Reproductive failure of dominant males in the poeciliid fish *Limia perugiae* determined by DNA fingerprinting

(reproductive success/sexual selection/size polymorphism/social dominance/simple repetitive sequences)

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ABSTRACT Hierarchical structures among male individuals in a population are frequently reflected in differences in aggressive and reproductive behavior and access to the females. In general, social dominance requires large investments, which in turn then may have to be compensated for by high reproductive success. However, this hypothesis has so far only been sufficiently tested in small mating groups (one or two males with one or two females) due to the difficulties of determining paternity by conventional methods. DNA fingerprinting overcomes these problems by offering the possibility to determine genetic relationships and mating patterns within larger groups [Burke, T. (1989) *Trends Ecol. Evol.* **4**, 139–144]. We show here that in the poeciliid fish *Limia perugiae*, in small mating groups the dominant male has a mating success of 100%, whereas in larger groups its contribution to the offspring unexpectedly drops to zero.

Sexual dimorphism is the most apparent result of sexual selection (1, 2). The same applies for some forms of conspicuous male courtship behavior. Similarly, sexual selection is made responsible to favor the occurrence of more than one male morph and alternative mating behavior in certain animal populations.

In the live-bearing poeciliid fish, many species are polymorphic for male body size (3–5). Large males outcompete smaller ones and become dominant in the social structure of a given group (6). In at least one genus, *Xiphophorus*, it has been conclusively shown that differences in body size result primarily from allelic variation of a single polymorphic Y chromosome-linked locus (known as *P* locus; refs. 4, 7–9). Large males reach sexual maturity at a much later age than smaller ones, imposing a cost in form of an increased risk of prereproductive mortality (5) due to predation, etc. The different size classes differ with respect to their sexual behavior. Large males have a pronounced courtship behavior that precedes copulation attempts, whereas small males show simple "sneaking" behavior (3, 10, 11). Additional costs of a courting male include a higher risk of becoming predated because of being garish due to the more brilliant coloration (12, 13). High rank males also invest the energy input required to defend the hierarchy and protect females from the mating attempts of subordinate males. In female choice tests, large males are preferred (5, 11, 14). In *Xiphophorus nigrensis* using phenotypic markers in progeny tests of two females with one large and one small male, the dominant large male was found to be rewarded by a greater reproductive success (11). In the guppy, *Poecilia reticulata*, a dominant male was more successful even when the female showed preference for

the subordinate male (15). These findings are in perfect agreement with the expectations from the hypothesis that large investments are rewarded by high reproductive success. The large and sometimes spectacularly pigmented male morphs are regarded to be the result of sexual selection. Behavioral polymorphisms as well as the accompanying phenotypic polymorphisms are maintained or balanced by natural selection.

Here, we use *Limia perugiae*, a poeciliid fish endemic to the southeast of the Caribbean island Hispaniola, to study mating patterns in relation to male polymorphism and mating group size. These fish inhabit freshwater biotopes—clear springs as well as muddy creeks and polluted man-made ditches. Males are polymorphic for adult size ranging from 20 mm up to sometimes 60 mm in length. The onset of sexual maturation, which results in cessation of growth, is determined by a genetic system comparable to the *P* locus of *Xiphophorus* with sex chromosomal alleles for large and small size. However, additionally at least one autosomal modifier locus interacts with *P*, thus allowing intermediate size males to appear (C.E.-D., J.H.S., I.N., M. Schmid, J.T.E., and M. Schartl, unpublished results). Of course, environmental factors modulate to a certain extent the final size of each genotype. Females constitute a single size class with a mean adult size of 40 mm. Independent of males' social behavior, females prefer large over small males (C.E.-D., J.H.S., I.N., M. Schmid, J.T.E., and M. Schartl, unpublished results). Like all other poeciliid fish species, *L. perugiae* does not provide parental care, and males are not territorial. The sex ratio is on average one to one. The highest rank males are marked by a very intense coloration: blue body contrasted by a black dorsal fin and a bright yellow caudal fin with black margin.

In the natural habitat, the social and reproductive groups are larger than the two male/two female situation that could be studied so far with the help of phenotypic markers for paternal traits (e.g., see refs. 11 and 15). Simple repeat oligonucleotides represent useful tools to study genetic relationships even in the absence of phenotypic markers (26) within all species tested at all levels of eukaryotic organismic evolution (17). We therefore used this method to determine the reproductive success of males of different social status in small and large mating groups. We unexpectedly find that with increasing size of the social groups the largest and dominant male becomes less successful in siring offspring.

MATERIALS AND METHODS

Experimental Animals. Our *L. perugiae* stocks are descendants from fish collected in 1978 at Piedra Blanca (K stock),

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central Dominican Republic, and at a karst spring (G stock), west Dominican Republic. Fish were kept as randomly bred population stocks under standard conditions described for maintenance of poeciliid fish in the laboratory (18).

Mating Experiments and Behavioral Tests. Behavioral and mating tests with large mating groups were performed in aquaria containing 180 liters of water with a sandy bed and containing several plants and hiding places to simulate the habitat found in nature (ref. 19; M. Meyer, personal communication) at a 12-h day/12-h night artificial light cycle. For the social groups, males and females from both K and G stocks were mixed. Exclusively young males and juvenile virgin females were assembled for the mating experiments to allow the social structure to establish before successful mating attempts could occur. For the two males/two females situation, 30-liter tanks were used with otherwise the same conditions. Newborn fish were collected from the experimental tank and raised separately. After termination of the mating experiments (3–4 months), females were isolated, and their next brood was raised separately. The male fish are referred to as α, β, γ, and ω in decreasing order of social rank.

DNA Fingerprinting. DNA was extracted from all adult fish used in the mating experiments and from a representative number of randomly selected offspring as described (20). Determination of paternal and maternal relationships by simple repeat oligonucleotide fingerprinting was performed as described (21).

RESULTS

After evaluating different restriction enzyme/probe combinations, the $(GGAT)_4$ probe on HinfI-digested DNA was found to be the most informative with respect to individualization in *L. perugiae* even for closely related individuals of the same stock (Fig. 1). Additional information was obtained

by rehybridization of the same gel with probes $(CA)_8$, $(GACA)_4$, $(GAA)_6$, and $(TTTC)_4$.

In a first series of experiments, one large and one small male were tested with two females. Offspring from two different broods in two independent experiments were tested for paternity. In one experiment, 13 of 14 animals were attributable to the large dominant male and one was of uncertain paternity. In the second case, all 12 F_1 fish tested were unequivocally offspring of the α male.

In the second set of experiments, four males ranging in size from 25 mm to 49 mm were assembled with four or five juvenile, virgin females. In three trials within a few days, the male fish established a size-dependent ranking that remained stable throughout the duration of the experiment. DNA fingerprinting of a representative number of offspring (Fig. 2) revealed that most females had contributed to the offspring generation and that generally individual broods were of mixed paternity. In the first mating group, all offspring tested were from one of the subordinate males. In the second and third group, paternity was assigned to both the β and γ male. In all cases, the smallest and most subordinate male never had offspring. This is in agreement with its exclusively defensive behavior. Surprisingly, none of the progeny were fathered by the dominant male (Table 1). After experiments 2 and 3, full fertility of the α males was confirmed by mating them without competition to virgin females. In the fourth mating group, males were assembled that did not establish a pronounced hierarchy, although size differences allowed some ranking. The lowest rank male did not exhibit hiding and the α male was far less aggressive than in the three other mating groups. In this experiment, all four males produced offspring.

The size of the group used in the second set of experiments is more similar to the situation found in nature (ref. 19; M. Meyer, personal communication). To relate the data on
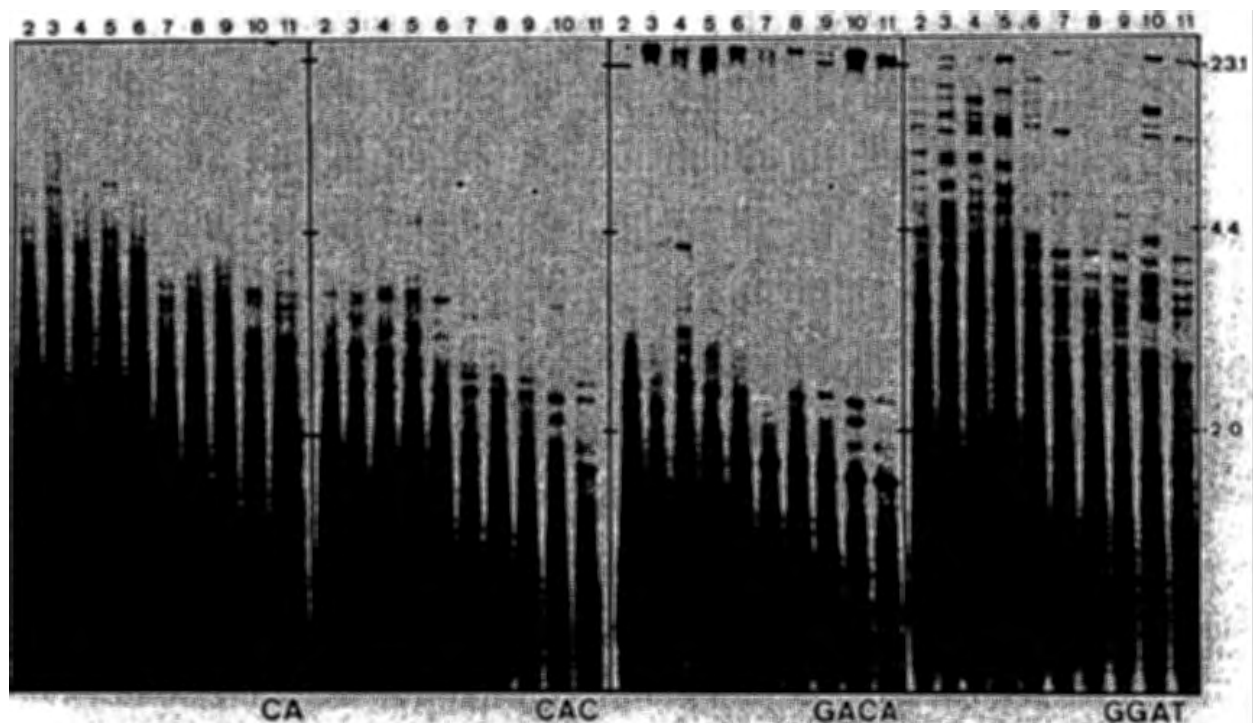

FIG. 1. Fingerprints of different individuals of *L. perugiae* as obtained with the oligonucleotide probes $(CA)_8$, $(CAC)_5$, $(GACA)_4$, and $(GGAT)_4$ by in-gel hybridization. For further methodological details, see *Materials and Methods* and ref. 21. Because of the many polymorphic signal bands, the probes $(GGAT)_4$ and $(CA)_8$ are most informative with respect to individual identification in *L. perugiae* specimens. No mutations have been detected in extensive family studies. Therefore, these two oligonucleotides were preferentially used for paternity (and maternity) determination in the subsequent experiments on mating success.

reproductive success to the agonistic and sexual activity, the behavior of the males was quantified (Table 2). It revealed that the dominant male spent most of its time (approximately one-third) with agonistic behavior and also courtship display. All other males were considerably less occupied by these activities ($\approx$5% of the observation time). On the contrary, the number of copulation attempts of low rank males was higher than that of high rank males.

DISCUSSION

In small competitive mating groups, we observed a pronounced reproductive success of the large dominant male. These observations are in agreement with findings using
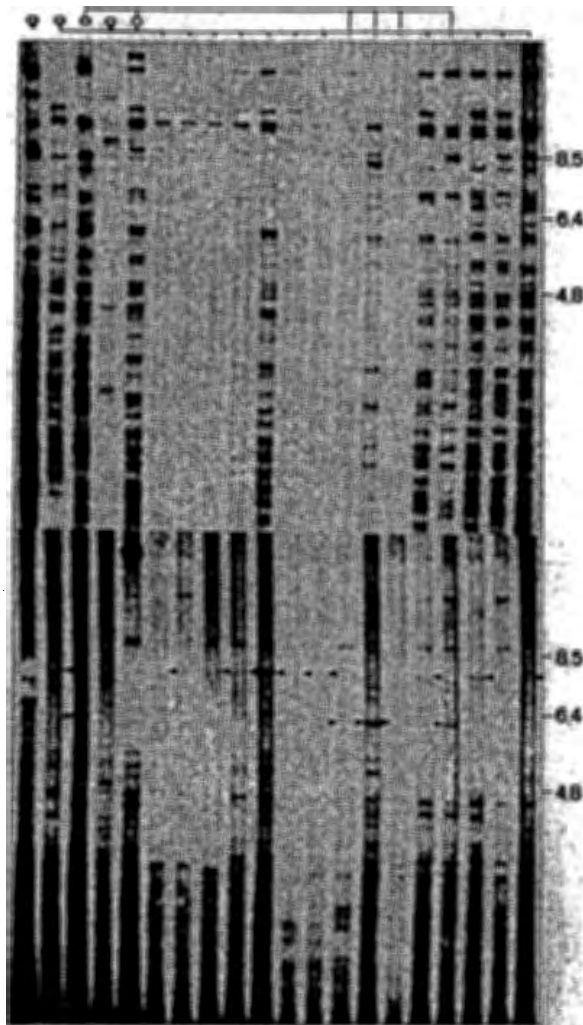

FIG. 2.   Determination of paternity in large mating groups of *L. perugiae* by DNA fingerprinting of the possible fathers (□; from left to right, α, β, γ, and ω males), the mother (○; lane 5 from left), and their offspring as obtained with the probes (GGAT)$_4$ (*Upper*) and (CA)$_8$ (*Lower*). Representative diagnostic bands could only be transmitted by one of the possible fathers (◄, β male; ►, γ male). Paternity was determined after three or four oligonucleotide hybridization steps by comparing all multilocus fingerprint patterns of each child with those of the mother, defining the paternally inherited bands. The latter were compared to the patterns of the putative fathers, thus sequentially excluding each of the nonfathers. Note that the DNA fingerprinting pattern does not indicate high levels of interrelatedness that could have been achieved through several generations of breeding in laboratory stocks. Fragment length markers are indicated in kilobase pairs on the right.

Table 1.   Paternity of F_1 offspring ($n = 177$) of large mating groups of *L. perugiae*

Mother	Father				
	α	β	γ	ω	Uncertain
Experiment 1					
1	0	6	0	0	0
2	0	10	0	0	0
3	0	4	0	0	1*
4	0	8	0	0	0
Uncertain	0	1	0	0	0
Total	0	29	0	0	1
Experiment 2					
1	0	0	1	0	0
2	0	7	1	0	4†
3	0	15	0	0	1‡
4	0	0	1	0	0
5	0	25	6	0	3†
ND	0	6	6	0	0
Total	0	53	15	0	8
Experiment 3					
1	0	0	0	0	0
2	0	1	0	0	0
3	0	11	7	0	3†
4	0	11	4	0	0
5	0	1	1	0	0
Total	0	24	12	0	3
Experiment 4§					
1	7	0	0	5	0
2	5	5	0	0	0
3	0	2	5	0	0
4	0	1	0	0	0
5	0	0	2	0	0
Total	12	8	7	5	0

ND, mother not determined. Because of the similarity of the fingerprint pattern of females (high inbreeding coefficient) in the second experiment, this precluded in some cases unequivocal determination of maternity.
*Not unequivocally ascribable to one of the four possible fathers because of too few paternal bands transmitted.
†Attributable either to β or γ.
‡By assumption of one mutation attributable to the β male.
§Social hierarchy less clearly established, see text.

similarly sized mating groups in the pygmy swordtail *X. nigrensis* (11) and in the guppy (15). The reproductive success of the large male in this kind of competition experiment must be attributed to the selective advantage gained by its dominance and the pronounced courtship behavior. It is in perfect agreement with the expectation that a high commitment of energy and cost into sexual and social behavior leads to high fitness. If the aforementioned test situation would relate to anything determining the evolutionary history of *L. perugiae* in feral populations, this, however, would predict that the small phenotype, which is invariably connected to subordinateness, is prone to disappearance by negative selection. For the pygmy swordtail, it has been reported, however, that in the natural habitat the small male morph is much more frequent (11). The authors also noted that in some of the competitive matings the subordinate male did quite well in siring offspring, which they explained by possible differences in female mate choice.

In our experiments using larger mating groups, the dominant male failed to reproduce. This observation is in direct contradiction to the expected results and the behavioral data on fish reported so far. However, the only component of fitness that has been possible to monitor in earlier studies on larger mating groups is the number of observed matings or mating attempts by each male, but even this may be misleading as the number of successful fertilizations may be

Table 2. Quantitative analysis (*n* = 30) of male behavior in large mating groups of *L. perugiae*

Behavioral element*	Male											
	α			β			γ			ω		
	Median	Quartile		Median	Quartile		Median	Quartile		Median	Quartile	
		Upper	Lower		Upper	Lower		Upper	Lower		Upper	Lower
Courtship display												
Time, min	1.44	7.43	0.83	0.11	0.58	0	0.04	0.47	0	0		
No. of actions	14	21	7	2	8	0	2	6	0	0		
Following												
Time, min	1.06	1.45	0.63	0.15	0.94	0.02	0.8	1.85	0.31	0.94	2.71	0.17
No. of actions	21	26	15	3	15	1	10	21	7	12	17	7
Nibbling												
Time, min	0.31	0.47	0.22	0.04	0.19	0	0.15	0.4	0.06	0.15	0.4	0.03
No. of actions	6	9	5	3	5	0	3	8	1	4	7	1
Copulation attempts	2	4	0	1	4	0	6	16	2	7	13	3
Agonistic attacks												
Time, min	2.36	3.26	0.73	0.03	0.25	0	0.16	0.35	0.05	0		
No. of actions	34	40	10	1	7	0	4	8	2	0		

*According to ref. 22. The total observation time was 20 min.

substantially different. After analyzing the genetic relationships of the progeny with the males, it became apparent that intermediate size males, which exhibit little courtship but more simple sneaking behavior as an alternate mating tactic, are more effective reproductively than the more extreme social and size classes (represented by the α and ω males), which are practically excluded from reproduction.

The behavioral data may help to explain the result obtained from determining the mating success. As population size increases, the dominant male must spend more time fighting and less time pursuing females. Furthermore, attacks on nonaggressive subordinates decrease as aggressive high rank males devote proportionally more time fighting each other, allowing lower rank males a greater opportunity to mate successfully.

The question that arises is how in the mating system identified for larger social groups with a pronounced hierarchy can a size polymorphism in males be maintained if the genes of the extreme size class males are only rarely transmitted to the progeny. If size is determined by polygenic systems and/or environmental conditions, the maintenance of polymorphisms is readily explained. However, if a single locus, such as the *P* locus of *Xiphophorus* (4, 9), is of major importance in determining the onset of maturation and therefore adult size, only a balanced system of different modifier alleles for *P* present in males and females will guarantee the reappearance of all size classes in the offspring generations.

Our findings in *L. perugiae* are not compatible with the current understanding that social dominance leads to high reproductive success. However, several arguments have to be taken into consideration. First, alternative mating tactics leading to equal fitness have been proposed to exist as an evolutionary stable strategy (23, 24), and we cannot exclude that the mating system that we observed in *L. perugiae* will be unstable in the long run as an evolutionary strategy and will lead to changes in morph frequencies. Second, equal fitness of large and small male morphs has been predicted on the basis of the reasoning that the large, courting males, which were thought to be more successful reproductively, should have higher postmaturation death rates. This simply may not be true as pointed out for *X. nigrensis* by Ryan *et al.* (25). Third, a possible explanation for the observed phenomenon would be "inclusive fitness" (16). Populations of *L. perugiae* like most poeciliid fish live in limited habitats. Therefore, the chance that a subordinate male is a reasonably close relative to the dominant male is high. Another point concerns female choice. Differences in male behavior with

respect to frequency and duration of courtship display in large and small groups could influence female choice. However, it was found that females of *L. perugiae* do not prefer courting versus noncourting males. They choose only with respect to body size (C.E.-D., J.H.S., I.N., M. Schmid, J.T.E., and M. Schartl, unpublished results). Finally, it should also be taken into consideration that *L. perugiae* like most teleosts produce a large surplus of offspring (several hundred) of which only a minute number (several tens and less) reach maturity due to heavy predative pressure, etc. Especially, if the general expectation is that the population size is stable over ecologically long periods of time, only very few descendants per pair of parents will become efficient transmitters of parental genes. This may counteract or bias the gene pool of the survivors as compared to the newborn population. However, in all these cases the biological significance of aggressiveness, social hierarchy, and courtship behavior in *L. perugiae* would remain obscure.

From all our knowledge on the biology of *L. perugiae*, we can exclude that the total lifetime reproductive success will significantly deviate from that of the 3- to 4-month period in the life history of a given male investigated here. The social rank is determined mainly by body size. This in turn is fixed by the time of sexual maturity and does not considerably increase later. Thus the rank position of a male usually is not subject to change.

The difference observed in reproductive success of the dominant *L. perugiae* male in small and large groups documents the need to apply molecular biology methods to investigate mating success in other species in groups larger than the three or four individuals that can be studied by conventional methods. It will also be important to determine the paternity relationships in field studies. Very large sample sizes will be needed to determine the frequency of different male morphs and to calculate their fitness on the basis of differential mating success, age at maternity, and sexual and aggressive behavior in the natural habitat (25). Such approaches were illusory with the conventional repertoire of methods but appear now practical with the help of DNA fingerprinting.

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