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Chemical Communication in the Courtship of the Small Sulphur Butterfly *Eurema lisa* (Lepidoptera, Pieridae)

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Summary. 1. A receptive *Eurema lisa* female assumes a rigid posture and extends her abdomen out from between the hindwings in response to male courtship behavior.

2. Abdominal extension can be elicited from a virgin female in the laboratory by rubbing the antennae and thorax of a female with a live male restrained in forceps. An assay was developed using this response.

3. This behavioral assay showed that a male scent is required, in part, to elicit abdominal extension from virgin females.

4. The source of the scent is a patch of differentiated scales on the ventral surface of the base of the forewing. There are cells in the integument of the forewing which are in close association with the attachment points of these scales.

Introduction

Male butterflies of the family Pieridae (whites, sulphurs, and orange tips) are well known for possessing structures believed to be involved in scent production (Barth, 1950; Bergström and Lundgren, 1973; Jarvis, 1953; Silberglied, 1973; Taylor, 1973; Warren, 1961). In the pierid subfamily Coliadinae (the sulphurs), these structures, often called sex brands, are typically in the form of patches of specialized scales (androconia) near the wing base on the ventral surface of the forewing or on the dorsal surface of the hindwing (Common and Waterhouse, 1972; Silberglied, 1973). Although such structures are believed to produce chemical signals that are used during the courtship to stimulate the female, there is only a single study in the literature of chemical communication in pierids (Taylor, 1973). In that study, it was documented that males of two sympatric species, *Colias eurytheme* and *C. philodice,* emit a scent used by females for species discrimination. However, the morphological source of the scent was not localized, nor was the scent's effect on the behavior of the female precisely defined.

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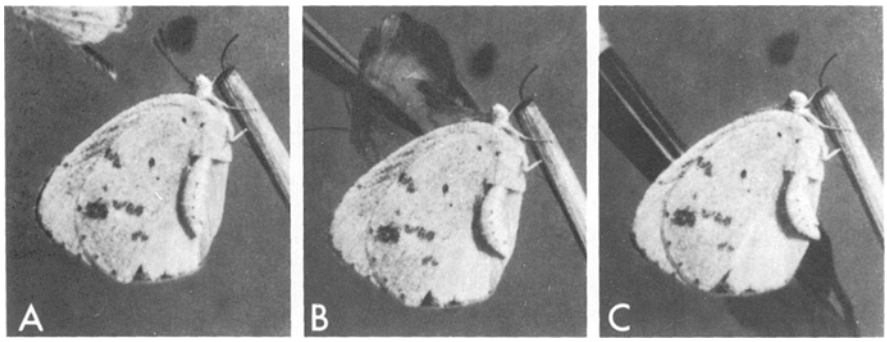

Fig. 1A–C. *E. lisa* female extending her abdomen in response to stimulation with the edges of the wings of a male held in forceps. Part of the female's hindwing has been cut away to expose the abdomen. **A** Before stimulation. **B** During stimulation, abdomen beginning to curl. **C** After stimulation. Note the extension of the abdomen beyond the edge of the hindwing

During a study of the courtship of the small sulphur, *Eurema lisa,* it became apparent that a stereotyped behavior, performed by the female during the courtship, might be elicited by a chemical scent given off by the male. In the courtship of *E. lisa,* the male buffets the perched female with his wings. She responds to this by assuming a rigid, wings-closed posture and extending her abdomen out from between the hindwings, thereby making her genitalia available to the male for copulation (Rutowski, in prep.). In the lab it was found that an identical response could be elicited from virgin females by stimulating their antennae and thorax with the distal edges of the wings of a live male restrained in forceps (Fig. 1). The virgin females responded much less frequently to identical stimulation with a live female. This preliminary observation was then formalized into a bioassay which, in this study, was used to determine whether the males of *E. lisa* produced a chemical signal and, if so, to localize its source.

It should be mentioned that although congeneric males had been noted to have sex brands in the typical coliad positions (Common and Waterhouse, 1972), no patches of specialized scales or other apparent scent producing structures were noted in *E. lisa* males prior to the experiments.

Materials

This study was carried out during the summer of 1973 and during the spring and summer of 1974. All eggs, larvae, and adults of *E. lisa* were obtained within a 110 km radius of the Archbold Biological Station in Lake Placid, Florida. Virgin females used as subjects in the bioassay were hand-reared from larvae or eggs found in the field or from eggs oviposited by captive females in outdoor cages. Larvae were fed on sprigs of the partridge pea (*Cassia fasciculata*) which were kept fresh in vials filled with water. In the laboratory used for rearing and experimentation, the air temperature was about 23° C, the humidity uncontrolled and variable, and the photoperiod regimen approximated 17–18 h of light and 6–7 h of darkness.

Methods

A. The Bioassay

A live male was used as a standard against which other stimuli were compared for their ability to elicit abdominal extension. To make this comparison, a protocol was developed in which a stimulus was lightly rubbed against the antennae and thorax of a virgin female once every 30 s for 5 s, or until she extended her abdomen from between the hindwings, whichever came first. The stimulus used in each case alternated between a live male ("control stimulation") and another specific stimulus ("experimental stimulation"). A response was recorded only if at least the tip of the abdomen appeared from between the female's hindwings. In tallying the results for a specific stimulus, such as a live female, only experimental stimulations, immediately preceded and followed by control stimulations which elicited a response, were scored. This served as a criterion for establishing the responsiveness of a virgin female during an experimental stimulation. Statistical comparison of the effectiveness of two stimuli in eliciting abdominal extension was made with a chi-square test for homogeneity at the 0.05 level of significance with one degree of freedom.

When used to stimulate a virgin female, intact butterflies, dead and alive, were held in forceps with the wings closed. Only the outer margins of the wings were used to contact the female. All dead animals had been dead at least one week at the time of their use. Other stimuli will be described as the results are presented.

Females were first stimulated 17 to 65 min after eclosion (mean, 41 min). In most cases, an experiment was ended when stimulation resulted in the female flying away, or when abdominal extension could not be elicited with any stimulus. When females were cooperative, experimentation was stopped after 30–40 min to avoid biasing the results with large amounts of data from individual females. Data from a single female never constituted more than 35% of the total data for a given stimulus.

B. Histology

Wings of live and dead *E. lisa* males and females were cleared in a dilute solution of $NaHCO_3$ (2–3 drops of 5.25% $NaHCO_3$ in about 15 ml of H_2O). The wings were left in the solution only long enough to bleach out all yellow color, and then dehydrated in ethyl alcohol, mounted on a slide with Zeiss mounting medium L 25 ($n_D = 1.525$), and examined with phase contrast microscopy.

Male wings were prepared for sectioning via fixation and embedment according to the methods of Ghiradella (1974) except that phosphate buffer (Millonig, 1962) was used throughout. Thick sections were made with an American Optical microtome, stained with basic fuchsin (Dawes, 1971), mounted in balsam, and observed using phase contrast microscopy.

Results

Of 90 virgin females stimulated with a live male, 25 never responded with abdominal extension, but either sat still, crawled away, flew away, or fluttered their wings. In several cases a second live male was used to stimulate an unresponsive female without success. All unresponsive females were discarded.

The remaining 65 females extended their abdomen in response to stimulation with a male. The protocol outlined in the methods section was then initiated and 2 experimental stimulations performed with an object drastically different from the live male – the butt of a pair of forceps. Twelve females responded to the forceps and were discarded as non-discriminating. The other 53 females did not respond and were used in further experimentation.

A. Demonstration of the Male Scent

The first stimulus compared with a live male was a live female held in forceps with the wings closed (Table 1). The virgin females responded to the restrained female in only 20% of the experimental stimulations.

As controls for the highly repetitive nature of the assay and for testing a variety of stimuli on the same female, two randomized experiments were performed. In both randomizations, the scoring technique was the same as that used in all experiments, i.e., there had to be a positive response to a male 30 s before and after each stimulation with a female that was tallied. In the first randomization, the order of presentation of a male and a female was completely randomized using digits drawn from a random number table (Snedecor and Cochran, 1967). If the number drawn was even, a male was used to stimulate; if odd, then a female was used. This had no effect on the response pattern to the restrained female ($\chi^2=0.40$, $p=0.4$–0.5). In the second experiment, the same method was used to randomize the presentation of a male and female in each experimental stimulation of an otherwise normal protocol. Again, the female received the same number of responses as in the initial experiment (Table 1; $\chi^2=0.11$, $p=0.2$–0.3).

Why was the female so much less effective than the male in eliciting abdominal extension? Two strong possibilities were that (1) the female's ventral wing coloration differed from the male's, lacking key visual stimuli necessary to elicit a response, or (2) the female lacked some chemical factor possessed by the male. To distinguish between these alternatives a dead male with his wings closed was used to stimulate virgin females. The response to this dead male was identical to the response to the female (Table 1; $\chi^2=0.12$, $p=0.2$–0.3). It is assumed that the dead male was visually and tactilely identical to a live male, since the colors of butterflies do not, in the short run, fade with death, and the edges of the wings are nearly as brittle and stiff in live animals as dead. The low effectiveness of a dead male and a female in eliciting abdominal extension seems to be due to a lack of some chemical factor or scent associated with the live male.

To further demonstrate this, a dead male was used to stimulate the female tactilely while an intact, live male was held with the wings closed 3 to 5 mm in front of the virgin female (Dead male + Live male − no contact). This procedure significantly increased the frequency of responses to a dead male (Table 1; $\chi^2=47.4$, $p=0.9995$). The importance of the tactile stimulus in this assay was shown by simply holding a live male 3 to 5 mm in front of the female (Live male − no contact). In only one out of 80 cases did the female respond (Table 1).

B. Source of the Male Scent: Localization

By keeping tactile stimuli as constant as possible and testing various parts of the male's anatomy, an attempt was made to localize the source of the male's scent. The wings and body of a male were cut apart and tested separately. The wings were held in forceps and their outer margins used to stimulate

Table 1. Results of experiments demonstrating the existence of a male scent and localizing its source to the wings

Stimulus	Total stimulations	Number of females	% responses	% no responses
Live female	80	12	20	80
Randomized: Every stimulation (Responses to female)	23	5	26	74
Randomized: Every other stimulation (Responses to female)	58	4	22	78
Dead male	84	9	18	82
Dead male + Live male − no contact	80	8	71	29
Live male − no contact	80	12	1	99
Live male-wings	80	12	99	1
Live male-body	80	10	30	70
Live male-body + Dead male − no contact	40	6	28	72
Live male-forewings	40	7	95	5
Live male-hindwings	40	8	95	5

the virgin female. The body was pinned laterally through the thorax and the tip of the abdomen used to contact the female during stimulation. The wings alone were 99% effective in eliciting abdominal extension while the response to the body was not significantly different from that to a live female ($\chi^2 = 2.14$, $p = 0.75$–0.9). A control was run in which the visual stimulus lost by removing the wings from the body was simulated by holding a dead male 3 to 5 mm in front of females while stimulating them with the isolated male body (Live male − body + Dead male − no contact). This procedure did not significantly change the frequency of responses to the male body (Table 1; $\chi^2 = 0.09$, $p = 0.2$–0.3). These experiments suggest that the wings are the source of the male's scent.

Forewings and hindwings were separately tested and proved to be equally good at eliciting a response (Table 1).

The source of the scent was localized further by experiments in which the wings were cut in half. In the first such experiment, the forewings and hindwings of a male were cut in half along a line perpendicular to the wing veins creating apical and basal pieces. Basal pieces from all 4 wings were stacked so that only ventral wing surfaces were visible ("Male-basal"), held in forceps, and used to stimulate the virgin females. Apical pieces ("Male-apical") were handled in an identical manner. The basal pieces elicited significantly more responses than the apical pieces (Table 2; $\chi^2 = 11.1$, $p = 0.999$–0.9995) and were as effective as whole wings in eliciting a response ($\chi^2 = 0.254$, $p = 0.25$–0.50). In a complementary experiment, the forewings and hindwings were cut in half along a line almost parallel to the medial wing veins. As before, stacks of wing pieces were made and used to stimulate the female. Pieces from the leading edge of the forewing and trailing edge of the hindwing were combined in one stack, called "Male-outer wing pieces" or "Male-outer." The other pieces were stacked and called "Male-inner wing pieces" or "Male-inner". A significant difference

Table 2. Results of experiments localizing the source of the male scent to the ventral surface of the base of the forewing

Stimulus	Total stimulations	Number of females	% responses	% No. responses
Cuttings:				
Male-apical	40	3	70	30
Male-basal	40	3	97.5	2.5
Male-inner	40	5	95	5
Male-outer	40	5	74	26
Brushings:				
Brush	15	5	13	87
Male – ventral hindwing	20	5	20	80
Male – dorsal forewing	21	5	52	48
Male – dorsal hindwing	21	5	76	24
Male – ventral forewing	20	5	95	5

was observed in the ability of these two wing parts to stimulate female abdominal extension (Table 2; $\chi^2 = 6.28$, $p = 0.975$–0.99), only the inner pieces being as effective as intact wings ($\chi^2 = 1.54$, $p = 0.7$–0.8). The results of these experiments indicate that the basal areas along the inner margin of the forewing and the costal margin of the hindwing are the most likely source of the scent. These are the only areas common to the wing pieces which were most effective in eliciting abdominal extension, i.e. the male inner pieces and the male basal pieces.

If certain parts of a male's wing were brushed with a small paint brush (M. Grumbacher, 2017, size 0) and the brush then used to stimulate a virgin female, abdominal extension could be reliably elicited. This fortuitous observation was then formalized into an assay, in which response patterns to brushings from specific surfaces of the wing bases could be determined. The surface to be tested was brushed for 10 s starting 15 s before the brush was to be used to stimulate the female on the antennae and thorax. Immediately after stimulation the brush was wiped clean of scales and other material.

The response pattern obtained with brushings from the ventral forewing was not significantly different from that obtained with the male wings (Table 2; $\chi^2 = 2.58$, $p = 0.8$–0.9). However, brushings from all other surfaces were significantly less effective than intact wings. In fact, the ventral hindwing was no more effective than an intact live female in eliciting responses ($\chi^2 = 0$, $p = 0$). The only two surfaces not significantly different from one another were the dorsal hindwing and the dorsal forewing ($\chi^2 = 1.75$, $p = 0.75$–0.9). The ventral surface of the forewing is clearly implicated as the primary, if not the only, source of the male's scent.

C. Source of the Male Scent: Description

Observations with a stereo microscope and a compound microscope (Epi-illumination) revealed distinct differences between males and females in coloration

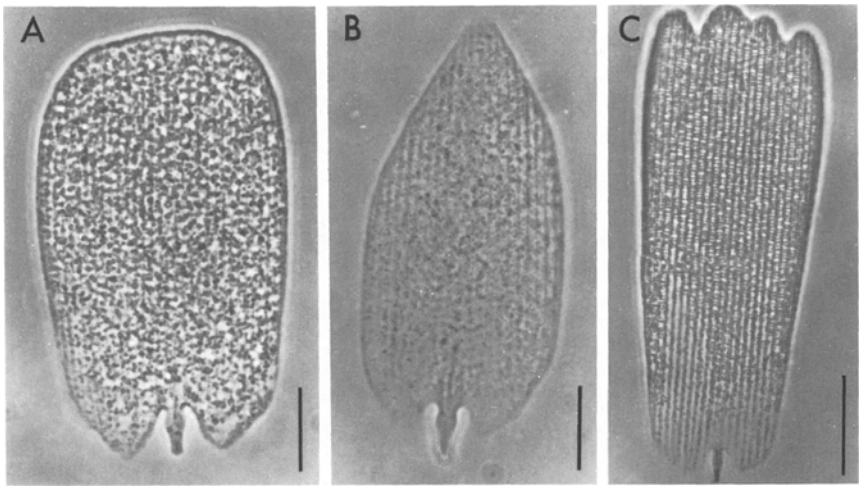

Fig. 2. Scales found on the ventral surface of the *E. lisa* male forewing. See text for details. Scale line = 20 micra

and in the distribution of scale types on the ventral surface of the base of the forewing. The greatest difference is in the area between the cubitus and the second anal vein, from the common origin of these two veins half way out to the wing margin. In the male this area is covered by cream-colored, apically rounded scales about 100 micra in length which are different in color and form from other scales on the ventral wing surface (Fig. 2A). The area below the second anal vein, from the wing base to a place below the first bifurcation of the cubitus, is characterized by transluscent ovoid scales (Fig. 2B). Other parts of the ventral forewing are covered mostly with longer (up to 150 micra) yellow scales, often scalloped apicad (Fig. 2C).

The female differs from the male in that she has none of the cream-colored scales in the basal area of the ventral forewing. Instead, the yellow scales found over the rest of the ventral wing surface extend posteriorly to a point halfway between the cubitus and the second anal vein, at which point they are replaced by transluscent, ovoid scales identical to those found in the male. These scales extend to the inner margin of the forewing. No other external differences were noted between males and females in this area of the forewing.

In the cleared preparations, cells with distinctly visible nuclei were found in the integument of wings taken from freshly killed males. The cells occurred in a well-defined area of the forewing (Fig. 3) which coincided precisely with the distribution of the male-specific scales. Moreover, each cell was associated with the location of a scale attachment insertion on the ventral surface of the forewing (Fig. 4A and C). Cells were not seen in other parts of the forewings (Fig. 4B) or in any part of the hindwing. Remnants of cells were found in the integument of the forewings of dead and dried males in the same area as whole cells were found in the wings of freshly-killed males. These remnants were not found in the wings of dead and dried females.

Sections from male wings confirmed the observation that the cells in the integument are associated with the point of attachment of scales on the ventral

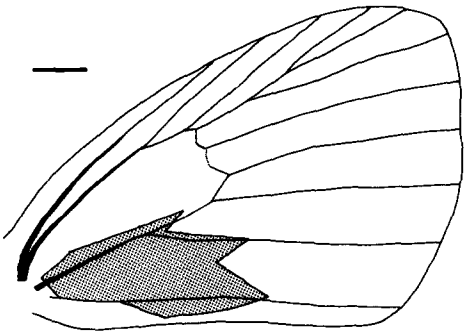

Fig. 3. Forewing of a male *E. lisa*. The stipling indicates the distribution of cells and ventral scales unique to that part of the wing. Scale line = 2 mm

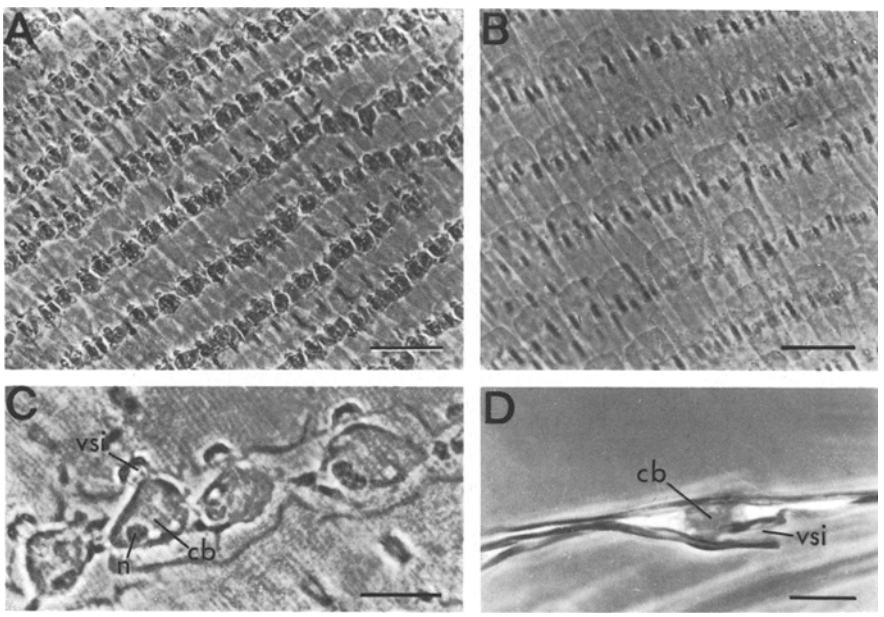

Fig. 4A–D. Photomicrographs from the forewing of an *E. lisa* male. **A** Area near the base of the forewing. **B** Area in the discal cell of the forewing. **C** Detail of cells (*cb*) and their nuclei (*n*) showing their close association with the ventral scale insertions (*vsi*). **D** Cross section of the forewing through the ventral scale insertion (*vsi*) and the cell (*cb*) within the wing integument. Scale lines: **A** and **B**, 65 micra; **C** and **D**, 20 micra

wing surface. It appeared that each cell resides within an expanded space between the dorsal and ventral wing integument (Fig. 4D).

Discussion

Chemical signals that are used by male butterflies to "seduce" females during the courtship have been experimentally documented for only a few species.

Best known is the extensive literature on the hair pencil secretion of the queen butterfly (*Danaus gilippus berenice* Cramer) and its allies (most recently see Schneider et al., 1975). It has been shown that *Danaus* males produce a pyrrolizidinone that is disseminated by the hair pencils and used to suppress the flight of females during the courtship, thereby facilitating copulation. A similar function was suggested for the scent believed to be produced by the sex brands on the wings of the Grayling *Eumenis semele* (Tinbergen et al., 1942). In preliminary experiments, males with their sex brands removed had difficulty keeping females in a quiescent state. Taylor's (1973) work with *Colias eurytheme* and *C. philodice* demonstrated a male scent that is used by females for species discrimination but the assay employed did not show how such discrimination was mechanically or behaviorally effected.

In this study, I have demonstrated with a laboratory assay that males of the small sulphur *Eurema lisa,* produce a chemical signal which is, in part, necessary to elicit abdominal extension reliably from virgin females. Since this response is a necessary prerequisite for copulation, the scent is probably an integral part of the stimuli presented to the female during the courtship, and used by her in deciding whether or not to accept a courting male. Thus the male scent functions as an aphrodisiac as in other species; however, it does so not only by causing the female to become quiescent but also by eliciting abdominal extension. The male scents of other species may evoke this behavior as well since female abdominal extension has been reported in the courtships of a number of species of butterflies including *Limenitis camilla* (Lederer, 1960), *Argynnis paphia* (Magnus, 1950), *Eumenis semele* (Tinbergen et al., 1942), *Heliconius erato* (Crane, 1955), *Colias eurytheme,* and *Colias philodice* (Silberglied, 1973). The male scent may have additional functions including involvement in male-male interactions, as occurs in some ithomiines (Pliske, 1975).

The results show that the chemical signal is only part of the stimulus required to elicit abdominal extension as tactile cues are also needed. Furthermore, the fact that some females tested did not respond at all to stimulation in the laboratory suggests that some other cues may have been lacking. Visual cues are a prime candidate based on experiments performed with *Colias eurytheme* (Silberglied, 1973) in which females made species discriminations on the basis of visual features of the males. A detailed analysis of the interplay of these various stimuli is required in a more natural situation.

How is the scent disseminated, both mechanically and behaviorally? In this regard it is interesting that, although the scent-producing structures are localized to a small patch on the ventral forewing, brushings from other wing surfaces are also quite effective in eliciting abdominal extension. This can be easily explained if one assumes that the scent is spreading from wing surface to wing surface by contact. When perched, butterflies hold the forewings within the hindwings. The only direct contact with the ventral forewing is made by the dorsal hindwing and, in fact, this is the surface which is second to the ventral forewing in eliciting responses. The dorsal forewing never contacts the ventral forewing but it could contact the dorsal hindwing on the contralateral side while the butterfly is sitting. Indeed, this wing surface ranks third in ability to elicit a response. The ventral hindwing never contacts any surface which

produces or carries the scent and, as might be predicted, it only elicits responses as often as a live restrained female. Thus, the brushing experiments show not only the source of the male scent but also suggest that the scent is spread to various wing surfaces via contact.

That the scent spreads out away from the bases of the wings is indicated by the experiments in which the wings were cut. In the first such experiment, the wings were cut into apical pieces (not bearing any scent-producing structures) and basal pieces (bearing the scent patch). Since the basal pieces were 75% effective in eliciting a response from virgin females the scent must spread out from the wing bases toward the wing tips.

The entire wing then appears to function as a disseminator of the male's scent which is presented to the female during the courtship as the male buffets the female with his wings. This relatively gross means of sending the chemical signal is by no means the only technique employed in the genus. In particular, *Eurema daira*, a species sympatric with *E. lisa* that presumably has scent-producing structures in the same location, has evolved a courtship which leaves less to chance. In that species the male alights alongside a perched female, extends his forewing on the side nearest the female, and strokes her antenna with the inner margin and ventral surface of his forewing (Myers, 1930; pers. observ.).

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