5A sieves at liquid nitrogen temperature, and then injected after heating for 1 minute at 100°C. Elution times for the N_2, (Ar-O_2), Ar, and outer column N_2 peaks were, respectively, 4, 9.5, 13.5, and 25 minutes at 0°C, with a flow rate of 0.03 liter/min. The analytical precision was ~0.3%, and accuracy was 0.5%. The GC values for O_2 were ~6% lower than the titration results because of bacterial consumption in the flasks and therefore were not used. When GC data for all three gases are used in Fig. 2, leakage of air into a flask moves a point parallel to the air injection slope, so that the ($\Delta O_2 - \Delta Ar$) increment above the air injection baseline is unaffected. When O_2 is analyzed separately, leakage into a flask affects only N_2 and Ar, and a point in Fig. 2 moves on a vector of 130° (Table 1). This means that the $(\Delta O_2)_J$ values will always be lower limits if air leakage occurs.

15. A plot of ΔN_2 versus ΔAr provides limits on the Δ_P and Δ_T effects because the vectors for Δ_P (slope is 1) and Δ_T (slope is 0.9, 15° to 23°C) are similar to each other, but quite different from that for air addition (slope is 2.2, 15° to 23°C). Subtracting the air component from the ΔN_2 and ΔAr values leaves the combined (Δ_P and Δ_T) anomalies ("$\Delta_{P,T}$") for each sample, which are necessarily identical for all three gases. For the subsurface samples at 28°N, $\Delta_{P,T}$ varies from 0.7 to 1.4%, corresponding to a range in Δ_P of that same magnitude or a Δ_T range of +0.4° to 0.8°C or a combination of both effects. At 40°N the subsurface range of $\Delta_{P,T}$ is −1.7 to +0.8%, corresponding to a Δ_P of that magnitude or a Δ_T of −1° to +0.5°C or a combination. These values are not unexpected. However, the surface water values of $\Delta_{P,T}$ at 28°N and 40°N are −5.3 and −2.2% for depths of 1 to 2 m at midday. (At 28°N this effect makes $F_J > 1$.) These negative values represent bulk loss of gases that causes negative Δ_P anomalies, possibly due to cavitation from the cycloidal propellers of the R.V. *Melville*. A final effect is partial solution of gases from bubbles, which results in preferential injection of gases according to the $(\beta D)_i$ values in Table 1, that is, in a component enriched in the order of Ar, O_2, and N_2 relative to air, and thus intermediate in composition between an unfractionated Δ_P component and actual air (*10*). In Fig. 2, partial solution of bubbles moves a point on a vector with a lesser slope than that shown for bulk air injection, so that the value of $(\Delta O_2)_J$ is slightly underestimated when the bulk air baseline is used. This effect is probably never significant for O_2, but experiments on this process would be of interest.

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17. We thank J. Costello for the hydrographic and O_2 measurements aboard the R.V. *Melville*, and K. Rubin for the N_2 and Ar analyses at Scripps Institution of Oceanography; we also thank Y. Horibe and V. Craig for help both on land and at sea. Research supported by NSF grants OCE84-03111 (H.C.) and OCE85-09839 (T.H.).

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Identification of a Juvenile Hormone-Like Compound in a Crustacean

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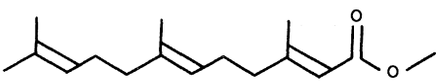
Juvenile hormone (JH) has central roles in the regulation of insect development and reproduction but has not previously been identified in other arthropod classes. The hemolymph of a crustacean, *Libinia emarginata* (Leach), has now been analyzed for JH-like compounds. Samples contained 0.003 to 0.030 nanogram of JH III per milliliter and 10 to 50 nanograms of methyl farnesoate per milliliter; methyl farnesoate is a compound structurally related to JH III that has JH bioactivity. Several tissues were examined for synthesis and secretion of JH-like compounds. Of these tissues, only the mandibular organs produced and secreted JH III and methyl farnesoate. However, microchemical analysis revealed that this JH III was racemic, and thus likely an artifactual oxidation product of methyl farnesoate. Secretion of methyl farnesoate was related to reproduction in females, with the highest rates observed in *Libinia* near the end of the ovarian cycle when oocyte growth and vitellogenesis are greatest. These results indicate that JH-like compounds such as methyl farnesoate have regulatory roles in crustaceans.

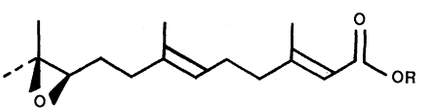
THE JUVENILE HORMONES (JHs) ARE a family of sesquiterpenoid compounds that regulate both metamorphosis and gametogenesis in insects (*1*). With the discovery that ecdysteroids regulate molting in both insects (*2*) and crustaceans (*3*) investigators have speculated that Crustacea might also contain a compound related to JH. This possibility is supported by the detection of JH biological activity in extracts of crustacean eyestalks (*4*) and data on the effects of synthetic JH analogs on crustacean metamorphosis and reproduction (*5–8*). These results have proven difficult to interpret, however. For example, a number of compounds that occur widely in nature have JH activity in bioassays. Thus, the juvenilizing activity found in crustacean extracts (or vertebrate and plant extracts) has generally been attributed to the presence of such compounds (*9*). Likewise, the high concentrations of JH analogs used in many studies with crustaceans suggest that the observed responses may be nonspecific toxic effects.

In an attempt to identify a crustacean JH directly, we obtained hemolymph from three groups of adult spider crabs (*Libinia emarginata*): females, males, and males from which eyestalks had been removed. Acetonitrile extracts of these samples were processed (Fig. 1) and analyzed by gas chromatography–mass spectrometry (GC-MS) with selected ion monitoring (*10*). Extracts exhibited peaks at mass-to-charge ratios (*m/z*) of 76 having the same retention time as the d_3-methoxyhydrin derivative of JH III (Fig. 1, A and B), but no substance was detected consistently at *m/z* 90 with a retention time identical to that of the corresponding derivative of JH 0, JH I, or JH II. Furthermore, we were able to detect JH III acid (Fig. 1C) in the samples by using a modification of the methodology described by Bergot *et al.* (*10*) (see legend to Fig. 1).

We also analyzed hemolymph samples for methyl farnesoate (MF), a compound structurally similar to JH III but lacking the epoxide moiety (see structures). MF is produced by the corpus allatum of some insect species (*11–13*), has JH activity (*12–14*), and may function as a JH at some developmental stages (*12, 13*). GC-MS analysis of these samples revealed the presence of a material eluting with the same retention time as MF and exhibiting peaks at *m/z* 69, 114, and 121 (Fig. 2) in the ratios characteristic of this compound.



Methyl (2*E*,6*E*)-farnesoate



(10 *R*) Juvenile hormone III (R = CH_3)
(10 *R*) Juvenile hormone III acid (R = H)

The concentrations of JH-like compounds in the above hemolymph samples are shown in Table 1. The relative concentrations of these compounds were similar in each group, with MF exceeding JH III by about a factor of 1000. The lowest concentration of MF and related compounds was found in adult females; these compounds were present in about three times this concentration in hemolymph of adult males. The highest levels of these compounds (five to ten times that of the concentrations in adult females) were observed in males from which eyestalks had been removed 14 days

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earlier. Removal of eyestalks, an important neuroendocrine center in crustaceans, affects the progression of larval metamorphosis (*15*) and adult vitellogenesis (*16–18*), results reminiscent of those produced in insects by elevations in JH levels. Thus, the effects of eyestalk removal correlate well with our observations that this surgery increases the concentration of JH-like compounds in hemolymph, and they may reflect the lack of a factor secreted by the eyestalk that normally inhibits the secretion of JH-like compounds.

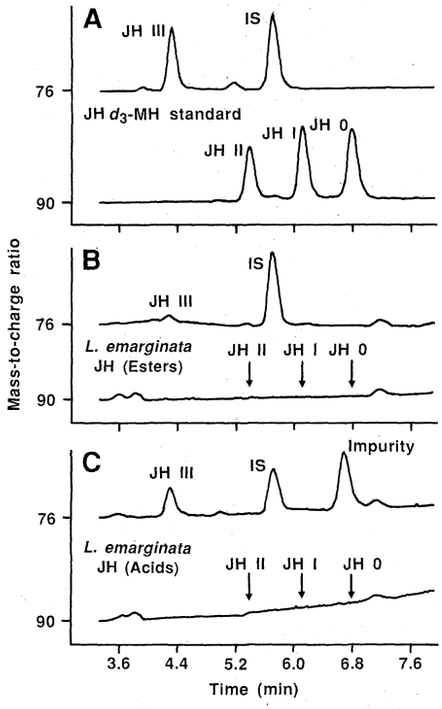

Fig. 1. Analysis of hemolymph for JHs and JH acids. Hemolymph from several animals was pooled and added to an equal volume of cold acetonitrile. A small amount (0.1 μCi) of [10-^{3}H]iso–JH II ethyl ester (5.8 Ci/mmol) (*10*) was added to each sample as an internal standard (IS), and the acetonitrile extract was partitioned between *n*-pentane and 4% brine (recovery ~95% in the pentane layer). Another portion of internal standard was added to the aqueous lower phase, which was extracted with ethyl acetate to remove JH acids (recovery ~70%). Samples were dried and stored under N_2 at −20°C until processed and analyzed by the procedure of Bergot *et al.* (*10*). This procedure consists of separating JH from major lipid classes by solvent partitioning and two filtration-type chromatographic steps. The JHs are then converted to their d_3-methoxyhydrin (MH) derivatives by solvolysis of the epoxide moiety in acidic d_4-methanol. These derivatives are purified by a group separation on normal-phase LC before their final analysis by GC-MS. JH acids were converted to the corresponding methyl esters with diazomethane, followed by purification and analysis as above of the JHs so produced. (**A**) d_3-Methoxyhydrin standards, including JH 0, JH I, JH II, iso–JH II ethyl ester, and JH III. Upon GC-MS analysis with selected ion monitoring, the first three gave intense peaks at *m/z* 90, and the last two gave peaks at *m/z* 76. (**B**) GC-MS analysis of JH d_3-methoxyhydrins derived from extracts of male *Libinia* hemolymph. Only JH III was detected. (**C**) GC-MS analysis of JH d_3-methoxyhydrins derived from JH acids extracted from male hemolymph. Only JH III was detected, indicating the presence in the hemolymph of JH III acid. GC conditions: 2 m by 2 mm 3% OV-101/Ultrabond II; temperature programmed from 200° to 260°C at 5°C per minute. Electron impact mode.

To determine the source of these compounds in the spider crab, we used an approach similar to that used for the study of JH biosynthesis by the insect corpora allata (*19, 20*). Tissues were incubated in vitro with methyl-labeled methionine, and incorporation of the methyl group into JH-like compounds was determined. Among the tissues tested were the mandibular organs (MOs), which have been implicated in the regulation of crustacean reproduction (*21–23*). MOs were located following published descriptions (*24, 25*); electron microscopy revealed ultrastructural features similar to those reported by others (*24, 26*).

Mandibular organs secreted a major radiolabeled product that eluted with MF on normal-phase liquid chromatography (LC) (Fig. 3), and several minor products, one of which eluted with JH III. When an MO from an adult male was incubated with 20 μ*M* [*methyl*-^{3}H]methionine (1 mCi/ml; 50 Ci/mmol), the observed rate of secretion was 2 and 0.005 ng/hour per gland for MF and JH III, respectively. When MOs from male animals were incubated with 250 μ*M* [*methyl*-^{14}C]methionine (10 μCi/ml; 45 mCi/mmol), the observed rate of MF secretion (from 0.4 to 12 ng/hour per gland) was about seven times that of the rate observed in contralateral glands incubated with [^{3}H]methionine. Higher concentrations of [*methyl*-^{14}C]methionine had no additional effect on the observed rate of MF secretion. This result suggested that the low concentration of the [^{3}H] precursor was limiting the secretion of radiolabeled MF. The rate of JH III secretion was too low for detection with [^{14}C]methionine.

When radioactive materials eluting with MF and JH III on normal phase LC were collected and analyzed by reversed-phase LC, more than 95% of the material eluted with the appropriate standard. Extracts of medium alone, or of medium from cultures of other tissues (muscle, hepatopancreas, eyestalk, and epidermis) of *Libinia* did not contain radioactivity that eluted with either MF or JH III, suggesting that these are unique products of the MO.

The identity of these two products secreted by the MO was further confirmed by GC–MS. Incubation media from MO cultures analyzed by the method of Bergot *et al.*(*10*) contained JH III and JH III acid (see

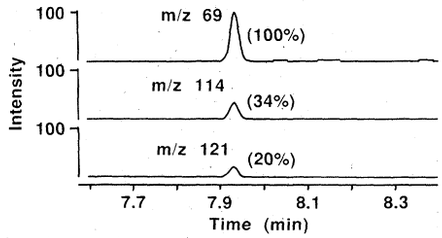

Fig. 2. Analysis of hemolymph for MF. A small amount (0.05 μCi) of (2*E*,6*E*)-[10-^{3}H]MF (1.6 Ci/mmol) was added as an internal standard to a portion (~20%) of the pentane extract of each hemolymph sample. Samples were processed as for Fig. 1, except that treatment with acidic d_4-methanol was omitted and the solvent for LC purification was 1% ether in pentane (solvent 50% saturated with water); zones corresponding to the region of (2*E*,6*E*)-MF ($k' \sim 3.0$) were collected for GC-MS analysis. Detection was by GC-MS with selected ion monitoring at *m/z* 69, 114, and 121. Quantification was by external standard with the *m/z* 69 ion (base peak). Correction was made to 100% recovery of radiolabeled internal standard and for its contribution to the mass of MF detected (only about 5% in the sample shown which is from male *Libinia* without eyestalks). GC conditions: 30-m DB5 capillary column (J.&W. Scientific); temperature programmed from 60°C (0.5-minute hold) to 180°C at 30°C per minute, and then to 270°C at 10°C per minute. Electron impact mode.

legend to Fig. 1C for GC conditions). For the identification of MF, MOs from several animals were incubated with [^{14}C]methionine overnight, and approximately 250 ng of secreted product ([^{14}C]MF) was purified by normal-phase LC, reversed-phase LC, then normal-phase LC once more, and analyzed by GC-MS. A full mass spectrum of this product was identical with that of a (2*E*,6*E*)MF standard, except that the predominant molecular ion had an *m/z* of 252, which is consistent with the occurrence of ^{14}C in the methyl group of this product.

Because JH III was present in such low amounts relative to MF, we tested the possibility that JH III was an artifact of MF oxidation (*27*). A small mass (134 ng) of [10-^{3}H]MF was highly purified by LC, under conditions that completely removed JH III, and then processed by the JH-titer methodology, including the final LC purification step, but not analyzed by GC-MS. Up to 0.3% of the radiolabel migrated in the region corresponding to JH III d_3-methoxyhydrin. Thus, it is possible that the low level of JH III detected in these biological samples resulted from the artifactual oxidation of MF at some step in the sample preparation. Likewise, when the radiolabeled JH III secreted by MOs was analyzed for chirality [by LC, after dilution with racemic JH III and conversion to its diastereomeric derivatives (*28*)], only 59% of the radiolabeled material had the 10*R* configuration, the

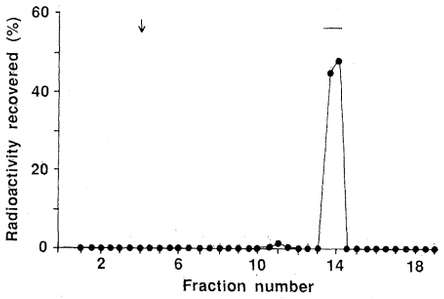

Fig. 3. The secretion of radiolabeled MF by MOs in vitro. Whole MOs or pieces of other tissue of a similar size were incubated in Pantin's saline (*31*) supplemented with radioactive methionine (in each milliliter, either 1 mCi [*methyl*-^{3}H]methionine or 10 μCi [*methyl*-^{14}C]methionine at 50 Ci/mmol and 60 mCi/mmol, respectively). After incubation for 2 hours, the medium was removed and the tissue rinsed and weighed. The culture medium plus ethanol rinses of the chamber were adjusted to 4% saline with NaCl and then extracted with pentane. Radiolabeled material in the pentane fraction was separated by normal-phase LC (Alltech, 5-μm silica gel, 250 by 4.6 mm; solvent, 1.5% ether in pentane) and the radioactivity in each fraction determined by liquid scintillation spectrometry. In some cases fractions eluting with MF were collected, redissolved in 70% acetonitrile in water, and analyzed by reversed-phase LC (μBondapak C_{18}, Waters). The figure shows the analysis of products secreted by an MO from a male *Lifinia emarginata* incubated with [^{3}H]methionine and analyzed by normal-phase LC. Radioactivity co-eluting with MF (dark bar) (arrow, solvent front) represents 97% and 64% of the amount eluted and injected, respectively.

Table 1. JH-like compounds in hemolymph of *Libinia*. Male and female *Libinia* were obtained from the Marine Biological Laboratory Resources Center (Woods Hole, MA) during the summer and maintained in running seawater for a total of 3 weeks. The eyestalks of some males were removed, and 14 days later 4 to 5 ml of hemolymph was removed from each animal and pooled.

Source	*n*	Carapace length (mm)*	(2*E*,6*E*)-MF (ng/ml)	JH III (ng/ml)	JH III acid (ng/ml)
Female	5	59 ± 2	11	0.003	0.10
Male	5	65 ± 6	24	0.01	0.11
Male, without eyestalks	6	51 ± 2	55	0.03	0.83

*Means ± standard deviations.

natural isomer in insects, while 41% was the 10*S* form. The absence of stereochemical purity at the epoxide implies that most of this product may be formed by nonbiological oxidation of MF. Taken together, these results strongly suggest that the trace levels of JH III detected in both hemolymph and culture medium are artifacts created during the work-up and analysis of these samples. The JH III acid detected in these samples may represent a similar oxidation product of farnesoic acid, a compound that was not measured in this study.

The rates of MF secretion varied considerably in MOs from different animals, suggesting that secretion might vary with the physiological state of the donor. However, the anatomical location of the MO makes it difficult to ablate this tissue for a direct investigation of its function. Therefore, we have studied the relation of MF to oogenesis by measuring MO secretory rates at different stages of reproduction in *Libinia*.

The in vitro secretory rates of MF by MOs from these animals were closely related to the stages of ovarian growth (Fig. 4). MF secretion was lowest, about 0.5 ng per gland per hour in juvenile animals and in adult females with orange and red embryos. The secretory rate was significantly [$\alpha = 0.05$ by comparison of rank sums (*29*)] higher, over 3 ng per gland per hour, in animals with brown embryos when oocyte development and vitellogenesis were occurring. The secretory rate of MF then dropped to intermediate levels, about 1.5 ng per gland per hour, in animals with gray embryos or with empty brood pouches when ovarian development was being completed.

This relation between secretion of MF by the MO and vitellogenesis in *Libinia* is in all likelihood a cause-and-effect response, since Hinsch (*23*) has already shown that implants of MOs into juvenile females stimulate ovarian development.

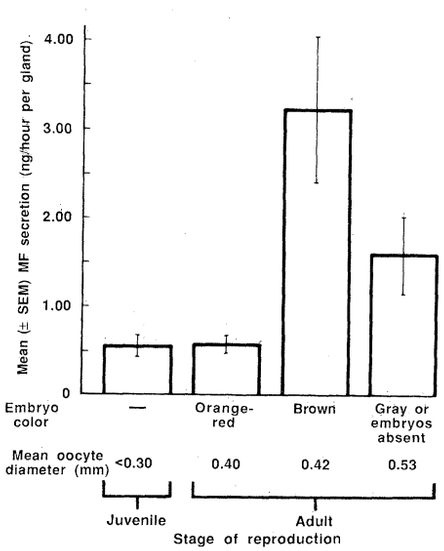

Fig. 4. Rates of MF secretion by MOs from female *Libinia* at different stages of reproduction. Juvenile females that were reproductively inactive were distinguished from adult females by the external morphology of their aprons. Stages in the ovarian cycle, approximately 20 days long, of adult females were estimated by the color of the embryos from the previous cycle (*32*). These embryos remain in the brood pouch until hatching and are initially orange, and then become red, brown, and finally gray. Gray embryos normally hatch within 2 days, and the brood pouch remains empty for about a day before the next batch of embryos is laid. Oocyte diameter was measured to confirm the stage of oocyte development and was found to rise at the end of the cycle, increasing from 0.40 mm in animals with orange or red embryos to 0.53 mm in animals with gray embryos or with an empty brood pouch. MOs were removed from females at the indicated stages, and MF secretion was determined by incubation with [^{14}C]methionine (as described in the legend to Fig. 3).

The results demonstrate that hemolymph from *Libinia* contains an appreciable amount of MF. To our knowledge, this is the first time a JH-like compound has been identified in a crustacean. In addition, we have demonstrated that the MO is a major source of MF in this species. There have been speculations concerning the function of the MO, including its possible homology to the insect corpus allatum (*21–23*), but this is, to the best of our knowledge, the first identification of a secretory product of this tissue. Finally, we have shown that the rate of MF secretion by the MO is related to vitellogenesis in the female, which suggests that this compound has a role in crustacean reproduction similar to that of JH in insect reproduction. Although a small amount of JH III was also found, this is probably the product of the artifactual oxidation of MF to JH III. While a role for JH III in this species cannot be ruled out, MF is clearly the major circulating JH-like compound. Whether MF is (i) itself a hormone acting directly on target tissues, (ii) a prohormone requiring conversion to JH III, or (iii) some other compound in target tissues has yet to be determined.

The occurrence of MF as an MO secretory product is not unique to *Libinia*. We have detected MF synthesis by MOs from other crabs and from the lobster *Homarus americanus* (*30*).

Our results provide strong evidence that the regulation of postembryonic development and reproduction by insects and crustaceans is similar. It seems likely that JH-like compounds have equivalent roles in other arthropod classes as well. Such information will be useful in predicting the potential impact on nontarget arthropods of JH analogs for third-generation insecticides. In addition, knowledge about the regulation of

crustacean development and reproduction will eventually be useful in the mariculture of beneficial species, such as shrimp and lobster, and in the control of deleterious species, including predators, parasites, and fouling organisms among the crustaceans.

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Evolution of Male Pheromones in Moths: Reproductive Isolation Through Sexual Selection?

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Central to our understanding of the species concept is knowledge of the nature and evolution of reproductive isolating mechanisms. The once widely accepted model of Dobzhansky, which holds that isolation evolves through selection against hybrids of differentially adapted populations, is now largely rejected. This rejection is due to both theoretical difficulties and a paucity of examples of the predicted pattern of reproductive character displacement. From a survey of five families of Lepidoptera, entailing more than 800 species, evidence is given that male courtship pheromones have evolved within the context of sexual isolation as an adaptive response to mating mistakes between differentially adapted populations; however, distinct from the natural selection model of Dobzhansky, this report suggests the mechanism for change to be sexual selection.

THE MECHANISMS BY WHICH INDIviduals of two noncompatible populations are prevented from mating are among the most important characteristics of species, representing the cornerstone of the biological species concept (*1, 2*); elucidation of how such mechanisms arise is important to our complete understanding of the speciation process. Barriers to mating that arise due to incompatibilities in behavior are termed ethological isolating mechanisms and represent the most important group of isolating mechanisms in animals (*1*). Theories that have been proposed to explain the origin of ethological isolating mechanisms may be characterized as either adaptive or incidental. According to the former group, ethological isolation arises as a response to selection against mating with an individual whose genotype is adapted to a different niche (*3, 4*). Thus, the production of either infertile offspring or hybrids that are not optimally suited to either niche brings about an increased tendency to mate assortatively, that is, with individuals of similar genotype. Proponents of incidental models, on the other hand, argue that species-specific mating patterns are only an incidental by-product of the genetic divergence that occurs when two populations become isolated in time or space (*5, 6*). Accordingly, if the potential for interbreeding is reestablished, the two populations will already be sufficiently distinct in their mating behavior that individuals from different populations will not recognize each other as potential mates, and interpopulational matings will be rare. Within each of these categories, differences also arise as to whether the vehicle for change in mating patterns is natural selection (*3, 5*) or sexual selection (*4, 6*). Here we present evidence from five families of Lepidoptera that male courtship pheromones in moths have evolved within the context of reproductive isolation through sexual selection.

The presence of male scent–emitting structures in Lepidoptera, represented by various brush organs, hair pencils, and wing folds, is usually associated with a courtship sequence that allows their presentation to the female before mating; the importance of male pheromones to mating success has been documented for a number of diverse groups (*7*). We examined the relation between the occurrence of male scent–disseminating structures and the probability of contact with closely related species to test the predictions of the adaptive response and incidental models for the origin of lepidopteran male pheromones within the context of reproductive isolation. Interspecific contact was indexed by the sharing of a common host plant by two members of the same genus, on the assumption that species that utilize the same host are at greater risk of making mating mistakes than those that do not. However, when seasonal or geographical information was available on adult activity, it was used as further evidence for the probability of interspecific contact. Thus, with regard to moth mating systems, the distinction between the predictions of the models is clear and testable. If male pheromone–emitting structures have arisen as an adaptive response to mating mistakes, then they should be preferentially found in spe-

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