and alkaloids) based plant species utilised by butterflies as a part of their larval and adult resource. Incidence of polyphagy and estimation of niche breadth would be done to emphasise on the utilization of cardiac glycoside based larval resource of butterflies. Hierarchial Clustering would be used to illustrate the formation of clusters among species based upon the dietary preferences of their larval stages. Exploration of dried or damaged pyrrolizidine alkaloid based plant materials by adult danainae would be represented by regression analysis. The amount of information content (expressed as Shannon-Wiener Function) regarding adult resources as emanating from such plant substrates would also be ascertained.

II. METHODS:

2.1. Sampling Design

The entire study was conducted across study sites established in the Jalpaiguri and Darjeeling district of West Bengal, India. Initial survey of the study area to identify larval host plants of Danainae (Danainii and Euploii) was done. Dead, decayed, damaged or withered plant species with large assemblages of butterflies were also recognised. On field identification of plants from specially constructed quadrats was done and their association with larvae was also recognised in few cases. These results

were interpreted based on reliable records from literature [30,31]. Identification of dried plants and plant parts with large assemblages of adult butterflies was done along with assistance from taxonomist.

Sampling of larval host plants was undertaken weekly in a month with the help of two trained field assistants. The sampling procedure was repeated at an interval of seven days. Importantly, sampling of plants with adult butterflies was conducted on days other than those used for sampling of their larval host plants. The butterflies were observed and photographed occasionally for subsequent identification from published literature [30,31].

2.2. Data Analysis

The incidence of polyphagy among butterfly species was measured as **polyphagy index (PI)=$(AXB)^{1/2}$**; where A= number of genera of host plants and B=taxonomic diversity of hosts (where 1=one plant species; 2=one plant genera; 3=one plant family; 4=one plant order; 5=two or more plant orders) [32].

Niche breadth of butterfly species was estimated using **Levin's Measure of Niche Breadth**. It is represented as **$B=1/\sum p_j^2$** (where p_j is proportion of individuals found or utilizing a resource "j" [33].

Significantly Cluster Analysis is widely recognized as a group of multivariate mathematical algorithm involving grouping of species into closed clusters based on their strong similarity [34]. Hierarchial Clustering using Ward's Method was employed in this study to create a dendrogram for identifying the number of probable clusters formed among danainae species based upon their similarity in their larval resource utilization. Importantly, Hierarchical Clustering (using hclust function of the program-R) was used to illustrate such similarity among species across the study area.

In order to ascertain the size of feeding guild of adult butterflies associated with damaged plant parts, the average number of butterflies as observed was regressed on the average duration of their visits on dead/damaged/withered plant parts.

The species diversity of visiting butterfly species was determined using Shannon-Wiener Function [35] for quantification of information content of plant-butterfly interaction. Since, base 2 log was used; the units of information content of sample were bits per plant species. Shannon-Wiener Function (H′) was calculated using the following formulae: **$H'=\sum pi\ (\log_2 pi)$** (where, pi=ni/N; ni is the number of individuals of ith species and N=∑ni)

For better interpretation of results regarding the information content of communication, Shannon-Wiener Function values in the range of 3.500 bits per plant species or above was said to be indicating greater species diversity, thereby entertaining higher number of species. Similarly, Shannon-Wiener Function values below 3.500 bits per plant species are said to be representing lesser species diversity and in turn attracting lesser number of species.

III. RESULTS AND DISCUSSION

Relationship between herbivore insects and their respective food plants constitute the central theme of any investigation on community ecology [36,23,37,38]. Perhaps more specifically these herbivores are known to prefer taxonomically defined subgroup of plant communities [39,40]. Strategies as developed by larvae to avoid noxious plant chemicals, and in turn attain morphological, physiological and behavioural specialization to exploit such resources are significant [41]. Phylogenetic conservatism in adaptation to different phytochemical profile of their host plants probably forms the basis of such preference among lepidopterans [42].

Several centauries of research has unfurled enormous information on variation in sequestration across *Danaus* [43], variation in their ontogenic pattern [44] along with phylogenetic alteration in milkweed butterflies across the globe [45,46,47]. Although reports regarding the utilization of larval host plants by Danainae are available from the Indian perspective [30,31], any investigation on the host plant chemistry mediating such choices is lacking. Thus the present study attempts to provide a preliminary report based on the utilization of cardiac glycosides (CGs) based primary host plant by danainae larvae and pyrrolizidine alkaloids (PAs) based secondary host plants by adults. Significantly a total of 16 Danainae (Danaini and Euploeini) were observed across the entire study area. 16 plant species belonging to Asclepiadaceae, Apocynaceae and Moraceae were identified as larval resources of butterflies (**Table 3.1**). Additionally, nine plant species (Asteraceae, Boraginaceae, Fabaceae, Verbenaceae) were recognized for their assemblages of adult butterflies. Dead/ decayed/withered/ bruised parts of these plants attracted huge aggregation of species (**Table 3.1**).

Table 3.1: **List of daninae butterfly species along with their primary (larval) and secondary (adult) host plants**

Observed Butterfly species	Primary host plant of Danainae (Larval Resource)	Secondary host plant of Danainae (Adult resource obtained from dried/damaged plant substrates)
Tirumala limniace (Cramer)	*Asclepias curassavica, Calotropis procera, Tylophora indica, Wattakaka volubilis* **(Asclepiadaceae)**	*Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara*
Tirumala septentrionis (Butler)	*Asclepias curassavica, Wattakaka volubilis* **(Asclepiadaceae)**	*Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara, Tridax procumbens*
Danaus genutia (Cramer)	*Asclepias curassavica, Tylophora indica, Ceropegia woodii* **(Asclepiadaceae)**	*Chromolena odorata, Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara, Tridax procumbens*
Danaus melanippus (Cramer)	*Tylophora indica* **(Asclepiadaceae)**	*Chromolena odorata, Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara*

Danaus chrysippus (Linnaeus)	*Asclepias curassavica, Calotropis procera, Calotropis gigantea, Tylophora indica,Ceropegia woodii* (**Asclepiadaceae**)	*Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara, Tridax procumbens*
Parantica melaneus (Cramer)	*Asclepias curassavica, Calotropis gigantea, Ceropegia woodii* (**Asclepiadaceae**)	*Chromolena odorata, Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara, Tridax procumbens*
Parantica aglea (Stoll)	*Calotropis gigantea, Tylophora indica, Wrightia arborea, Tylophora tenuis, Ceropegia woodii, Cryptolepis buchanani* (**Asclepiadaceae**)	*Chromolena odorata, Crotalaria retusa, Heliotropium indicum., Lantana camara, Tridax procumbens*
Parantica sita (Kollar)	*Tylophora tenuis* (**Asclepiadaceae**)	*Chromolena., Crotalaria retusa, Heliotropium indicum, Lantana camara, Tridax procumbens*
Euploea sylvester (Fabricius)	*Ichnocarpus frutescens* (**Apocynaceae**), *Hoya parasitica* (**Asclepiadaceae**), *Ficus glomerata, F.hispida, F.hirta, F.retusa* (**Moraceae**)	No species were recorded
Euploea mulciber (Cramer)	*Ichnocarpus frutescens, Nerium odorum* (**Apocynaceae**), *Ficus glomerata, F.hispida, F.hirta, F.retusa* (**Moraceae**)	*Chromolena odorata, Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara, Tridax procumbens*
Euploea midamus (Linnaeus)	*Nerium odorum* (**Apocynaceae**), *Ficus hispida, F.hirta, F.retusa* (**Moraceae**)	*Chromolena odorata, Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara, Tridax procumbens*
Euploea klugii Moore	*Ficus glomerata, F.hispida, F.retusa* (**Moraceae**)	No species were recorded
Euploea algea (Godart)	*Ichnocarpus frutescens* (**Apocynaceae**), *Ficus glomerata, F.hispida, F.hirta* (**Moraceae**)	*Chromolena odorata, Ageratum conyzoides. Crotalaria retusa, Heliotropium indicum, Lantana camara*
Euploea radamanthus (Fabricius)	*Ichnocarpus frutescens* (**Apocynaceae**), *Ficus glomerata, F.hispida, F.hirta, F.retusa* (**Moraceae**)	*Chromolena odorata, Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara*
Euploea doubledayi C. & R. Felder	*Ichnocarpus frutescens* (**Apocynaceae**), *Ficus glomerata, F.retusa* (**Moraceae**)	No species were recorded
Euploea core (Cramer)	*Ichnocarpus frutescens, Holarrhena pubescens, Nerium odorum* (**Apocynaceae**), *Tylophora indica, Cryptolepis buchanani* (**Asclepiadaceae**), *F.hispida* Linn., *F.hirta* Vahl., *F.retusa* Linn. (**Moraceae**)	*Chromolena odorata, Ageratum conyzoides, Crotalaria retusa, Heliotropium indicum, Lantana camara, Tridax procumbens*

However the secret of such preferences among different stages of butterflies lies in the biochemical profile of their food plants. Hierarchial Clustering revealed the formation of two clusters i.e. Asclepiadaceae and Apocynaceae-Moraceae based on similarity in food plant families. Creation of subclusters with these two clusters with lesser intercluster distance suggested a closer similarity in the larval resource utilization (**Figure 3.1**).
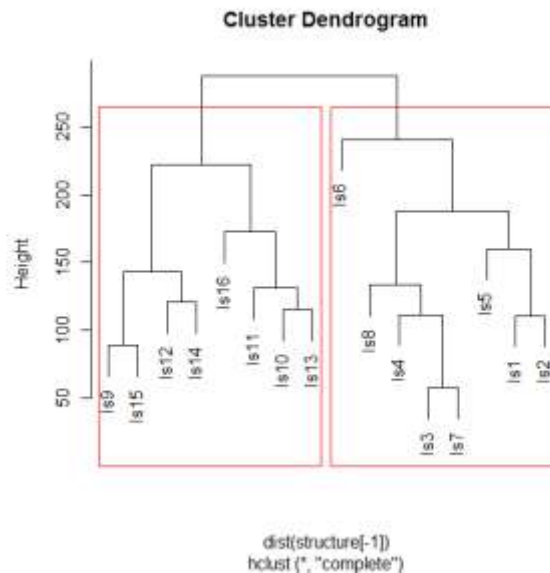
Figure 3.1: Cluster Dendrogram representing the utilization of larval plant resource by danainae butterflies (where:ls1: *Asclepias curassavica,* ls2: *Calotropis procera,* ls3:*Calotropis gigantea,* ls4:*Tylophora indica,* ls5: *Hoya parasitica,* ls6: *Wattakaka volubilis,* ls7: *Cryptolepis buchanani,* ls8: *Ichnocarpus frutescens,* ls9: *Holarrhena pubescens,* ls10:*Wrightia arborea,* ls11: *Ceropegia woodii,* ls12; *Ficus glomerata,* ls13: *Ficus retusa,* ls14: *Ficus hispida,* ls15: *Ficus hirta,* ls16: *Nerium odorum*)

Danaus plexippus were the pioneers in the studies on cardenolide sequestration since 50 years [48,49]. Such butterflies are blessed with an ability of modification based upon specific amino acid substitutions in cardenolide binding site of Na$^+$/K$^+$ ATPase. Later, several workers believed that most beneficial amino acid substitutions in such binding sites occur at 111,119 and 122 along with 786 and 797 in some specific clades thereby providing them with enhanced resistance [50.47]. [51] attempted to summarize the processing and storage of cardenolides by hypothesizing that monarchs are engaged in selectively storing cardenolides of higher and intermediate polarities. However cardenolides with lesser polarities are subsequently metabolised into compounds of intermediate polarities for storage. Such regulation in cardenolide sequestration is probably linked with their association with a diversity of milkweed species encountered both within and between generations [44]. An interesting switchover in the sequestration mechanism among monarchs was found to be shaped by its ontogeny. While caterpillars sequestered larger amount of cardenolides as early instar larvae, fifth instar compensated by feeding on lower cardenolide diet with substantial sequestration. Significantly voruscharin was reported to constitute 40% of leaf cardenolides. Reports on the conversion of voruscharin to calotropin, and calactrin thereby imposing an additional burden on growth are available [52]. Voruscharin was also recognised for being highly inhibiting (more than 50 fold times) in monarch neural Na$^+$/K$^+$ ATPase thereby exhibiting an immensely popular defensive potential. Notably oviposition was reported to be greatest on plants with intermediate cardenolide content in turn creating a trade-off between costs and benefits of sequestration.

Since sequestration was predominantly driven by predators or parasitoids, the components of third trophic level, it provides a picture on the involvement of higher trophic levels in any co-evolutionary interaction [46,53] in turn influencing insect host plant interactions. Specialist caterpillars are believed to be less palatable to predatory ants as compared to generalist caterpillars [54]. So predation could rightly be recognised as a selective force shaping specialist feeding behaviour. Accordingly, specialist herbivores with narrow diet breadth probably sequester more efficiently as compared to species feeding from a wider repertoire of host plants [55]. Thus in the present study sequestration could probably be highly recognised in monophagous species possessing narrow niche breadth (*Parantica sita, Danaus melanippus*) .

However specialist species with narrow diet breadth may not provide restricted opportunities for host shifts or speciation. It was reported that switching over of host plant based sequestered phytotoxin to a newer host plant possessing a different set of defensive toxin may be governed by "dual defence system" [56]. Such episodes of host shifting made on distantly related plants with similar phytochemistry or sequestration physiology may probably support the occurrence of polyphagy as reported among the studied species. *Euploea core* with highest niche breadth and polyphagy index (NB: 7.930; PI: 5.477) was indicative of their utilization of widest repertoire of larval food plants (**Figure 3.2**). Additionally *Danaus chrysippus* (NB: 5.7012; PI: 3.464), *Parantica aglea* (NB: 5.995; PI: 3.464), *Euploea sylvester* (NB: 5.981; PI: 3.873) and *E. mulciber* (NB: 5.921; PI: 3.873) were also significant (**Figure 3.2**).
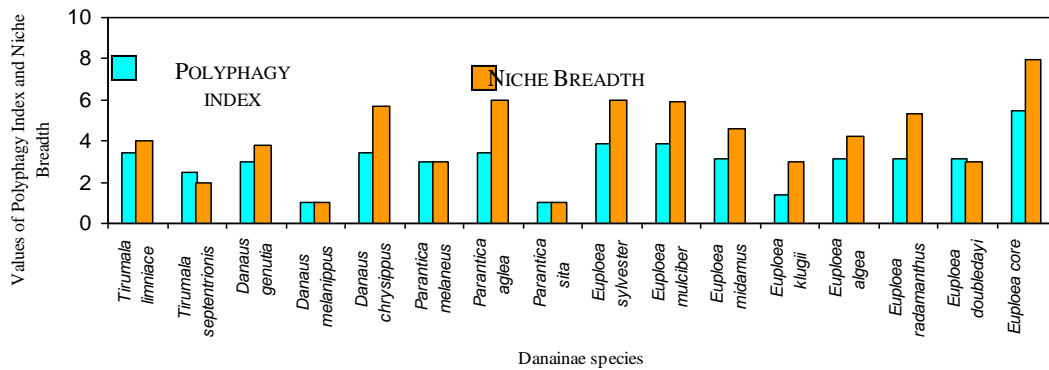
Figure 3.2: Polyphagy Index and Niche Breadth values of larval danainae species

Thus sequestration can not be considered to be an evolutionary deadend, because it opens up newer avenues for species exploring a wider range of host plants (with expanded diet breadth) sharing a diverse phytochemical profile. Also evident in the present investigation, besides *Danaus*, *Euploea* also explore a wide range of host plant species with varied level of sequestration. While *Danaus* accumulate cardenolides exclusively in the midgut lumen, non-sequestering *Euploea* degrade and eliminate such toxins selectively starting from midgut and extending up to hindgut. [57,55]. Such spatial separation along with compartmentalization in the utilization of cardenolides among different milkweed species appears noteworthy. Importantly cardenolide resistance of Na^+/K^+ ATPase also exhibit a phylogenetic event among milkweed butterflies beginning with *Euploea core* (being highly sensitive) leading to *Danaus gilippus* (possessing intermediate resistance) and finally ending with *D. plexippus* (being highly resistance), the level of sequestered cardenolides at three discrete levels of resistance is quite worth mentioning. Although the present study also reports the association of *Tirumala* and *Parantica* with their larval host plants, but their role in sequestration requires further refining. Therefore cardenolide chemistry appears to be the major determinant in shaping the tritrophic interaction between butterflies, their host plants and their natural enemies.

Pyrrolizidine alkaloids (PA)-pharmacophagous: a well established syndrome amongst lepidopterans i.e. Danaini, many Ithomiini and Arctiini is an iconic event with particular relevance. Insects exhibiting pharmacophagy gather PAs from plant species belonging to Apocynaceae, Asteraceae, Boranginaceae and Fabaceae [58,59,60,61]. Significantly, 13 out of 16 Danainae species were observed aggregating on the dried or injured plant substrates belonging to nine species (**Table 3.1**). Assemblages of *Danaus, Tirumala* and *Euploea* as observed in the present study were also reported from *Crotalaria, Artemisia, Heliotropium, Eupatorium, Senecio* and *Chromalaena* [62,63,64,29,65]. PAs are beneficial as components of male courtship pheromone [66,67,68,69] but fail to attract females, instead stimulating them at close range thereby mediating mate choice. Thus PA-pharmacophagy appears to be male biased behaviour for danainae butterflies. Such alkaloids being important precursor for pheromone production are required for the biosynthesis of danaidone and hydroxyl- danaidone [67]. Pre-copulatory ritual is visualised as hovering males over receptive females with hair-pencils extruded thereby disseminating pheromones to be recognised by their prospective mates [70,71]. PAs are transferred by the males as nuptial gifts packed within spermatophore to females to be incorporated into their eggs [72]. Thus a successful courtship could be equated with mating success and fecundity [73,74]. Although being a temporary event, sequestration of PAs is said to provide a biological advantage to such species. Being a costly affair, concerned species are found to be involved in locating a suitable dry plant substrate, then dissolving the matter by application of a fluid (regurgitated gut content or saliva), which is finally re-imbibed [75,76]. Regression analysis as observed in the study depicted the formation of a feeding guild involving adult butterflies based on their utilization of PAs. A significant relationship was found between the number of visits by butterfly species and the duration of their visits on the dried/withered/damaged/injured plant substrates (R^2:0.79; N=9) (**Figure 3.3**).
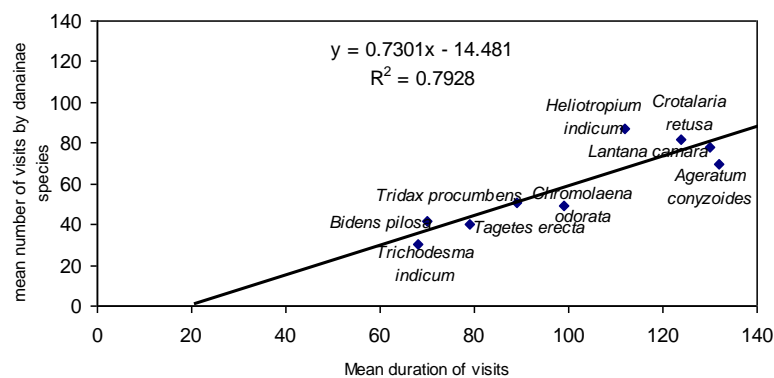

Figure 3.3: Variation in the number of visits by danainae species in relation to the duration of such visits

Butterflies are known to gather PAs from "secondary or adult host plants" by repeated "scratching" with their tarsal claws. However, PAs are non-volatile and thereby remain undetectable over a distance, but attractive odours are released when PAs are degraded by hydrolysis in a humid environment [29]. Such odour are in turn probably known to generate an information entropy

(expressed as Shannon-Wiener Function values) to be received by the other danainae butterflies in turn favouring the creation of larger aggregations. *Ageratum conyzoides* (H':3.631), *Crotalaria retusa* (H': 3.517), *Heliotropium indcum*(H':3.548) and *Lantana camara* (H': 3.546) with higher values of Shannon Wiener Function (H'>3.5) probably possess a rich PA source in turn generating a greater information entropy attracting a larger aggregation of butterflies **(Figure 3.4).**
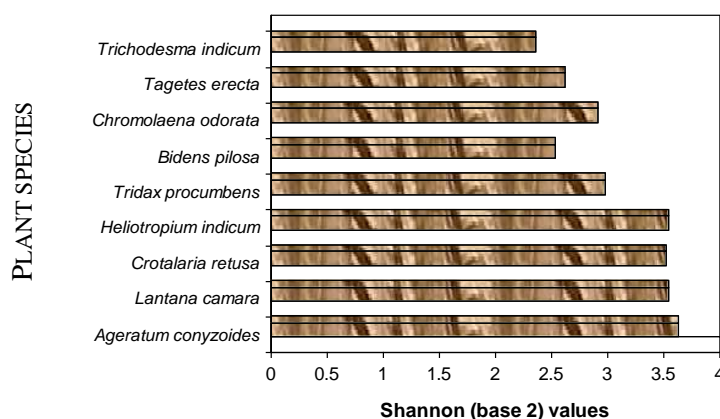

Figure 3.4: Shannon-Wiener values of plant species attracting assemblage of adult butterflies

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