



## Mating preferences in a species flock of Mexican pupfishes (*Cyprinodon*, Teleostei)

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Female mating preferences were examined in three members of an evolutionarily young *Cyprinodon* lineage from Laguna Chichancanab, Yucatan, Mexico. In separate experiments, females of *C. beltrani* and *C. labiosus*, and *C. beltrani* and *C. maya* were simultaneously presented with conspecific and heterospecific males and given the opportunity to spawn with either or both. These three species showed different degrees of conspecific mate recognition: complete for *C. maya*, intermediate for *C. labiosus*, and indiscriminate for *C. beltrani*. We conclude that only *C. maya* is reproductively isolated and that *C. beltrani* and *C. labiosus* still hybridize in nature. Whereas between *C. beltrani* and *C. maya* mate choice is symmetric, it is asymmetric between *C. beltrani* and *C. labiosus*. The results of the mating trials are consistent with mitochondrial DNA data which indicate that *C. maya* is genetically the most distinct species in the flock. They are also supported by experiments on visually and chemically mediated mate preferences.

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ADDITIONAL KEY WORDS:—sympatric speciation – sexual selection – female choice – premating isolating mechanisms.

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## INTRODUCTION

Critical to understanding the process of speciation is discovering when and how isolating mechanisms develop (Mayr, 1963; Endler, 1989). Most studies to date examine mate choice patterns of allopatric species in zones of secondary contact (Cobb & Ferveur, 1996), or of sympatric species showing distinct morphological, behavioral and ecological adaptations (e.g. McPhail, 1994). Thus these studies examine the end product of speciation rather than the initial phases.

We investigated female mating preferences in a species flock of pupfishes of the genus *Cyprinodon* in Laguna Chichancanab, Yucatan, Mexico. The laguna is small and shallow, with a surface of about 7.5 km<sup>2</sup>. The geological history of the laguna includes a desiccation phase 8000 years b.p. (Covich & Stuiver, 1974; Hodell, Curtis & Brenner, 1995). Since then the lake filled and its level has been relatively stable. The water cannot be tolerated by most species because of the very high levels of calcium sulphate. Besides the endemic pupfishes, the native fish fauna includes only the live bearer *Gambusia sexradiata*.

Five species of *Cyprinodon* were described based on differences in morphological characters, especially in the structure of their feeding apparatus (Humphries & Miller, 1981; Humphries, 1984a). This might suggest trophic divergence and exploitation of different feeding niches. The most common species, *C. beltrani* (Fig. 1A), is a substrate feeder with a diet consisting primarily of detritus and algae. It is morphologically and ecologically very similar to the coastal-dwelling Yucatan pupfish, *C. artifrons*, the sister species of the Laguna Chichancanab pupfishes. Therefore we assume that *C. beltrani* is the basal member of the flock. The other four pupfishes—*C. maya* (Fig. 1B), *C. labiosus* (Fig. 1C), *C. verecundus*, and *C. simus*—have significantly shorter guts indicating a carnivorous diet. Gut content analysis has confirmed this in general but the diet of *C. simus* is similar to that of *C. beltrani* (Stevenson, 1992; Hortskotte, Wilkens & Strecker, in prep.). All five endemics live in complete syntopy and are dispersed throughout the lake. The comparatively small size of the lake, its homogeneous shoreline, and the ubiquity of all species suggest the occurrence of sympatric speciation. Despite the morphological differences between the five species, genetic differentiation is minimal or absent (see below) (Humphries, 1984b; Strecker *et al.*, 1996). This, along with the geological record, suggests that the species flock is very young and still in the process of speciation.

Phylogenetic relationships among the species have yet to be resolved. The occurrence of specimens which cannot be discriminated by their morphology brings into question the existence of reproductive isolation among all species. Postmating isolating mechanisms are absent or minimal, in the laboratory interspecific crosses are fertile to the second generation (Strecker, 1996). Allozyme studies have shown that genetic similarity between these species is high (the range of Nei's index is 0.972–1.00), and more typical of the variation found within a species (Humphries, 1984b). Comparison of mitochondrial DNA sequences of the D-loop indicate that the species flock is monophyletic, and that all five species are genetically very similar. Only *C. maya* has a unique haplotype and may be reproductively isolated (Strecker *et al.*, 1996).

As is typical of other *Cyprinodon* (Barlow, 1961; Itzkowitz, 1978; Kodric-Brown, 1978), the Chichancanab pupfish have a polygynous, lek-like breeding system with female choice. The males establish breeding territories over suitable spawning substrates. Experiments in the laboratory resulted in a preference for loose gravel

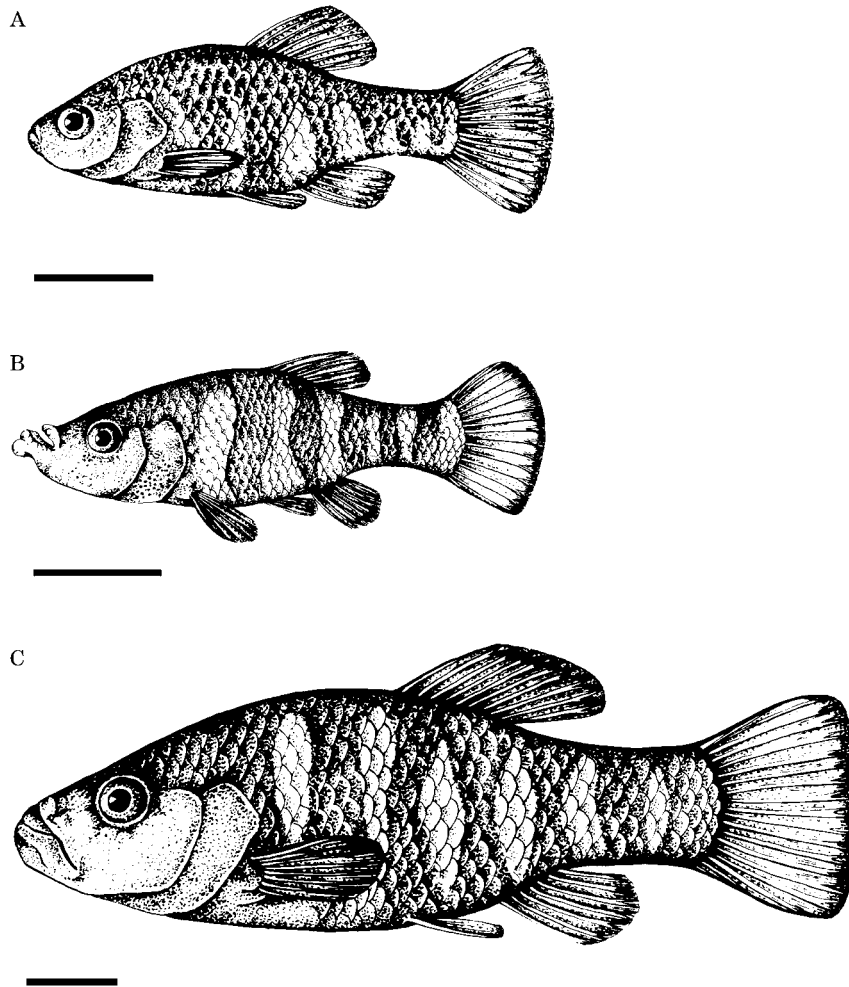


Figure 1. A, *C. beltrani*. B, *C. labiosus*. C, *C. maya*. Scale bar = 1 cm.

as spawning substrate (Strecker, 1996). The breeding coloration of *C. labiosus* and *C. beltrani* males is nearly identical, with a black body and fins, metallic blue shoulder, and yellow caudal peduncle and tail. *C. maya* males usually show a less intense breeding colouration. Females of all three species show the typical cryptic colour patterns of buff background with brown vertical bars characteristic of the genus (Humphries & Miller, 1981). For pupfish in general, gravid females visit the territories, spawn with the resident male, and release either a single egg or no egg each spawning act (Barlow, 1961; Itzkowitz, 1974; Mire & Millett, 1994). The eggs are cryptic and demersal. There is no direct overt male parental care for eggs nor offspring but they receive some protection while in the defended territory. Both males and females mate with numerous sexual partners (Kodric-Brown, 1983). In the Chichancanab pupfishes, reproduction occurs throughout the year (U. Strecker, pers. observ.). The male courtship display of the different species is qualitatively similar.

We tested for premating isolating by examining mating preferences in three species: *C. beltrani*, *C. labiosus*, and *C. maya*. We examined the mating preference of *C. maya* females when given a choice between conspecific and *C. beltrani* males. We chose *C. maya* because it is the largest (30–95 mm) and genetically the most distinct member of the species flock and is most likely to be reproductively isolated from the other species (Strecker *et al.*, 1996). *C. beltrani* is smaller and the least morphologically differentiated species compared with the general pupfish phenotype. We also chose *C. beltrani* and *C. labiosus* because they are similar in size (33–37 mm), and their males often establish breeding territories with contiguous boundaries. Thus females of both species may be courted by, and mate with, conspecific as well as heterospecific males.

## METHODS

### *Study animals*

In the experiments, we used the first and second generations of wild-caught fish. The species were kept in separate aquaria of different sizes (25–100 l) at a temperature of 25–27°C, salinity of 5‰ and a 12-hour light–dark cycle. Fish were fed ad libitum with Tetramin dry food, supplemented with live cladocerans, *Tubifex*, and *Artemia*. Prior to each experiment the test females were isolated from males for a minimum of three days to insure gravidity.

### *Experimental design*

Mating preferences of *C. beltrani* and *C. maya* females were tested using the following experimental design. All trials were conducted in a 36-l aquarium (50 × 26 × 28 cm). One glass petri dish, 15 cm in diameter, filled with multicoloured gravel, was placed at each end of the aquarium. A centrally-positioned plastic plant provided a territorial boundary between males and ensured that territories were of the same size. Three males of both species were placed in the aquarium to ensure that one of the males would be territorial. We conducted female choice trials only if one male of each species defended a territory over the spawning substrate. A female of each species was simultaneously introduced into the experimental tank. This design was chosen because it is more realistic, and approximates the breeding system in the lake. Generally, groups of females visit the breeding area and mate with more than one male. The spawning act is very brief, usually lasts less than a few seconds, and females do not interfere with each other's choice of mating partner. Observation started after the first spawning for each female and lasted for 20 min. We noted the number of times each female spawned with the conspecific and the heterospecific male. Preferences were recorded only in trials where the female spawned at least twice. We used six different sets of males and scored 27 and 24 females for *C. beltrani* and *C. maya*, respectively.

For mating preference tests on *C. beltrani* and *C. labiosus* females, we used a 25 l aquarium (60 × 21 × 20 cm) with two gravel-filled petri dishes (each 10 cm in diameter) at each end. We performed this experiment with 16 different male sets and recorded spawnings from 45 *C. beltrani* and 35 *C. labiosus* females.

TABLE 1. Mean number of spawning acts the test females performed with their own males and males of the other species. The range is given in parentheses

(A) <i>C. beltrani</i> – <i>C. maya</i>		
<i>C. beltrani</i> females		
<i>C. beltrani</i> male	<i>C. maya</i> male	Total
15.5 (2–56)	0.6 (0–10)	16.1 (2–66)
<i>C. maya</i> females		
0	10.4 (2–21)	0.4 (2–21)
(B) <i>C. beltrani</i> – <i>C. labiosus</i>		
<i>C. beltrani</i> females		
<i>C. beltrani</i> male	<i>C. maya</i> male	Total
7.3 (0–21)	9.1 (0–45)	16.4 (2–55)
<i>C. labiosus</i> females		
2.5 (0–8)	6.4 (0–22)	8.9 (2–27)

Sneak matings by non-territorial males, of which there were 20 (1.1%) in all experiments, were not considered because they circumvent female choice.

#### Statistics

Since the data were not normally distributed, we employed paired Wilcoxon-signed-ranks tests.

### RESULTS

#### *C. beltrani*–*C. maya*

When given a choice between *C. beltrani* and *C. maya* males, *C. beltrani* females consistently preferred their own males ( $P=0.0001$ ,  $Z=-4.541$ ,  $n=27$ ). The mean number of spawning acts with the conspecific and heterospecific male is presented in Table 1A. Almost all the spawnings (96.5%) were performed with conspecific males (Fig. 2A). We also examined the frequency of female spawnings with either conspecific or heterospecific males as well as with both males. The majority of the *C. beltrani* females (24 out of 27; 88.9%) spawned exclusively with their own males, just 11.1% (three females) spawned with both males, and none spawned exclusively with the *C. maya* males (Fig. 3A).

All *C. maya* females tested with conspecific and *C. beltrani* males spawned only with *C. maya* males ( $P=0.0001$ ,  $Z=-4.286$ ,  $n=24$ ; Figs 2A, 3A; Table 1A).

#### *C. beltrani*–*C. labiosus*

*C. beltrani* females did not differentiate between their own and *C. labiosus* males ( $P=0.8071$ ,  $Z=-0.244$ ,  $n=45$ ). The mean number of spawnings is even slightly higher with heterospecific *C. labiosus* males (Table 1B).

Although *C. labiosus* females did not uniformly choose conspecific males, they

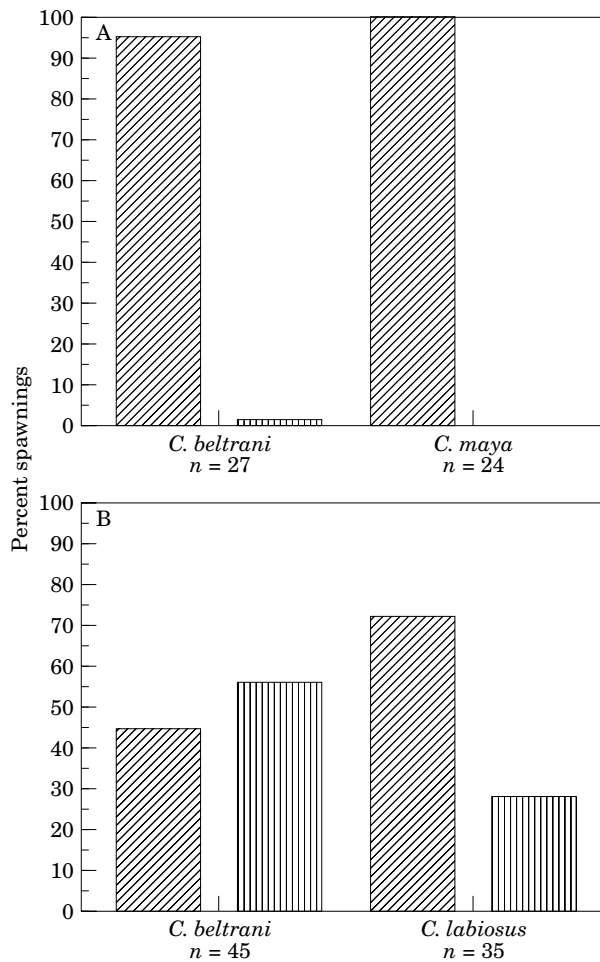


Figure 2. Percentage of spawnings by *C. labiosus* and *C. beltrani* females with conspecific (▨) and heterospecific (▤) males. A, *C. beltrani*–*C. maya*. B, *C. beltrani*–*C. labiosus*.  $N$ =number of females tested.

showed a preference for conspecific males ( $P=0.0109$ ,  $Z = -2.547$ ,  $n = 35$ ) and they spawned in the mean more often with conspecifics (Table 1B).

Also a comparison of the proportion of the spawnings shows that *C. beltrani* spawned even more often with heterospecific as with conspecific males, whereas *C. labiosus* females spawned proportionally more often with their own males than *C. beltrani* females (72% vs. 44.6%; Fig 2B).

Most females of *C. beltrani* and *C. labiosus* spawned with both males (66.8% and 51.4%, respectively; Fig. 3B). In cases where females spawned with only one species of male, *C. beltrani* females spawned 13.3% with the conspecific and 20.0% with the heterospecific male. By comparison, a higher proportion of the *C. labiosus* females spawned exclusively with their own males (34.3% vs. 14.3% with *C. beltrani* males; Fig. 3B).

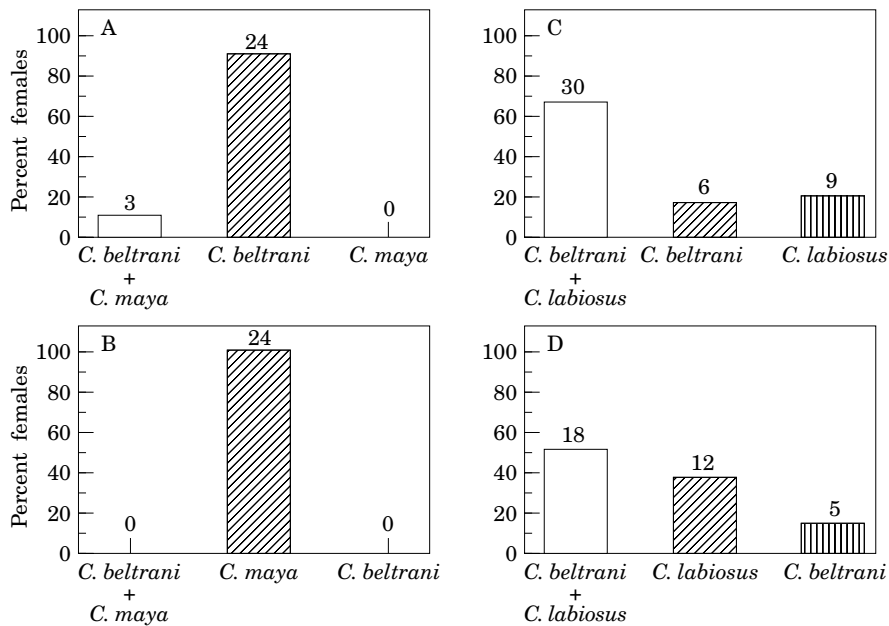


Figure 3. Percentage of females spawning in each replicate with conspecific (▨), heterospecific (▤) and both (□) males. A, *C. beltrani*. B, *C. maya*. C, *C. beltrani*. D, *C. labiosus*. Number above each column is the number of females.

## DISCUSSION

### *Mate choice between C. beltrani and C. maya*

Both *C. beltrani* and *C. maya* females discriminate against heterospecific males, suggesting that both use species-specific cues to choose mates. All *C. maya* females spawned exclusively with conspecific males. These results also agree with studies on mating preferences based on visual and chemical cues (Strecker, 1996; Strecker & Kodric-Brown, 1999). Furthermore, *C. maya* is the only member of the species flock that shows clear genetic differentiation based on mitochondrial DNA (Strecker *et al.*, 1996). Thus *C. maya* is reproductively isolated, at least from *C. beltrani*.

Our data indicate that premating isolation between *C. beltrani* and *C. maya* is symmetrical, since *C. beltrani* females also mate almost exclusively with conspecific males. Interestingly, results from mate recognition experiments using visual and chemical cues have shown that *C. beltrani* females could not discriminate between conspecific and *C. maya* males (Strecker & Kodric-Brown, 1999). We as yet do not know the proximate mechanism for mate recognition in *C. beltrani* but apparently, visual or chemical cues alone are not sufficient, when presented singly. However, one possibility is that predator avoidance behaviour by *C. beltrani* females causes them to avoid aggressive *C. maya* males, thus preventing heterospecific matings. *C. maya* are larger than *C. beltrani* as well as carnivorous and, in the laboratory, have been observed to chase and prey on smaller *C. beltrani* (Strecker, pers. observ.). Another possible explanation is that differences, even subtle in male courtship could be responsible. Without data on male courtship one cannot eliminate the possibility

that *C. maya* males are responsible for this low hybridization rate by not courting or courting less intensely *C. beltrani* females. In other words, it is possible that the only isolation in this system is *C. maya*, with *C. beltrani* not playing a role in the discrimination.

Furthermore, the existence of size-based assortative mating could be another explanation. Female preferences for males of a size similar to their own have been shown for *C. pecosensis* (Kodric-Brown, 1977) as well as for *Plecoglossus altivelis* (Osmeridae) (Iguchi & Mackawa, 1993). Thus a combination of size-assortative mating and predation risk may be sufficient to prevent heterospecific matings in *C. beltrani* even in the absence of a species-specific mate recognition system.

#### *Mate choice between C. beltrani and C. labiosus*

Both *C. beltrani* and *C. labiosus* mate with conspecific as well as with heterospecific males but to different degrees. Most females mated with both males (Fig. 3B). This suggests that many females may choose their mates at random even ignoring species-specific traits. Nevertheless, females of these two species differ in their ability to discriminate between conspecific and heterospecific males. While *C. beltrani* females divided their spawning acts almost equally among the *C. beltrani* and *C. labiosus* males, *C. labiosus* females spawned more than twice as much with their own males (Table 1B; Fig. 3B).

In the closely related pupfish *C. variegatus*, choice tests have shown that male competitive superiority is apparently not a factor in female mate selection (Itzkowitz, 1978). This lack of female choice is compensated by male–male competition for the suitable spawning sites on which the territories are established, and the females deposit their eggs in proportion to the size of these territories. To avoid this effect in our experiments male territory size was standardized. The *C. beltrani* females spawn almost equally with conspecific as with heterospecific males (Fig. 3B). This indicates that *C. beltrani*, the basal member of the flock, is still using this ancestral strategy for mate choice and that the females even do not distinguish between conspecific and heterospecific males. In contrast, *C. labiosus* females have developed a mate recognition system which biases their choice towards conspecific males.

These differences in ability to discriminate between conspecific and heterospecific males are supported by experiments on chemically mediated mate preferences (Strecker, 1996; Strecker & Kodric-Brown, 1999). In these olfactory choice experiments *C. labiosus* females preferred their own males. The results of both the mating (this study) and the olfactory choice experiments (Strecker, 1996; Strecker & Kodric-Brown, 1999), suggest that a species-specific mate recognition system, although imperfect, is present in *C. labiosus* but is absent in *C. beltrani*. The mitochondrial DNA data lend additional support to this interpretation, since 80% of the haplotypes in these two species are identical (Strecker *et al.*, 1996). It is likely that morphologically indeterminate forms, which are found in the natural habitat, may represent 'hybrids' between these two species.

The mate recognition system of *C. beltrani* and *C. labiosus* is not only incomplete, it is also asymmetrical. It is much better developed in *C. labiosus* than in *C. beltrani* (Figs 2B, 3B). This suggests that selective pressures acting on the two species differ. Since *C. beltrani* is the most abundant species in the lake (68–85% of all *Cyprinodon* captured in seine hauls; Humphries & Miller, 1981), thus, even if the females mated

randomly, most matings would be with conspecific males. In *C. labiosus* a more discriminatory mate recognition system could reflect selection against mate choices that produce hybrid offspring of an intermediate phenotype (Dobzhansky 1940, 1951; Hatfield & Schluter, 1999; but see Butlin, 1989; Coyne, 1992; Grant & Grant, 1996). Although hybrids raised in the laboratory are both viable and fertile, they may be less so under field conditions, where they must compete with the parental types for food as well as mates (Hatfield & Schluter, 1999).

#### *Modes of sympatric speciation*

Two mechanisms, alone or in combination, that can cause sympatric speciation are disruptive and sexual selection (Galis & Metz, 1998; Sturmbauer, 1998). The lack of competing fish species caused by the extreme chemical conditions in the Laguna Chichancanab may favour adaptive radiation by providing open ecological niches that can be invaded by species not originally adapted to these niches (Lack, 1947; Fryer & Iles, 1972; Futuyma, 1979; Dominey, 1984; Bush & Howard, 1986; Wilkens, 1988). Trophic factors can play an important role in the speciation process (Turner & Grosse, 1980; Galat & Vucinich, 1983; McPhail, 1984; Smith & Todd, 1984; Taylor & Bentzen, 1993). The pupfish flock in the Laguna Chichancanab shows exploitation of new niches. Asymmetrical disruptive selection could cause changes in feeding morphology from a generalized detritivore, such as *C. beltrani*, to a predator such as *C. maya*.

Also different female mating preferences in a population may lead to speciation. Body size, for example, has been shown to play a role in premating isolation in sympatric species (McPhail, 1994; Nagel & Schluter, 1998). This may operate here as well, since *C. maya* males are almost twice as large as *C. beltrani* males. Also olfactory cues might provide a similar cue in *Cyprinodon* (Strecker & Kodric-Brown, 1999).

Our data cannot test which is the driving force in this speciation process. However, the development of mating preferences does appear to be a critical step in the genetic differentiation of populations and the completion of the speciation process (Endler & Houde, 1995). Morphotypes, such as *C. beltrani* and *C. labiosus*, and reproductively isolated species, such as *C. maya*, may represent two different phases in the speciation process.

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