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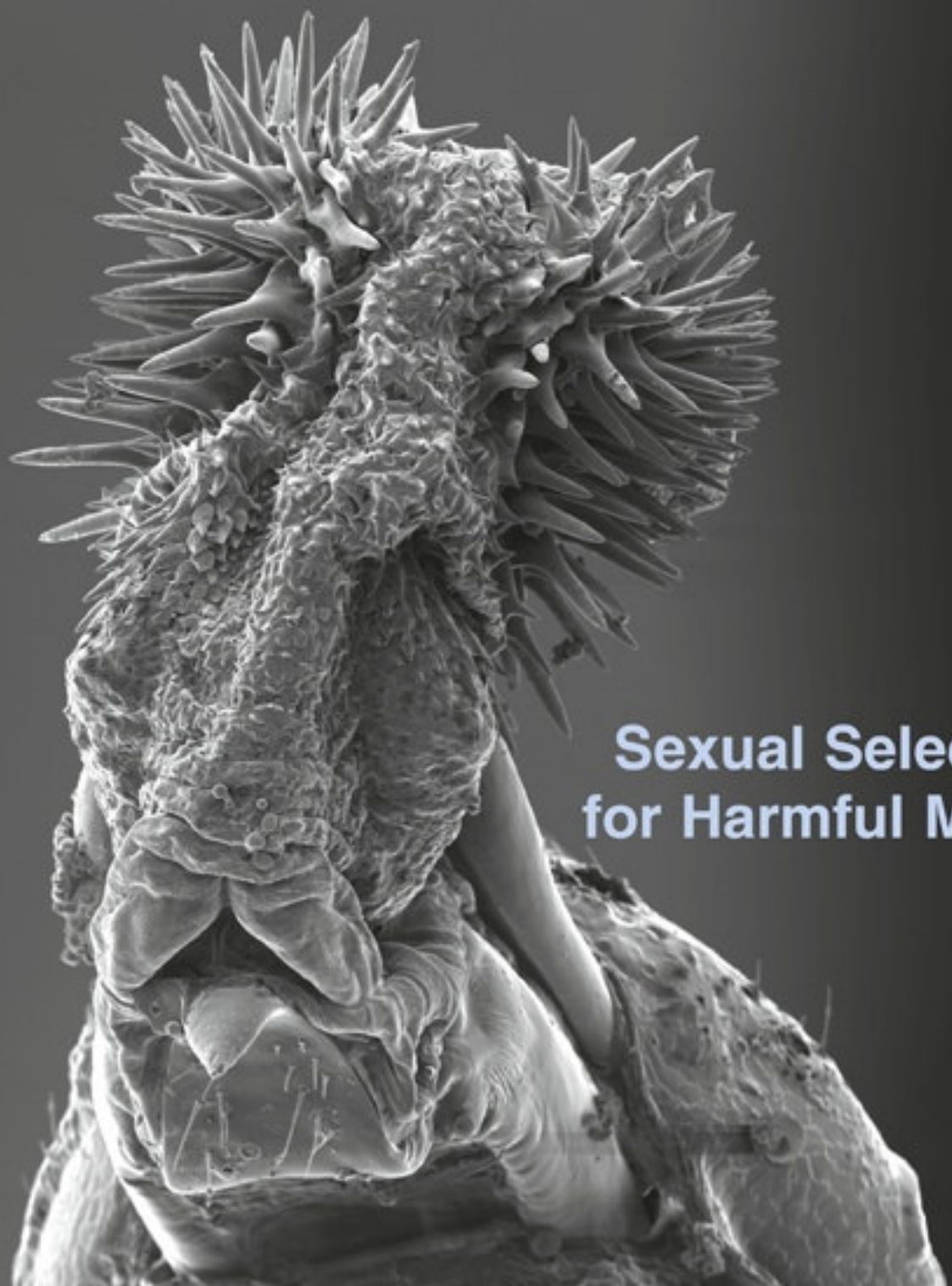

Sexual Selection for Harmful Males

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Report

Sperm Competition Favors Harmful Males in Seed Beetles

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Summary

One of the most enigmatic observations in evolutionary biology is the evolution of morphological or physiological traits in one sex that physically injure members of the other sex [1–3]. Such traits occur in a wide range of taxa [3] and range from toxic ejaculate substances [4–6] to genital or external spines that wound females during copulation [7–11]. Current hypotheses for the adaptive evolution of such injurious traits rest entirely on the assumption that they are beneficial to their bearer by aiding in reproductive competition [1, 3]. Here, we assess this key assumption in seed beetles where genital spines in males physically injure females. We demonstrate that male spine length is positively correlated with harm to females during mating but also that males with longer spines are more successful in sperm competition. This is the first complete support for the proposal that sexual selection by sperm competition can favor morphological traits in males that inflict injury upon females. However, our results suggest that harm to females is a pleiotropic by-product, such that genital spines in males elevate success in sperm competition by means other than by causing harm.

Results and Discussion

Difference among Populations

Because males accrue fitness through females, a male that bears a morphological trait that inflicts injury upon his mates will himself suffer from the harm inflicted. For this simple reason, understanding the adaptive evolution of injurious traits in males is a challenge [1]. In theory, such traits may evolve by two distinct routes [3]. First, if females show responses to harm that directly benefit the harming male, there may be direct selection on males to injure their mates [12–14] despite the fact that harm per se carries costs for the harming male (the "adaptive harm hypothesis" [15]). Such female responses may involve delaying remating, increasing sperm usage, or increasing the short-term rate of offspring production [12–16], and harming females is adaptive to males in the sense that harm triggers the female response that benefits males. Second, a trait that is beneficial to males in terms of intrasexual reproductive competition may be injurious to females as a negative pleiotropic side effect [1, 3] (the "pleiotropic harm hypothesis" [15]). Here, harm to females is not causally related to elevated success in reproductive competition among males. Under both hypotheses, therefore, sexual selection in males on traits that cause harm is assumed to outweigh negative direct selection in males, because of lowered fecundity of their mates, on the very same traits [3]. Currently, there is no direct support for either of these hypotheses [3, 15, 16].

To test these hypotheses, we employed the seed beetle *Callosobruchus maculatus* (Coleoptera, Bruchidae), a model system for studies of sperm competition and sexual conflict [7, 11, 15, 17–21]. Here, male genitalia are armed with conspicuous sclerotized spines that penetrate the wall of the female copulatory duct during copulation, leaving prominent melanized scars in these tissues [7, 11]. Females suffer costs as a result of such injuries [7, 11, 18, 19] but males seem not to benefit directly from the harm inflicted upon their mates [15]. Instead, harm to females has been suggested to be an indirect side-effect of these spines [18]. We quantified (1) the length of the genital spines, (2) the amount of harm caused to females during mating, and (3) male sperm competition success in 13 different populations of *C. maculatus*.

Males from different populations were distinct with regards to their genital morphology (Figure 1): the length of the genital spines and the size of the area covered with spines differed across populations, both in absolute terms (MANOVA of the three genital variables: Wilk's $\lambda = 0.591$, $F_{36,367} = 1.984$, $p < 0.001$) and relative to male body size (MANCOVA: population; Wilk's $\lambda = 0.573$, $F_{36,364} = 2.101$, $p < 0.001$, male body size; Wilk's $\lambda = 0.932$, $F_{3,123} = 2.991$, $p = 0.034$). Further, the amount of scarring to the copulatory duct that occurred in females from our standard reference population was affected by the population origin of their mate (Figure 1) (MANOVA of the two scarring variables: Wilk's $\lambda = 0.744$, $F_{24,334} = 2.217$, $p = 0.001$). Although large females suffered less injury during copulation (MANCOVA: female body size; Wilk's $\lambda = 0.962$, $F_{2,166} = 3.322$, $p = 0.038$), the effect of population origin of the male was even stronger when variation in female body size was accounted for (population; Wilk's $\lambda = 0.732$, $F_{24,332} = 2.337$, $p < 0.001$). Earlier studies of this species has shown that male success in sperm competition varies across populations [17, 20] and this was true also in our case ($\chi^2_{12} = 23.12$, $p = 0.027$), controlling for variance in P2 resulting from the larval competitive environment of the male ($\chi^2_3 = 9.998$, $p = 0.018$) and the number of eggs laid by the female prior to the second mating ($\chi^2_1 = 7.741$, $p = 0.005$) (see Experimental Procedures). Thus, males from the 13 populations were distinct with regards to (1) their genital armature, (2) the amount of injury and scarring they caused in females, and (3) their sperm competition success.

Pattern of Covariation across Populations

Both hypotheses for the evolution of injurious traits make the critical assumptions (1) that males with more injurious morphological traits are at an advantage, directly or indirectly, in reproductive competition between males and (2) that these benefits are large enough to offset the costs to such males that stem from harm caused to their mates [1]. Although males of many taxa show traits that carry costs for females [1–3], no previous study has identified an injurious male trait that is

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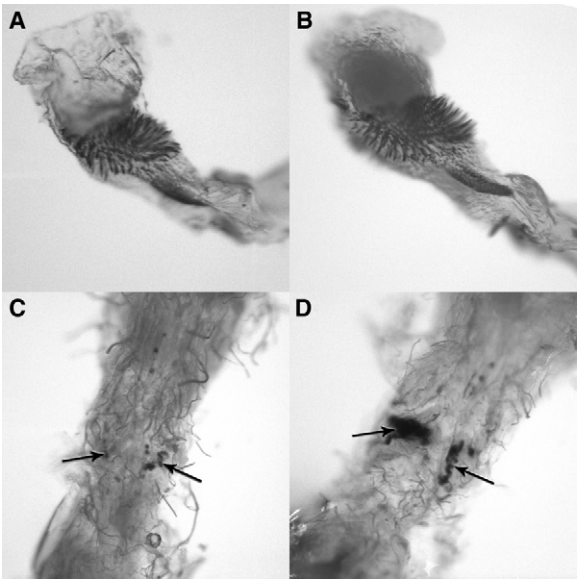

Figure 1. Male Genitalia and Injury to Females

The male genitalia of *C. maculatus* are armed with rigid spines that cause internal injuries in females during copulation. The ventral spines are here pointing toward top right and the dorsal spines in the opposite direction. Males from some populations ([A]; Oman) have spines that are on average almost 30% shorter than males from other populations ([B]; Volta). Males from populations with shorter genital spines leave females with less internal injuries ([C]; female mated once to a male from Oman) than do males from populations with longer spines ([D]; female mated once to a male from Volta). Arrows indicate melanized scars in the female copulatory duct.

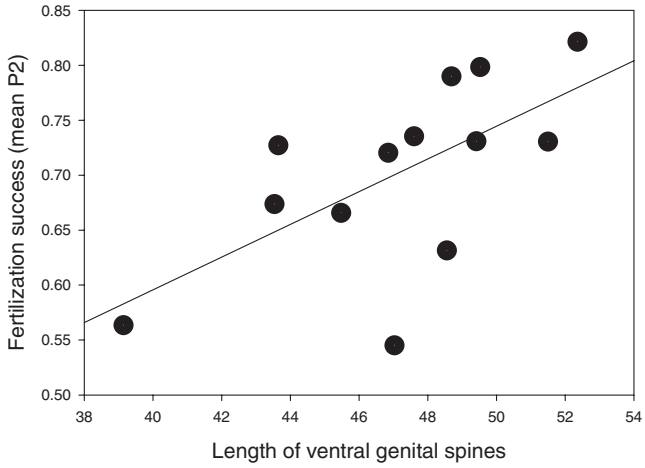

Figure 2. Covariation between Genital Spines and Sperm Competition Success

The relationship between the average length of the ventral spines of the genitalia and mean male fertilization success, here measured as the absolute proportion of eggs that are fertilized by the second (focal) male to mate with a female, across 13 populations of *C. maculatus* (r_p = 0.63, p_{dir} = 0.016; randomization test based on 10,000 random permutations). Line represents conventional linear regression.

also associated with differential reproductive success [22] and the strength of sexual selection on injurious traits is entirely unknown. Direct empirical support is presently limited to studies of physiological traits in *Drosophila* fruit flies. Civetta and Clark [23] showed a correlation between male sperm competition success and female postmating mortality in a comparative study of chromosome-extracted lines, although a male trait was not identified. By using knockdown mutant lines, Wigby and Chapman [24] showed that a particular male ejaculate accessory gland protein depresses female fitness. Although this protein has phenotypic effects in females that are likely to benefit males, its net effect on male reproductive success has not been documented [25].

Given that our populations differ, we adopted a comparative approach and used the pattern of covariation of mean values of these traits across populations to test three a priori predictions. First, we predicted that males with longer genital spines should cause more harm to females. This prediction was tested in a conventional multiple regression model of the area of the postcopulatory scars in the female copulatory duct, with the three measures of male genital spinosity as predictor variables. This model showed that the degree of genital spine elaboration was positively related to scarring in females ($F_{3,9}$ = 4.525, p_{dir} = 0.021). Second, we predicted that males with longer genital spines should be more successful in sperm competition. We tested this prediction in a multiple regression model of $P2_R$ (see Experimental Procedures) by using the three measures of male genital spines as predictor variables. Again, the prediction was corroborated: males with longer spines were more successful in sperm competition, whether variation male body size was accounted for ($F_{3,9}$ = 3.38, p_{dir} = 0.042) or not ($F_{3,9}$ = 3.30, p_{dir} = 0.044). We note that this was particularly true for the length of the genital spines on the ventral side of the male genitalia (N = 13, β' = 0.77, t = 3.05, p_{dir} = 0.009) (Figure 2), which are also the longest spines (Figure 1) (length of dorsal spines: β' = 0.15, t = 0.60, p_{dir} = 0.35; spiny area of genitalia: β' = 0.14, t = 0.54, p_{dir} = 0.37). Third, we predicted that high sperm competition success should be positively associated with infliction of harm to females. This prediction was tested in a multiple regression model of $P2_R$, by using the number of scars in the copulatory duct and their area as predictor variables. This model confirmed a positive relationship between $P2_R$ and harm ($F_{2,10}$ = 4.40, p_{dir} = 0.026).

The analyses above show that male genital armature and the harm that males inflict upon females are correlated and, more importantly, that both are positively related to male success in sperm competition. As in any comparative study, however, the pattern of correlated evolution we document here may involve confounding effects of other variables [26]. In our case, three facts suggest that male spines are causally related to both harm and sperm competition success. First, a causal link between spines and harm has been established in ultrastructural studies [7]. Second, a link between spines and sperm competition success has been explicitly predicted in several previous contributions [7, 11, 15, 18, 27]. Third, our analyses control for the potential effects of the most obvious putative confounding variables, notably male phenotypic condition and male and female body size.

Although our results provide the first complete support for the frequent suggestion that sperm competition can select for morphological traits in males that are harmful for their mates [1, 3, 28], they are consistent with both the pleiotropic harm and the adaptive harm hypotheses [15]. In order to better distinguish between these hypotheses, we assessed the independent effects of genital spines and harm to females on male sperm competition success. The pleiotropic harm hypothesis predicts that spines should show strongest independent effects and the adaptive harm hypothesis predicts that harm should show strongest independent effects. We tested this

by first fitting a full multiple regression model of $P2_R$, by using both measures of scarring and our two measures of genital spine length as predictor variables ($F_{4,8}$ = 4.01, p = 0.044). Dropping the two genital spine variables from this full model resulted in twice as large reduction in fit to data (partial $F_{2,8}$ = 3.29, p = 0.090), as did dropping the two scarring variables (partial $F_{2,8}$ = 1.73, p = 0.238). More importantly, the only variable significant at p_{dir} = 0.05 in the full model was the length of the ventral genital spines (t = 2.55, p_{dir} = 0.042) and a backward stepwise regression of the full model (α to remove/enter = 0.1) yielded a model containing only the length of the ventral genital spines (β' = 0.72, t = 3.43, p_{dir} = 0.004). These analyses show that genital spine morphology covaries with sperm competition success when statistically keeping harm to females constant, whereas harm shows no such covariation when genital spines are kept constant. It thus seems as if the genital spines, rather than the harm they inflict, are causing the elevation seen in sperm competition success, as predicted by the pleiotropic harm hypothesis.

Theory also assumes that intrasexual selection for harmful traits must be strong in order to compensate for the costs of harm inflicted [1]. In contrast to experimental studies with phenotypic or genetic engineering, studies of the pattern of covariation between phenotypic traits and reproductive success can be used to provide estimates of the strength of selection [29, 30]. Our design does not, however, allow the estimation of conventional standardized phenotypic selection coefficients [29]. Yet, the slope of the relationship between mean trait values across populations (Figure 2) may reflect phenotypic selection because male sperm competition success was measured in a common sperm competitive background for all males. With $P2_R$ as a dependent variable (i.e., a male fitness component), the standardized regression coefficient of ventral spine length was β' = 0.71 (r_s = 0.80, p_{dir} = 0.001) in a univariate regression and β' = 0.76 (t = 3.21, p_{dir} = 0.011) in a multiple regression, the latter controlling for covariation with all other genital traits, male body size, and harm inflicted upon females. Although these slopes should obviously be interpreted with caution, because they are estimated across rather than within populations, they do suggest that selection on genital spines by sperm competition may indeed be strong [31]. Comparative work in this group have suggested that females suffer fitness costs of harm [11], but several experimental studies have shown that the fitness reduction that females suffer as a result of injuries during copulation is restricted [7, 18, 19, 27], in part because of effective female resistance adaptations [11]. The large advantage in sperm competition suggested by the above analyses may thus outweigh the cost to males that stems from harming their mates. We note, however, that the exact balance between these two factors will be contingent upon factors such as the mating system, female life histories, and the sperm competition regime [1, 13, 14].

The proximate function of the genital spines is unknown. They may serve as an anchor during copulation, help position the male genitalia optimally within the female genital tract [18], and/or aid in removal or displacement of rival sperm [32]. Alternatively, harm could act to elevate sperm competition success, by for example increasing female uptake of accessory seminal substances by puncturing the wall of the copulatory duct [33], but we note that our analyses suggest that this is not the case because harm seemed to have no independent effect on male sperm competition success. Future studies with phenotypic manipulation of the genital spines may help unravel their proximate function.

Conclusion

Earlier comparative work in seed beetles has established that male genital armature and female resistance to these harmful male traits are involved in sexually antagonistic coevolution [11]. The present work shows that sperm competition seems to be the engine of this coevolution: males with more harmful genital spines are more successful in competition over the fertilization of ova within females. In more general terms, thus, our results provide novel support for the suggestion that sperm competition can spawn sexually antagonistic coevolution [1–3, 5, 28].

Experimental Procedures

Populations and Rearing

We used 13 geographically distinct focal populations of *C. maculatus* (Benin, Brazil [London], California, IITA [Nigeria], Lossa, Mali, Oman, Oyo, South India [London], Uganda, Upper Volta, Yemen, and Zaire) and, in addition, a standard reference population (Nigeria mix). Although these populations are genetically distinct [20] and differ to some extent in productivity and external morphology [21], they are fully reproductively compatible: hatching rate of eggs in crosses between populations is invariably very high ($\geq$95%). All beetles in this study were reared under standardized density on black-eyed beans (*Vigna unguiculata*) at 30°C, 60% RH, and a 12L:12D light cycle, and had been reared under this regime for at least 35 generations. We used digital image analyses to secure three measures of the male genital armature and two measures of the amount of harm males inflict upon females (i.e., scarring in the copulatory duct). Male sperm competition success was quantified in a standard competitive background, as P2 in a double mating experiment with a "sterile male technique" protocol.

Male Genital Spines

In order to quantify key aspects of the genital armature, male genitalia (N = 8–12 males per focal population) were inflated under CO_2 anesthesia, with an artificial inflator constructed by connecting a plastic micropipette tip to an adjustable water-jet vacuum pump. Once fully inflated, male genitalia were stabilized in 100°C water and photographed (lateral view) with a Lumenera Infinity 2-2 digital camera mounted on a Leica MZ8 dissection microscope. Elytron length (mean length of left and right elytra) was also measured for all males and used as a measure of body size. We then employed image analysis to measure three aspects of the genital armature for each male, with ImageJ (http://rsb.info.nih.gov/ij): (1) the average length of the five longest ventral spines, (2) the average length of the five longest dorsal spines, and (3) the length of the entire area of the genitalia bearing spines.

Harm to Females

To standardize the impact of variation across females in susceptibility to harm, this experiment involved only females from our standard reference population. Virgin females were each mated once only (at age 1 day postemergence) to a virgin male from one of the focal populations (N = 13–15 pairs per population). Females were then isolated individually for $\geq$6 days to allow for full melanization of internal injuries. We quantified the amount of harm to females by dissecting out the copulatory duct and the bursa copulatrix of all females. We then used image analysis (see above for setup) to record two variables for each female: (1) the number of discrete melanized scars in the wall of the copulatory duct [11] and (2) the total area of all scars. Repeated measures on a subset of our data (N = 30 females) showed that our measures of scarring were highly repeatable (repeatabilities 0.96 and 0.98, respectively). Again, elytron length was recorded to provide a measure of female body size.

Sperm Competition Success

Virgin females (1 day postemergence) of the reference population were each first mated to a randomly selected virgin sterile male from the reference population (sterilized by irradiation; 80 Grey, Caesium source). Females were then isolated with a single bean for 48 hr, after which each female was mated a second time to a virgin male from a focal population. Females (N = 14–17 per focal population) were then kept individually in Petri dishes containing 30 beans for oviposition for 7 days. After another 7 days of egg maturation, all eggs were scored as hatched or unhatched. Male sterility is higher than 99% at the irradiation dose used [20], and hatched eggs

were thus considered to have been fertilized by the focal second male. The proportion of hatched eggs thus forms our measure of focal male fertilization success (i.e., P2) for each female. Because variation in female prior oviposition [34] and male phenotypic condition [35] are known to greatly influence female sperm use and oviposition after a second mating in *C. maculatus*, we used (1) the number of eggs laid by females between the first and second mating and (2) a measure of the competitive environment during the larval stage for each focal male (the number of adults emerging from their natal host bean) as two covariates in our models of P2. Further, to control for individual level variation in these two variables in our analyses of variation across populations, we regressed P2 (square-root arcsine transformed) on the two variables and then used mean residual P2 (denoted $P2_R$) per population to characterize the sperm competitive ability of males.

Statistical Methods

We used general linear models for statistical inference, in all cases where the assumptions of such models (i.e., homogenous variances and normally distributed residuals) were upheld. However, variance in sperm competition success across males was analyzed with a generalized linear model, with binominal errors and a logit link function, of the number of hatched eggs with the total number of eggs laid after the second mating as the binominal denominator. To compensate for overdispersion, we implemented the method of Williams [36] prior to statistical inference.

When testing hypotheses in which the sign of the effect was predicted a priori, we used directed tests [37]. Directed tests enable detection of patterns that are opposite to predictions while retaining much of the statistical power of one-tailed tests. In all directed tests (denoted p_{dir}), we followed the convention of setting $\gamma/\alpha = 0.8$ [37].

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