that bind B_A and B_B, and the regions between the D and E helices that form most of the Q_A and Q_B binding sites.

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Correlated Evolution of Female Mating Preferences and Male Color Patterns in the Guppy *Poecilia reticulata*

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Sexual selection may explain why secondary sexual traits of males are so strongly developed in some species that they seem maladaptive. Female mate choice appears to favor the evolution of conspicuous color patterns in male guppies (*Poecilia reticulata*) from Trinidad, but color patterns vary strikingly among populations. According to most theory, correlated evolution of female mating preferences and preferred male traits within populations could promote this kind of divergence between populations. But mating preferences could also constrain the evolution of male traits. In some guppy populations, females discriminate among males based on variation in the extent of orange pigment in male color patterns, and populations differ significantly in the degree of female preferences for orange area. In a comparison of seven populations, the degree of female preference based on orange is correlated with the population average orange area. Thus male traits and female preferences appear to be evolving in parallel.

DARWIN (*1*) SUGGESTED THAT MATE choice by females can lead to the evolution of elaborate sexual display traits. Models of sexual selection (*2–6*) assume genetic variation in female preferences and suggest mechanisms for correlated evolutionary changes in both female preferences and male secondary sexual traits. Female mating preferences affect the evolution of male traits directly. Changes in female preferences may result from direct selection, indirect "good genes" selection (*5, 7*), or correlated effects of selection on other traits or functions. Environmental differences can affect how females perceive males, leading to nongenetic differences in the expression of preferences (*6*). In addition, the Fisher model of sexual selection (*2, 3*) predicts a within-population genetic correlation between female preferences and male traits because females with a particular preference tend to mate nonrandomly with males with the corresponding sexual display traits. This genetic correlation leads to changes in female preferences as an indirect response to selection on male traits, leading in turn to further changes in both characters. Each model predicts correlated evolution of female preferences and preferred male traits within populations. The direction in which male traits and female preferences evolve depends on stochastic and selective factors (such as direct selection on male traits by predators), so different populations are expected to evolve divergent suites of male traits and female preferences (*3, 4*). But it is also possible that female mate choice could limit the divergence of male traits if the evolution of mating preferences is constrained (*8–10*). Female choice for an ancestral type may explain why male butterflies are often nonmimetic in species with mimetic females (*8*), and conserved patterns of mate choice may promote hybridization in some fish species (*9, 10*). There is some evidence for genetic variation in female preferences (*11*), but there is little empirical information on the joint evolutionary dynamics of mating preferences and male secondary sexual traits to evaluate these ideas. We compared mating preferences of female guppies (*Poecilia reticulata*) from natural Trinidad populations that vary greatly in male color patterns to test the idea of parallel divergence in preferences and preferred male traits.

The color patterns of male guppies vary within and between populations, and some of this variation is related to differences in predation regime (*12*). Within populations, females have mating preferences based on variation in male color patterns, particularly orange spots (*7, 12–14*). Data from three streams show that female preferences differ among populations and suggest that male color patterns and female preferences may covary across populations (*15, 16*). We present data on preferences and color patterns from seven populations in six streams (Table 1).

All experiments were conducted with fish descended from individuals collected in Trinidad less than three generations previously and raised under standard conditions (*14*). Virgin females used in our experiments were reared in sibling groups and separated from males before male color patterns developed. Males were reared to maturity in sibling groups and then were allowed to interact with stock females for several days before we used them in experiments. Female choice is based on the relative area of orange pigment in male color patterns (orange area; *17*) and was studied by placing experimental groups of six males and six females in 1.0 by 0.5 by 0.3 m aquaria (with gravel on three

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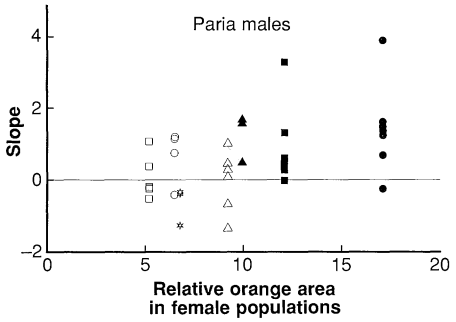

Fig. 1. Degree of female preference for orange among Paria males, as a function of the average orange area (×100) in the female source populations. Each point gives the degree of preference among six males in one observation session. Note that the number of observation periods is unequal among streams. The symbols indicate female source populations. Open square, Arima; open circle, Guanapo; star, Oropuche 1; open triangle, Oropuche 4; closed triangle, Quare; closed square, Marianne; and solid circle, Paria.

sides and the bottom), and observing the sexual responses of females to the courtship displays (*18*) of each male (*14, 16, 19*). The relative attractiveness of a given male to the females in the group was estimated as the fraction of his displays that elicited a sexual response (D). Individual females were not distinguished, so D is an aggregate measure of preference of all six females as a group. This behavioral measure of preference predicts male mating success (*20*). The degree of preference for orange area is estimated as the regression of D on orange area for all fish used in that session. The slope of this regression, which we call preference slope, is a measure of the overall degree of preference for orange area for females in a given observation trial. Previous experiments with guppies from Trinidad's Paria river showed a significant regression of D on orange area, indicating preference for males with greater orange area. Analysis of behavioral data and experimental manipulations suggest that the cue used by females is indeed the orange spots rather than correlated behavioral differences in males. There are no significant differences among orange area classes in either latency to court or courtship rate, and female preferences for orange disappear under orange but not other ambient light colors (*14*).

Each experimental group consisted of six males from one population and six females from the same or another population. Several replicate groups of males were drawn from each population during this study. Each male group was tested, in separate trials, with females from its own and one or more other populations, as well as the Paria population, which has the greatest development of orange (Table 1). Males were chosen so that each could be identified by unique color patterns, and their relative orange pigment area was recorded (*17*). Preference slopes were compared among females from different populations to test the hypothesis of correlated evolution of female preferences and male traits; we predicted that the preference slope of females should be correlated with the average orange areas of males from their own populations. Female preferences were compared in separate analyses for test males from each population (Table 1 and Figs. 1 to 3).

The average orange area of male color patterns varied from 5 to 17% of body area among populations (Table 1). Populations varied significantly in the strength of preferences based on orange area when females were tested with Paris, Marianne, and Quare males (Figs. 1 and 2 and Table 1); these are the three populations with the greatest mean orange areas. In all three cases the strength of female preferences was correlated with mean orange area of males from the female's population (Table 1). In tests with Paria males (Fig. 1), females from the three high orange populations consistently had positive preference slopes (16 of 17 trials), whereas females from the other four populations showed no consistent pattern of preference (8 of 18 trials positive; $\chi^2 = 10.01$, 1 df, $P < 0.001$). Tests with males from the Marianne and Quare populations showed a similar pattern but there are too few data from low orange populations for statistical tests. We interpret this as genetic variation because all fish tested were born in the laboratory and were reared under identical laboratory conditions, some for more than two generations.

Tests involving males from the remaining four low orange populations revealed no differences in female preferences between populations and no correlation with population orange area (Fig. 3 and Table 1). Females may have been unable to discriminate among males based on orange area in populations where there is little orange.

These results support a prediction of many different kinds of sexual selection theory: females from guppy populations with the most orange in males show the greatest degree of preference for orange, and those with little orange show little preference. Female preferences appear to have evolved in parallel with male color patterns in these populations. We do not know if variation in color patterns is simply a direct consequence of variation in preferences or if preferences themselves have evolved as an indirect re-

Table 1. Populations tested, amount of orange, and statistical tests for variation in female preferences shown in Figs. 1 to 3. Each row gives the results of experiments in which females from several populations were tested with males from one population. Population names are rivers in the northern range of Trinidad, where the stocks were collected; Oropuche 1 and 4 are different sites on the Oropuche river. Orange area is given as relative orange area (*17*) times 100. The abbreviation K-W is the Kruskal-Wallis test statistic for differences in degree of preference; r_s is the Spearman rank correlation between female preference slope and average relative orange areas of males from the population of females tested; and n is the sample size for orange area measurement (column 2) and total number of observation sessions (column 7).

Source of males tested	Orange area in male source [mean ± SEM (n)]	Source populations of females tested	Preference differences		Preference and color	
			K-W	df	r_s	n
Paria	17.0 ± 1.2 (41)	Paria, Arima, Oropuche 1, Oropuche 4 Guanapo, Quare, Marianne	16.8**	6	0.51**	36
Marianne	12.1 ± 1.2 (30)	Paria, Arima, Marianne	6.9*	2	0.69**	14
Quare	9.9 ± 0.5 (37)	Paria, Oropuche 4, Quare	6.3*	2	0.46†	15
Oropuche 4	9.2 ± 0.9 (29)	Paria, Oropuche 4, Quare	1.7‡	2	−0.08‡	14
Oropuche 1	6.4 ± 0.6 (23)	Paria, Oropuche 1, Guanapo	1.3‡	2	0.30‡	11
Guanapo	6.4 ± 1.0 (23)	Paria, Oropuche 1, Guanapo	0.6‡	2	−0.18‡	11
Arima	5.2 ± 0.4 (29)	Paria, Arima, Marianne	0.1‡	2	−0.05‡	13

‡$P > 0.10$; †$P < 0.10$; *$P < 0.05$; **$P < 0.01$.

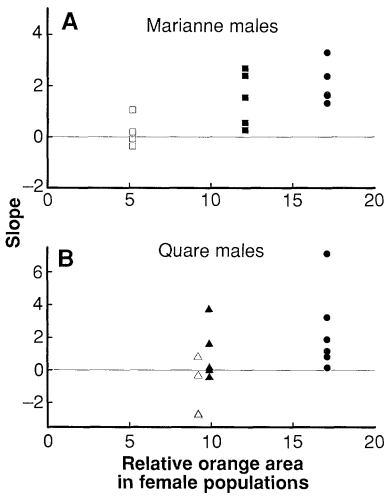

Fig. 2. Degree of female preference for orange among (**A**) Marianne and (**B**) Quare males, as a function of the average orange area (×100) in female source populations (defined in Fig. 1).

sponse to natural selection on color patterns as predicted by the Fisher model of sexual selection (*2, 3*).

Some published studies support (*15, 21*) and others contradict (*10, 13*) our results. Breden and Stoner (*15*) reported that female guppies from places with low predation intensity have a stronger preference for brighter and more actively courting males than do females from places with more severe predation. Males from low predation localities tend to have brighter color patterns than high predation males (*12*), but Breden and Stoner did not quantify the color patterns of the populations that they studied. Variation in the dominant frequency of advertisement calls of cricket frogs (*Acris crepitans*) is correlated with variation in the tuning of the female auditory system. This physiological difference among females is not a result of pleiotropy or body size allometry and results in preferential phonotaxis and possible assortative mating (*21*). In contrast to our results, Kodric-Brown (*13*) reported consistent preferences of individual female guppies based on differences in showiness of male color patterns and the amount of carotenoid (orange, red, and yellow) colors. Preferences were similar among females from two inbred laboratory strains and a domesticated common aquarium strain. This might reflect a genetic difference between domestic and wild guppy strains, which differ radically in history of inbreeding and selection for color patterns. In two other studies (*10, 22*), mating preferences are consistent between closely related species pairs, but preferred male traits differ, suggesting that preferences may precede the evolution of corresponding male traits. Females of both *Xiphophorus nigrensis* and *X. pygmaeus* fish prefer large-morph males with elaborate courtship, but such males are lacking in *X. pygmaeus*. Females of both *Physalaemus pustulosus* and *P. coloradorum* frogs are most sensitive to low-frequency chuck elements of male calls, but this element is lacking in *P. coloradorum* calls. In both *Xiphophorus* and *Physalaemus*, variation in the male trait is not associated with corresponding variation in female preferences, suggesting that female preferences may have predated the evolution of male traits and did not evolve in parallel with them (*22*). But our data are inconsistent with that hypothesis because we do find parallel variation in male traits and female preferences.

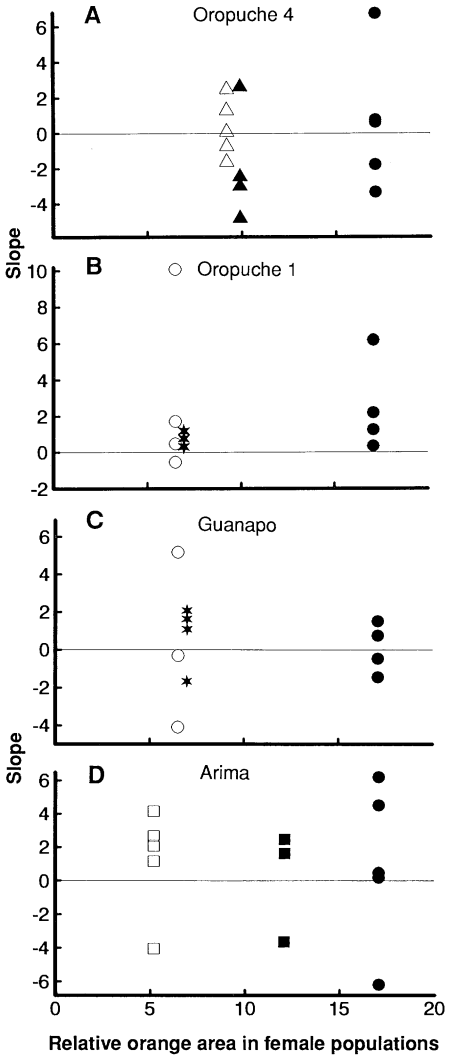

Fig. 3. Degree of female preference for orange among (**A**) Oropuche 4, (**B**) Oropuche 1, (**C**) Guanapo, and (**D**) Arima males, as a function of the average orange area (×100) in female source populations (defined in Fig. 1). These males have the smallest average of orange area among the seven populations used in this study (Table 1).

The variable results of other studies may simply reflect differences in evolutionary history of the few strains used in each of those studies. If we had studied only the Paria, Marianne, and Quare rivers, we might have concluded that females had consistent preferences, and if we had studied only the others, we would have concluded that orange was unimportant in sexual selection. A critical test of the predictions of sexual selection theory requires testing in many different populations differing as much as possible in male trait distributions.

Our finding of correlated evolution of color patterns and female preferences based on color patterns implies that sexual selection can be important in the divergence of populations. The divergence of mating preferences could represent a first step toward acquisition of reproductive isolation between populations, and the potential for future speciation and divergence (*3, 4*).

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17. Male color patterns were recorded by anesthetizing males in 0.03% methane tricainesulfonate (MS-222) solution and photographing them with Kodak Ektachrome 160 slide film under tungsten light. Color patterns were traced from projected slides and the areas of orange spots were calculated by a computer and digitizer. The relative area of orange in a color pattern (orange area) is the sum of the areas of all orange and orange-red spots divided by the total profile area of the fish's body, excluding fins. We used relative rather than absolute orange area to eliminate any potential effects of pleiotropy or allometry of body size.
18. In a courtship display, a male moves in front of a female, stiffens his body into an S-shape, and vibrates vertically and around its long axis. Courting males move among females, performing one or more displays to each. Females respond to male displays by moving toward the male with a gliding motion distinct from normal swimming. Displays were recorded only if they were observed from initiation, were uninterrupted, and were directed to a particular female [G. P. Baerends, R. Brouwer, H. T. Waterbolk, *Behaviour* **8**, 249 (1955); R. N. Liley, *Behavior Suppl.* **13**, 1 (1966)].
19. A female response to a male's display was scored if she ceased her previous activity, oriented toward the male, and glided unambiguously toward him. In a given observation session, males were observed courting females introduced to the males as virgins 24 hours previously. We used this delay because most virgins copulate almost immediately with the first male they see. Males and females usually cease all sexual activity for 30 min or more after copulation, making observations difficult. Females remain responsive to males for several days after they are first introduced to males, and copulations are less likely to interfere with observation sessions after the first 24 hours. For each male in turn, 5-min focal observations were made, recording the presence and frequency of his displays and female responses. To ensure that each male had been observed displaying to as many females as possible, we made an additional 20-min opportunistic observation in which we shifted attention rapidly from male to male, recording displays and responses as they occurred. Males that performed fewer than five displays in an observation session were eliminated from analyses. Four observation sessions in which females were unresponsive to male displays were excluded from the analysis.
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23. This work was supported by NSF grant BSR 86-14776 to J.A.E. We thank C. Jordan of the Trinidad Ministry of Food Production and Marine Exploitation, Fisheries Division for permission to collect and export guppies from Trinidad. We are grateful to G. Borgia, S. Emms, B. Lyon, P. Grant, R. Grant, P. Houde, T. McLellan, K. Long, and P. Ross for comments on the manuscript.

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Identification of an Inhibitor of Neovascularization from Cartilage

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Certain tissues such as cartilage are resistant to vascular invasion, yet no single tissue-derived molecule that can inhibit angiogenesis has been reported. A protein derived from cartilage was purified that inhibits angiogenesis in vivo and capillary endothelial cell proliferation and migration in vitro in three separate bioassays. This protein is also an inhibitor of mammalian collagenase. These findings may help elucidate the mechanisms by which neovascularization is controlled in both normal and pathological states.

ANGIOGENESIS, THE PROCESS OF new capillary formation, participates in numerous physiological events, both normal and pathological. Under normal conditions, angiogenesis is associated with wound healing, corpus luteum formation, and embryonic development (*1, 2*). However, a number of serious diseases are dominated by abnormal neovascularization, including solid tumor growth and metastases, diabetic retinopathy, neovascular glaucoma, and rheumatoid arthritis (*3*). The potential therapeutic benefit that a naturally occurring inhibitor of angiogenesis might have in controlling diseases in which neovascularization is involved has prompted a search for angiogenesis inhibitors.

Extracts of cartilage, one of the few avascular tissues in the body, can inhibit angiogenesis (*4, 5*). Although extracts from several different tissue sources have been shown to contain anti-angiogenic activity (*6*), no single tissue-derived macromolecule capable of inhibiting angiogenesis has been identified. Our data from three different bioassays now indicate that a specific protein derived from cartilage inhibited the proliferation and migration of capillary endothelial cells in vitro and angiogenesis in vivo. These three related bioactivities copurified with collagenase inhibitory activity throughout the purification. This molecule was extracted from bovine scapular cartilage with 2 M NaCl and was purified by a series of precipitation and column chromatography steps; NH_2-terminal protein sequence of this cartilage-derived inhibitor (CDI) was determined (Fig. 1).

We measured the effect of CDI on the proliferation of capillary endothelial cells (ECs) in vitro. Capillary ECs proliferate in response to an angiogenic stimulus during neovascularization (*7*). By using the specific cells involved in angiogenesis and stimulating them with a known angiogenesis factor, acidic fibroblast growth factor (aFGF) (*8*),

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1	2	3	4	5	6	7	8	9	10
---	Thr	---	Val	Pro	Pro	His	Pro	Gln	Thr
11	12	13	14	15	16	17	18	19	20
Ala	Phe	---	Asn	Ser	Asp	Val	Val	Ile	Arg
21	22	23	24	25	26	27	28		
Ala	Lys	Phe	[Val]	Gly	Thr	Ala	Glu		

Fig. 1. NH_2-terminal protein sequence of cartilage-derived inhibitor. Automated Edman degradation of CDI was performed on an Applied Biosystems Model 477A protein sequencer with the use of the manufacturer's standard program NORMAL-1. The phenylthiohydantoin–amino acid fractions were identified with an on-line (ABI) Model 120A HPLC. A search of the NBRF-PIR protein sequence database (*25*) revealed that this protein differs from a collagenase inhibitor isolated from human amniotic fluid (which itself is virtually identical to that of a human skin fibroblast inhibitor with the exception of one residue difference) in only two amino acids, at position 17 (Val for Leu) and at position 27 (Ala for Pro) for 28 NH_2-terminal residues (*26*). Samples were not reduced and alkylated, therefore, the dashes (------) represent bona fide blanks that align by similarity with expected cysteine residues of previously reported collagenase inhibitors; all three residues are in agreement (*26, 27*). Sequence differences between this collagenase inhibitor and others (*23, 26, 27*) may be ascribed to species variations, variable forms of this protein in the same tissue (*28*), or both. Brackets indicate determination with less than full confidence.

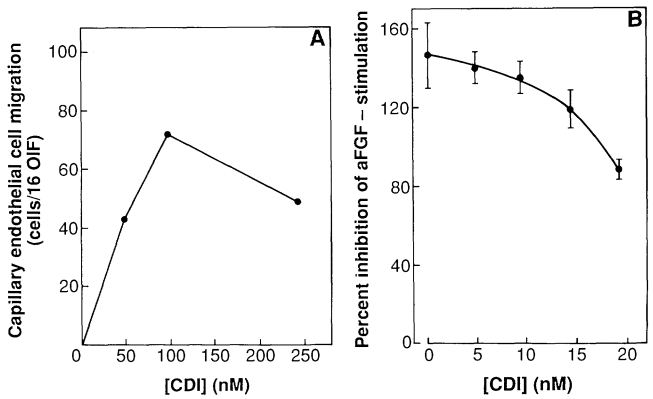

Fig. 2. (**A**) Inhibition of capillary EC proliferation by CDI. Capillary endothelial cells (2×10^3) in 0.2 ml were plated onto gelatin-coated 96-well tissue culture dishes (Nunc) on day 1. On day 2, cells were fed with Dulbecco's modified Eagle's medium (Gibco) with 5% calf serum (Hyclone) (DMEM/5) and aFGF (10 ng/ml) (FGF Company), and increasing concentrations of freshly purified CDI. Wells containing phosphate-buffered saline (PBS) (Gibco) alone and PBS + aFGF were included as controls. On day 5, media were removed and cells were washed with PBS and assayed for acid phosphatase activity (*12*). This assay exhibited a linearity between acid phosphatase activity and endothelial cell number up to 10,000 cells per well (*12*). We verified this linearity in the presence of the cartilage inhibitor and other inhibitors of capillary EC proliferation such as vitreous and platelet factor–4. Percent inhibition was determined by comparing wells exposed to stimulus with those exposed to stimulus and inhibitor. Each point represents the mean of quadruplicate control wells and triplicate inhibitor wells, and the SEM is less than 7% of the mean. (**B**) Effect of cartilage-derived angiogenesis inhibitor on capillary endothelial cell migration. Migration was measured with the use of blind well chambers (Neuroprobe, no. 025–187) and polycarbonate membranes with 8-μm pores (Nucleopore) coated with human fibronectin (6.67 μg/ml in PBS) (Cooper). Basic FGF (bFGF; 10 ng/ml; Takeda Company) diluted in DMEM/1 was added to the lower well. The upper wells received 2.5×10^4 capillary ECs and increasing concentrations of purified CDI (used within 24 hours of purification). Control wells receive DMEM/1, either with or without bFGF. The cells that had migrated through the membrane onto the lower surface were fixed, stained, and quantified by counting the number of cells on the lower surface in 16 oil immersion fields (OIF per well). Each point represents the mean ± SEM of four wells. In control wells without bFGF, the number of migrated capillary ECs was 61 ± 7 cells. IC_{50} values were 143, 38, and 8 μg/ml for CDI obtained at the A-1.5m, Bio-Rex 70, and Sephadex G-75 chromatography steps, respectively.