Courtship and Other Behaviors Affected by a Heat-Sensitive, Molecularly Novel Mutation in the *cacophony* Calcium-Channel Gene of Drosophila

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ABSTRACT

The *cacophony* (*cac*) locus of *Drosophila melanogaster*, which encodes a calcium-channel subunit, has been mutated to cause courtship-song defects or abnormal responses to visual stimuli. However, the most recently isolated *cac* mutant was identified as an enhancer of a *comatose* mutation's effects on general locomotion. We analyzed the cac^{TS2} mutation in terms of its intragenic molecular change and its effects on behaviors more complex than the fly's elementary ability to move. The molecular etiology of this mutation is a nucleotide substitution that causes a proline-to-serine change in a region of the polypeptide near its EF hand. Given that this motif is involved in channel inactivation, it was intriguing that cac^{TS2} males generate song pulses containing larger-than-normal numbers of cycles—provided that such males are exposed to an elevated temperature. Similar treatments caused only mild visual-response abnormalities and generic locomotor sluggishness. These results are discussed in the context of calcium-channel functions that subserve certain behaviors and of defects exhibited by the original *cacophony* mutant. Despite its different kind of amino-acid substitution, compared with that of cac^{TS2}, cac^{S} males sing abnormally in a manner that mimics the new mutant's heat-sensitive song anomaly.

THE normal forms of calcium channels exist in many forms. In vertebrates, for example, six classes of voltage-gated Ca^{2+} channels, which are distinguished by their voltage dependency and sensitivity to pharmacological agents, have been cloned (Hoffman *et al.* 1994; Catterall 1998). Several aberrant forms of calcium-channel polypeptides are also known. Most of these variants, the majority of which in turn are naturally occurring, exhibit unsurprising connections between altered genotypes and phenotypes. In part because functions of ion channels are ubiquitous among organisms and widely dispersed within a given animal, one imagines that mutations in the genes encoding them would obviously derange motor functions. In this regard, certain mammalian and *Caenorhabditis elegans* mutants speak to the whole-organismal and tissue-functional meaning of calcium-channel functions. However, the phenotypic defects associated with these channel variants are in the main neuropathologies: migraines, ataxia, lethargy, muscular dysgenesis, or myotonia (*e.g.*, Lee *et al.* 1997; Miller 1997; Doyle and Stubbs 1998; Jen 1999; Ashcroft 2000; Weinrich and Jentsch 2000). It is difficult to pit such phenotypes against discrete, measurable behaviors exhibited by wild-type humans, mice, or nematodes (although see Schafer and Kenyon 1995).

Rare among calcium-channel mutants are variants of Drosophila that exhibit relatively enticing—or at least nonpathological—abnormalities of behavior. The mutations in question turned out to have occurred in an *X* chromosomal gene called *cacophony* (abbreviated *cac*). One of the first courtship mutants that was deliberately induced in Drosophila is the original *cac* variant (Schilcher 1976, 1977). This mutation (cac^{S}, whose allelic designation stands for "song") alters the patterns of sounds produced by the male as he extends a given wing and vibrates it near the female to produce his courtship song: Pulses of tone, instead of each containing on the order of three cycles as in wild-type songs of this species, contain approximately five cycles; pulse amplitudes are also higher than normal (Schilcher 1977; Kulkarni and Hall 1987; Peixoto and Hall 1998). However, the songs generated by *cac* males are not pathologically degraded or otherwise aberrant; they are still highly patterned (Wheeler *et al.* 1989; Neumann *et al.* 1992). For example, *cac* males produce their tone pulses at a rate of ~30/sec as in wild type (Bernstein *et al.* 1992), and the mutant's intrapulse "carrier" frequencies (baritone notes) are largely normal (Wheeler *et al.* 1989).

High-resolution mapping of the *cac* locus led both to a complicated phenogenetic picture and to a determination of the molecular etiology of the mutation: The *cac*-induced song defect is uncovered in flies heterozygous for this mutation and lethal genetic variants that had been independently mapped to the locus. These lethals in turn fail to complement *nightblind-A* (*nbA*) mutations,

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which also co-map and by themselves cause defects in visually mediated behavior and the light-elicited electroretinogram (ERG; Kulkarni and Hall 1987; Homyk and Pye 1989; Smith *et al.* 1998b); however, *cac/nbA* heterozygotes are normal for courtship song and vision. (Complementation tests of songs generated by chromosomally female *XX* flies were permitted by turning them into males via introduction of the *transformer* mutation, whose general properties are reviewed by Baker *et al.* 2001.) Fine mapping of *cac*, *nbA*, and the lethal mutations permitted positional cloning of the mutated gene; it was found to encode an α1 subunit of a voltage-activated calcium channel, which was named Dmca1A (Smith *et al.* 1996). Chromosomal lesions at the locus (which are among the lethal *cac* variants) rupture the open reading frame (ORF; Smith *et al.* 1996). The original cac^{S} variant was shown by Smith *et al.* (1998b) to be a missense mutant that harbors an amino-acid substitution within a transmembrane region of the third intrapolypeptide repeat (see Figure 1B). A cac^{nbA} mutation was found to have suffered a premature stop mutation in an alternatively spliced cassette with respect to a *cac* mRNA isoform whose expression is enriched in the visual system (Smith *et al.* 1998b; see Figure 1B).

Additional findings stemming from analyses of the original *cacophony* mutants—along with other of the older ones and a newly induced mutation at the locus—have further broadened the phenotypic significance of the gene's action: cac^{S} (but not cac^{nbA} mutations) causes convulsions and other locomotor anomalies at an intermediate/high temperature (37°) and much faster-than-normal loss of all motor functions at an extremely high one (46°; Peixoto and Hall 1998). Induction of mutations that enhance temperature-sensitive (*TS*) paralytic defects of a *comatose* mutation ($comt^{ST53}$) in a gene encoding an *N*-ethylmaleimide-sensitive fusion protein (NSF) led to recovery of a new *cac* allele (Dellinger *et al.* 2000). It was subsequently shown that this cac^{TS2} mutant is by itself heat sensitive for paralysis (at a temperature milder than that required to paralyze cac^{S}) and causes failure of synaptic function at neuromuscular junctions (Kawasaki *et al.* 2000).

Additional molecular findings have been obtained from analysis of the *cacophony* gene and its expression: The primary *cac* transcript was inferred by analysis of multiple cDNAs to be subjected to RNA editing (Smith *et al.* 1996), one of the first examples of this process in Drosophila and for a gene that encodes a voltage-sensitive ion channel (*cf.* Seeburg 2000); the presumed adenosine-to-inosine edits (observed as **a**-to-**g** substitutions at the level of cDNA heterogeneity) were subsequently shown to occur in actual flies via extractions of RNA and RT-PCR analysis (Smith *et al.* 1998a; Palladino *et al.* 2000). Assessments of Dmca1A sequences in Drosophila derived from natural populations revealed a low level of polymorphism; five noncoding sites were found to be variable among *Drosophila melanogaster* lines, although no amino-acid substitutions in the channel polypeptide were observed between this species and its close relative, *D. simulans*, within ~1 kb of genomic sequence. However, courtship-song analysis of the *melanogaster* lines revealed a significant association between pulse amplitude and one of the polymorphic *cac* sites (Peixoto *et al.* 2000). As this site is within an intron, we entertained the possibility that it could be in linkage disequilibrium with a nonsynonymous (amino-acid-changing) polymorphism elsewhere in the gene. These preliminary interspecific Dmca1A comparisons were made in light of the fact that the cac^{S} mutation changes the normal song such that visual traces of the mutant sounds look to the human observer's eye as if they could be those generated by males of another Drosophila species (*e.g.*, Cowling and Burnet 1981; Hoikkala and Lumme 1987; Tomura and Oguma 1994), given the "nonpathological" nature of the cac^{S} phenotype. This supposition about the possibility of naturally occurring calcium-channel variations contributing to song variations, and even their evolutionary divergence, may be more than empty speculation because certain components of the song differences between *D. virilis* and *D. littoralis* have been genetically mapped to a region of the *X* chromosome that includes the *cacophony* locus (Paallysaho *et al.* 2001).

Against this background, we wondered whether the new cac^{TS2} mutant would exhibit a "patterned" song abnormality or a phenotype that parts company with that kind of defect in either direction. cac^{TS2} flies are defective in their gross locomotion by definition (Dellinger *et al.* 2000), but this isolation phenotype means that the songs of cac^{TS2} males could fall anywhere in the range from severely anomalous to normal. Whatever that behavioral outcome might be, along with those obtained from testing visually mediated responses, we also aimed to correlate the intragenic *cacophony* change with the new mutant's phenotypes, concentrating on behaviors that extend well beyond the doleful defect by which this calcium-channel variant was identified.

MATERIALS AND METHODS

***D. melanogaster* strains and basic fly handling:** Flies were raised on a sucrose/cornmeal/yeast medium supplemented with the mold inhibitor Tegosept. Most cultures were maintained in 12 hr:12 hr light:dark cycles (12:12 LD) at 25° and 70% relative humidity. Flies emerging from the cultures were collected as <1-day-old adults under ether anesthesia. Males for courtship or longevity tests were stored singly in food vials; females paired with males for such tests, and males subjected to other kinds of phenotypic characterizations, were stored 10–15 flies per vial. To determine whether cac^{TS2} flies would be sensitive to temperature changes, they (and parallel controls) were also reared at 18° and 29° in incubators programmed for 12:12 LD.

The strains from which flies were taken for behavioral (and in some cases physiological) tests were Canton-S wild type, cac^{TS2}, cac^{S}, *w* $comt^{ST53}$ (the *white*-eyed, *comatose*-mutated, cac^{+}

strain that had been mutagenized by Dellinger *et al.* 2000 to induce cac^{TS2}), $comt^{ST53}$, $comt^{ST17}$, cac^{TS2}, $comt^{ST17}$, and cac^{S} $comt^{ST17}$. The cac^{S} strain was the original one, in which factor(s) causing mediocre mating-initiation latencies (Schilcher 1977) were still present but did not affect the mutant male's courtship song (Kulkarni and Hall 1987). The $comt^{ST17}$ and $comt^{ST53}$ mutants (for which "*ST*" stands for "sensitive to temperature") are also known as $comt^{1}$ and $comt^{4}$ according to FlyBase (http://flybase.bio.indiana.edu), which provides a soft-copy paper trail about the origin and properties of these NSF mutants. The cac^{S}, $comt^{ST17}$, $comt^{ST53}$, and cac^{TS2} strains (for the latter, one of them) are true-breeding stocks in which males are hemizygous for any one of these *X* chromosomal mutations and females are homozygous for it. True-breeding *w* $comt^{ST53}$, cac^{TS2} $comt^{ST17}$, and cac^{S} $comt^{ST17}$ stocks, and a separate cac^{TS2}-including stock (a source of males for some of the song recordings) were each maintained in a situation in which hemizygous mutant males mated with attached-*X* females [*C(1)DX, y f*/Y].

Molecular characterization of cac^{TS2}: *Obtaining DNA and cDNAs:* DNA corresponding to the 34 exons that are distributed over ~45 kb at the *cac* locus was obtained in two ways:

By cDNA synthesis, for which, in a given such operation, total *w comtST53* or *cacTS2* RNA was extracted from an homogenate of ~50 whole adults (from 4- to 6-day-old males of either genotype) using TRIzol reagent according to the manufacturer's instructions (GIBCO BRL, Rockville, MD). A total of 5 μg of RNA (from a given extract) was reverse transcribed with random hexamer primers using the ThermoScript RT-PCR system (GIBCO BRL).

By obtaining genomic DNA, for which ~75 4- to 6-day-old whole-adult males (*w* $comt^{ST53}$ or cac^{TS2}) were collected and immediately frozen with liquid nitrogen. They were then homogenized in the following buffer: 5% sucrose, 80 mm NaCl, 100 mm Tris pH 8, 0.5% SDS, 50 mm EDTA.

The homogenate was treated with 8 m KOAc, and phenol extractions were performed to separate out the nucleic acids. DNA was precipitated from the supernatant using isopropanol and exposed to two 70%-ethanol washes. The pellet was resuspended in TE (10 mm Tris-Cl, 1 mm EDTA) overnight and then treated with RNase A for 3 hr at 50°–60°.

PCR and DNA sequencing: Primers were designed to amplify 100- to 1000-bp segments of genomic DNA and cDNA products for sequencing; 26 such primer pairs were designed, which in sum covered the entirety of the 5.6-kb Dmca1A ORF within the *cac* locus (*cf.* Smith *et al.* 1996; Peixoto *et al.* 1997). Primers were synthesized by and purchased from Integrated DNA Technologies (Coralville, CA). PCR was carried out in a PTC-100 (MJ Research, Waltham, MA) for 30 cycles (94° for 1 min, 55° or 60° for 1 min, and 72° for 1 min). Reactions were performed in 50-μl volumes that included 0.4 mm primers (0.2 mm each forward and reverse oligonucleotides), 0.2 mm dNTP mix, 10× PCR buffer, and Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis) or 0.4 mm primers, 0.2 mm dNTP mix, 10× PCR buffer without Mg^{2+}, 1.5 mm $MgCl_2$, and Taq DNA polymerase from another source (Promega, Madison, WI). PCR products were analyzed on 1 or 1.2% agarose gels and then purified with the QIAQuick spin PCR purification kit (QIAGEN, Valencia, CA) for direct sequencing. Single-stranded sequencing reactions in both directions were performed in 20-μl volumes that included 0.2 mm primer (forward or reverse), 5 μl eluant containing the purified PCR product, and 8 μl Prism Dye-Deoxy terminator cycle sequencing kit mix (Perkin-Elmer/Applied Biosystems, Foster City, CA). Such reactions were carried out in the PTC-100 for 24 cycles (96° for 30 sec, 50° for 20 sec, and 60° for 4 min). They were then purified in 50 mg/ml Sephadex G-50 fine DNA grade columns (Pharmacia Biotech, Piscataway, NJ) and electrophoresed in a Perkin-Elmer/Applied Biosystems model 373 Stretch XL DNA sequencer. PCR products to be cloned before sequencing were purified with the QIAEX II gel extraction kit (QIAGEN). Overnight ligations at 4° were set up using the pGEM-T Vector System I (Promega). Subcloning-efficiency DH5α-competent cells (GIBCO BRL) were used for bacterial transformations, as plated in Luria broth agar culture dishes containing 100 μg/ml ampicillin, 0.05 μg/μl X-gal (Fisher Scientific, Pittsburgh), and 110 mm isopropyl thiogalactoside. Colonies were isolated and grown in overnight cultures. DNA was then extracted and purified using the QIAPrep spin miniprep kit (QIAGEN). Restriction enzyme digest with *Eco*RI (Promega) was performed to ensure that the DNA contained the fragment of interest. During the first stage of obtaining DNA sequence data from various subsets of the *cacophony* gene, as carried by *w* $comt^{ST53}$ or cac^{TS2} flies, PCR products were cloned before sequencing (see above). Direct sequencing was performed for certain intragenic regions only to confirm or to deny putative nucleotide differences (between the two genotypes just given) that were observed in the first-stage sequencing operation. MacVector 6.0 software (Accelrys, San Diego) was used for analyses of nucleotide sequences and alignments as well as for conceptual translations (to generate the Dmca1A polypeptides inferred to be produced by *w* $comt^{ST53}$ or cac^{TS2} flies). The investigator performing these molecular operations (B. Chan) deliberately did not consult with R. W. Ordway and co-workers as various components of the sequence data were emerging in parallel at our and his institutions (see RESULTS).

Behavioral observations and analyses: *General courtship and mating performances:* Males were reared and stored at 25° as usual (see above), except that approximately one-half of the flies of a given genotype had their wings completely clipped off with fine-tipped scissors; then they and their intact brothers were stored individually in food vials. Single pairs of flies were placed in a courtship-observation apparatus at 25°; this device, known as a "mating wheel" (Hall 1979), contains 10 chambers (diameter, 1 cm; height, 1 cm), which are formed by rotating separate discs of the wheel such that 20 "half-chambers," 10 containing an individual male, the rest an individual female, are merged at one moment. The times elapsing between the moment of pairing and initiations of courtship and of (subsequent) mating were recorded, as were durations of copulation.

Courtship-song recordings and analyses: Males to be recorded for sound production in the presence of females were reared and stored individually as above. Before recording, the male-containing vials were pretreated at 20°, 25°, or 30° for 30–60 min before being paired with individual females at the same temperature used for a given pretreatment. Wild-type virgin females were reared and stored as noted in the previous subsection, except that their wings were clipped off upon collection so that only the wing-vibrational sounds generated by a courting pair would emanate from males. Single male-female pairs were recorded at a given temperature for 5 min or until they mated; in the latter case, <3-min recordings were excluded from subsequent analyses. Fly-produced sounds were picked up by an Insectavox (Gorczyca and Hall 1987) and interfaced with a Sony Hi8 video/audio camera. The temperature was monitored inside the Insectavox, before and after each recording, to verify that the temperature did not fluctuate more than 1°; if it did, that record was not analyzed. Songs that passed muster were digitized and logged using LifeSong software (Bernstein *et al.* 1992), essentially as in Villella *et al.* (1997) and Anand *et al.* (2001); singing bouts with ≤3 pulses were not logged. During the 30° recordings, some of the mutant males fell on their backs and exhibited seizure-like activities; these produced sounds that could be misinterpreted as song sounds. Thus, it was necessary to observe the

video record while logging a song produced by such a male, to confirm that the sounds entered at the keyboard into the male's soft-copy file were exclusively song pulses.

For each logged song file the following parameters were computed: cycles per pulse (CPP), pulse amplitude (in arbitrary units, but scaled in the same way among files as in Bernstein *et al.* 1992), intrapulse frequency (IPF), and interpulse intervals (IPI). IPI cutoffs were applied as in Peixoto and Hall (1998); thus, for example, a >90-msec interval of silence between a pair of pulses at 20° was defined as an interbout interval.

Distributions of song bout types, varying as to their CPP values, were generated as follows: Using the song file obtained from a *given male*, the average CPP for any bout that produced at least eight pulses was computed; five categories were defined in terms of average pulse cyclicity per bout (lowest: ≤2 CPP; highest: >5); then proportions of bouts falling into each category were determined for that individual's record (see Figure 4). Subsequently, the mean proportion (±SEM) of each category's content *among males* of a given genotype (*e.g.*, what part of 100% is the "≤2" or ">5" category in the average male?) was computed (see Table 3).

Nonreproductive behaviors and responses to stimuli: *Phototaxis:* A Y-tube apparatus (Kulkarni and Hall 1987) was used to measure fly movements toward light. One clear plastic arm of the Y and its stem were thoroughly wrapped with black tape; the other arm was so wrapped except at the tip. An incandescent light source (at the end of a fiber-optics lead) was placed 2–3 cm from the open tip of that arm (resulting in an ~2000-lux stimulus). Five to 10 males of a given genotype, which had been raised and stored as adults at 25° (see above), were placed in the stem of the Y and allowed to walk toward the light for 2 min at room temperature (22°–23°). At the end of this time, numbers of flies distributed in each arm and those remaining in the start tube were counted. A second set of phototaxis measurements was obtained after exposing a different set of flies (of the various genotypes) to 29° for 20 min and then testing them at room temperature immediately afterward.

Electroretinograms: These light-elicited voltage changes were recorded extracellularly, basically as in Rendahl *et al.* (1992, 1996), as augmented and modified somewhat by Stowers and Schwarz (1999). Moreover, one set of cac^{TS2} *vs.* cac^{+} comparisons was made at room temperature (22°–23°) and another set in a recording chamber preheated to 30°–31°.

Locomotor activity: Individual males of a given genotype, which had been raised and stored as adults at 25° (see above), had their general locomotor activity measured at room temperature by placing a given fly in a cylindrical plastic chamber (diameter: 1 cm, height: 1 cm), which was divided across the diameter by a straight line. After introducing the fly into this chamber and allowing it a 3-min "accommodation" period, the number of times it crossed the line in the next 2 min was counted (*cf.* Kulkarni and Hall 1987; Villella *et al.* 1997). Additional line-crossing counts were made (for two separate groups of males) at 25° and at that temperature immediately after exposure of males to 29° for 20 min.

Responses to mechanical shock: Males that were 3- to 5-day-old Canton-S wild type, cac^{TS2}, and $comt^{ST53}$ and had been reared at 18°, 25°, or 29° (and stored at these respective temperatures) were individually placed in a series of empty culture vials at 25° and vibrated using a Vortex Genie 2 (Fisher Scientific) at top speed for 20 sec. Recoveries were measured by noting the amount of postvortexing time required for the first fly within a given genotypic group ($n = 10$ for each test) to regain its ability to crawl along the inside surface of the vial; the other flies within the group rapidly followed suit. The test for each genotype was repeated 10 times, using different sets of 10 flies each time. A second set of vortexings and recovery-time assessments was made after first exposing the flies to 29° for 30 min.

Statistics: JMP Version 3.1.5 (SAS Institute, Cary, NC) software (for Macintosh) was used to analyze data from the behavioral tests and recordings. Statistical analyses were carried out for the different kinds of metrics after transforming them to approximate normal distributions by testing the Studentized residuals (*cf.* Villella *et al.* 1997; Anand *et al.* 2001). These data were then subjected to ANOVA to compare the numerical results influenced by the various genotypes. Subsequent planned pairwise comparisons were performed, and α's were adjusted appropriately for experiment-wise error (*cf.* Sokal and Rohlf 1995). ERG parameters were analyzed nonparametrically using Kruskall-Wallis tests.

RESULTS

Dmca1A nucleotide-sequence divergences from the norm in the cac^{TS2} mutant: We sequenced cDNAs and segments of genomic DNA from the cac^{TS2} mutant and compared the results to those obtained from the cac^{+} allele carried on the *w*-marked, $comt^{ST53}$-bearing *X* chromosome in the strain used to induce this *cacophony* mutation. Several differences were found (Table 1), most of them involving synonymous base-pair changes or disparities between the published sequence for Dmca1A coding information (Smith *et al.* 1996; Peixoto *et al.* 1997; GenBank accession no. U55776) and both the cac^{TS2} and cac^{+} ($w\ comt^{ST53}$) sequences. One mutant *vs.* normal difference was found for which the amino acid specified is different between cac^{TS2} and cac^{+}($w\ comt^{ST53}$), and the codon in the latter (normal) sequence is identical to the published one at this position (Figure 1A). A **tcg** codon was found in cac^{TS2}, which would substitute serine for proline (**ccg**). This conclusion is fully consistent with mutant *vs.* normal sequence data generated independently by R. W. Ordway (personal communication), in whose laboratory cac^{TS2} was induced (Dellinger *et al.* 2000). The proline present in the relatively C-terminal region of CAC referred to above is three residues downstream of a calcium-channel motif known as the EF hand (Figure 1). This is a stretch of ~30 amino acids (Figure 1A) found in many different kinds of calcium-binding proteins (Lewit-Bentley and Réty 2000; Burgoyne and Weiss 2001).

One further case of CAC sequence heterogeneity was encountered in conjunction with the molecular characterization of cac^{TS2}: An **atg** (methionine) codon was found in a mutant-derived cDNA (Table 1), which at first blush was different from **ata** (isoleucine) at the corresponding position in cac^{+}($w\ comt^{ST53}$) or in the archived sequence (GenBank accession no. U55776). However, this turned out to be a case of cDNA heterogeneity (see legend to Table 1), which corresponds to a site within the extracellular loop between the third and fourth transmembrane segments of the first intra-Dmca1A repeat (Figure 1B). This feature of the sequencing results implies an additional instance of aden-

TABLE 1

Sequence analysis of *cacophony*

Base pair	Published codon	cac^{TS2} codon	Amino-acid change
	A. Base-pair differences leading to amino-acid changes		
936[a]	ata	atg	I128M
4714	ccg	tcg	P1388S
Base pair	Published codon	cac^{TS2} and $w\ comt^{ST53}$ codon	Amino acid
	B. Silent base-pair differences		
1890	ttt	ttc	F446
2778	ccg	cca	P742
3786	ccc	cca	P1078
3795	cgg	cga	R1081
3927	aag	aaa	K1125
4878	gac	gat	D1442
5649	cca	ccg	P1699
Base pair	Published codon	cac^{TS2} and $w\ comt^{ST53}$ codon	Amino-acid change (re. Smith *et al.* 1996)
	C. Base-pair differences in common with cac^{TS2} and $w\ comt^{ST53}$ (cac^{+})		
2092	ggt	agt	G514S
2997	atg	ata	M815I
3068	agt	aat	S839N
3361	ggt	agt	G937S
3598	gtg	agt	V1016M
4106	agc	aac	S1185N
5290	gat	aat	D1580N

Sequencing and resequencing the *cac* ORF (via RT-PCR, use of PCR'ed genomic DNA fragments, or both) led to establishment of various nucleotide differences among three Dmca1A (calcium-channel-encoding) sequences: the published one (Smith *et al.* 1996) and the two determined here, cac^{TS2} and the cac^{+} allele in the $w\ comt^{ST53}$ strain (the so-called parental one) in which this newest *cacophony* mutation was induced. The base-pair and amino-acid numbers are as reported and archived by Smith *et al.* (1996) (GenBank accession no. U55776). Nucleotide differences for which the three sequences do not all agree are indicated by underlining. (A) Base pair differences that led to amino-acid substitutions in cac^{TS2} compared with its parental strain and the published sequence. For the cac^{TS2}, the thymidine at base pair 4714 (per Smith *et al.* 1996 and GenBank accession no. U55776) was observed in four independent cDNA amplicons, resulting from RT-PCR of RNA from this mutant, and the expected cytosine (*cf.* Smith *et al.* 1996) was observed in two independent amplicons whose starting material was $w\ comt^{ST53}cac^{+}$. (B) Silent base-pair differences (compared with the synonymous published codon) at the seven positions indicated. (C) Base-pair differences observed in both cac^{TS2} and $w\ comt^{ST53}$ (which therefore were the same as each other), compared with the results of Smith *et al.* (1996) for these 7 bp and codons.

[a] Denotes heterogeneity at base pair 936, for which the canonical adenosine (**a**) was observed in three cDNA amplicons from cac^{TS2}, but a substitution to guanine (**g**) was observed in six independent ones. The corresponding coding information in genomic DNA from this mutant (three amplicons) was found to be **a**, as expected (GenBank accession no. U55776). With regard to position 936 as interrogated for $w\ comt^{ST53}\ cac^{+}$, the results were **a** in all three cDNA amplicons generated (*i.e.,* no putative editing-mediated substitution to **g** for these three particular transcripts) and **a** as well in a genomic-DNA amplicon.

osine-to-inosine RNA editing (observed as an **a**-to-**g** change at the level of cDNA analysis)—one that happened not to be encountered in the earlier cases of sequencing library-derived or RT-PCR'ed cDNAs involving the *cac* ORF (Smith *et al.* 1996; Peixoto *et al.* 1997; Smith *et al.* 1998a; Palladino *et al.* 2000).

General features of reproductive performances: Courtship-initiation latencies, mating-initiation ones, and mating durations were measured to assess the overall reproductive behavioral performances of *cac* mutant males, compared with those of genetically normal ones and males hemizygous for $comt^{ST53}$. Half of the tested males of a given type had their wings removed to assess the contribution of courtship song to female receptivity. For example, if an intact mutant male type exhibited a longer-than-normal mating-initiation latency (in part, a measure of the wild-type females' receptivity), this subnormality would not be solely the result of any singing abnormality that the male might exhibit (see *Courtship song*) if the wingless individuals were also less successful than wingless wild-type males (see below).

Proportions of males that courted and mated are

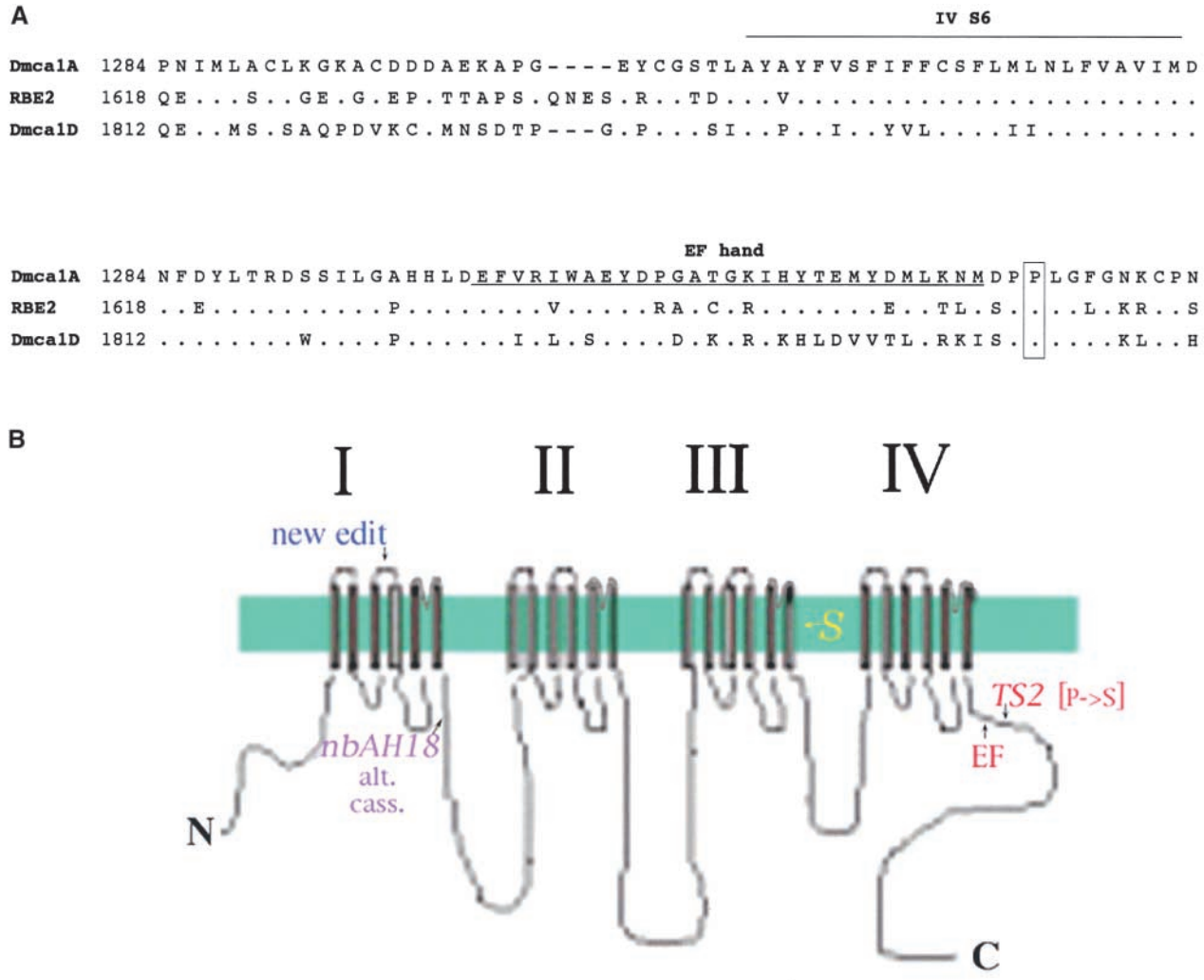

FIGURE 1.—Sequence analysis of *cacophony*. (A) Proximity of the cac^{TS2}-defined proline (P) to Dmca1A's EF hand (see tall thin box three residues downstream from the C-terminal end of that motif). An excerpt of amino-acid sequence for the CAC polypeptide (GenBank accession no. U55776) depicts the region containing the deduced region of the EF hand (*cf.* SMITH *et al.* 1996). This Dmca1A sequence is compared (as in SMITH *et al.* 1996) to portions of amino-acid sequences for a rat-brain calcium-channel α1 subunit (rbE2) and a polypeptide of this kind from Drosophila (Dmca1D) encoded at a locus separate from *cac* (ZHENG *et al.* 1995); identical amino acids are indicated by dots. (B) Diagram of a generic calcium-channel α1 subunit used to depict sites of intra-Dmca1A changes associated with *cacophony* mutations or post-transcripitional modification of *cac* RNA. Thus Dmca1A contains four intrapolypeptide repeats (I → IV), each of which courses back and forth between the cellular membrane (green) six times (S1 → S6, numbered in the N- to C-terminal direction). The amino-acid substitution in cac^{TS2} (*TS2*) is as described for A (also see Table 1). The approximate site of the F → I missense mutation in cac^{S} (*S*) is depicted where this substitution occurred within IIIS6 (*cf.* SMITH *et al.* 1998b). A *cac/nightblind* mutant (*nbAH18*) is accounted for by a nonsense mutation in a region of the *cac* ORF shortly 3′ to that which encodes IS6, which is alternatively spliced (with respect to two mutually exclusive cassettes, thus "alt.cass."), such that only a "visual-system-enriched" CAC isoform would be eliminated by cac^{nbAH18} (SMITH *et al.* 1998b). Among the RNA-edited nucleotides within *cac*'s transcript that lead to amino-acid changes, with reference to the genomic ORF (see text), a newly defined such site is shown for a region of the transcript that encodes residues located between IS3 and IS4 (see Table 1).

noted in Table 2. The percentage-courted values are all rather high, although slightly lower overall for the wingless males and for the *w* $comt^{ST53}$ males of either type. The *w* mutation impairs Drosophila's optomotor behavior (by eliminating screening pigment from the compound eye), and this visual-response defect is reflected in subnormal "tracking" of females by *white*-eyed males (*e.g.*, COOK 1980); so this *comatose* mutation may not be the cause of the mild decrements shown in Table 2. The mating success of cac^{TS2} males alone was slightly subnormal for the intact flies and more markedly impaired for the wingless version of this mutant (Table 2), *i.e.*, almost threefold lower than the percentage mated (within 60 min) for wild-type or $comt^{ST53}$ males (implying that the w^{+}, wingless version of the latter mutant mated as well as the corresponding normal males). The results for this *cacophony* mutant are similar to those obtained in flies expressing the original mutation in this gene:

TABLE 2

Courtship performances and successes

Genotype	*n*	Courtship initiation: % courted	Courtship initiation: Latency (min ± SEM, *n*)	Mating initiation: % mated	Mating initiation: Latency (min ± SEM, *n*)	Copulation duration (min ± SEM, *n*)
			Intact wings			
cac^{TS2}	20	100	3.8 ± 1.2 (20)[a,*]	80	11.9 ± 2.5 (16)	14.8 ± 0.6 (16)
cac^{S}	19	100	0.5 ± 0.1 (19)[a,b,*]	58	11.9 ± 3.2 (11)	24.0 ± 2.3 (11)[a,b,***]
w $comt^{ST53}$	20	85	13.7 ± 3.7 (17)[a,*]	0	—	—
w^{+} $comt^{ST53}$	20	100	1.6 ± 0.2 (20)	90	9.7 ± 2.0 (18)	14.4 ± 1.2 (18)[a,***]
WT	32	100	2.7 ± 1.1 (32)	94	9.7 ± 2.2 (30)	18.3 ± 0.7 (30)
			Wingless males			
cac^{TS2}	22	91	4.0 ± 1.8 (20)	18	28.0 ± 7.3 (4)	13.7 ± 0.5 (4)
w $comt^{ST53}$	22	82	11.7 ± 2.7 (18)[a,**]	5	55.2 (1)	13.0 (1)
w^{+} $comt^{ST53}$	20	95	1.1 ± 0.2 (19)[b,**]	50	34.9 ± 7.2 (10)	7.6 ± 2.3 (10)[a,****]
WT	36	94	2.4 ± 0.7 (34)	53	19.4 ± 3.6 (19)	18.1 ± 0.8 (19)

After introducing male-female pairs to each other (see MATERIALS AND METHODS), the times elapsing between the moment of pairing and the initiation of courtship and initiation of mating (if any for a given pair, as indicated by the percentage-including column headers) were recorded as latency values; copulation durations were measured for all the pairs that mated (the *n*'s to the right of those in the leftmost data column, which are for the total numbers of courtship pairs, indicate the numbers of pairs that courted or mated). See RESULTS for the reason that separate groups of males were either left intact (top of table) or dewinged (bottom). Using the courtship-initiation latency values, a one-way ANOVA was performed (on log-transformed data) for males with intact wings, using genotype as the main effect, which revealed significant differences among groups ($F_{[4,107]} = 23.26$, $P < 0.0001$). Subsequent planned comparisons (P values significant if adjusted $\alpha < 0.009$) were performed to compare all genotypes (cac^{TS2}, cac^{S}, and both *w* and w^{+} $comt^{ST53}$) to wild type (WT) and cac^{TS2} to w^{+} $comt^{ST53}$ and cac^{S}. These pairwise comparisons revealed that all genotypes except for $w^{+}comt^{ST53}$ were significantly different from WT males; in addition, cac^{TS2} males took significantly longer to initiate courtship compared with cac^{S}, but the former did not behave differently from w^{+} $comt^{ST53}$ males. A second one-way ANOVA was performed on the courtship-initiation latency values (log-transformed) for wingless males, using the same genotypes as above (except for cac^{S}) as the main effect, which revealed significant differences among groups ($F_{[3,90]} = 21.60$, $P < 0.0001$). Subsequent pairwise comparisons (adjusted $\alpha = 0.013$) revealed that only *w* $comt^{ST53}$ males showed longer courtship latencies compared with WT; moreover, wingless cac^{TS2} behaved differently from wingless w^{+} $comt^{ST53}$. To address whether differences in courtship latencies occurred for males with wings *vs.* males without wings, a third one-way ANOVA was performed to compare all genotypes above, except for cac^{S} males (which were not tested in wingless form); the outcome revealed significant differences among groups ($F_{[7,179]} = 15.56$, $P < 0.0001$). However, subsequent pairwise comparisons revealed that all genotypes had courtship latencies that were not different when the fly had its wings intact *vs.* wingless (all $P \geq \alpha = 0.013$). Transformed courtship-initiation values were subjected to a two-way ANOVA with genotype X wing condition (intact wings *vs.* dewinged) as the main effects. There was a genotype effect ($F_{[3,179]} = 33.94$, $P < 0.0001$), but no wing condition or interaction effects ($F_{[1,179]} = 0.01$, $P = 0.91$ and $F_{[3,179]} = 2.55$, $P = 0.06$, respectively). Using the mating-initiation latency values (except for *w* $comt^{ST53}$, only one male of which mated), two one-way ANOVAs were performed (on log-transformed data) for both males with intact wings and for wingless ones. Both ANOVAs revealed no significant differences in mating latencies among genotypes: intact wings, $F_{[3,74]} = 1.01$, $P = 0.39$; wingless, $F_{[2,32]} = 1.34$, $P = 0.28$. A third ANOVA was performed to compare mating latencies of bewinged *vs.* wingless males and revealed differences among groups ($F_{[5,96]} = 5.37$, $P = 0.0002$). Subsequent planned pairwise comparisons (adjusted $\alpha = 0.017$) showed that both WT and $w^{+}comt^{ST53}$ showed mating latencies that were different between their (respective) intact *vs.* wingless versions. In contrast, cac^{TS2} exhibited similar mating latencies for males of these two types ($P \geq 0.017$). A two-way ANOVA on mating-initiation latencies, with genotype and wing condition (see above) as the main effect, revealed a wing condition effect ($F_{[1,96]} = 20.33$, $P < 0.0001$), but not a genotype or an interactions effect ($F_{[2,96]} = 2.04$, $P = 0.14$ and $F_{[2,96]} = 0.28$, $P = 0.76$, respectively). Using the copulation-duration values, two one-way ANOVAs were performed, with genotype as the main effect, which revealed significant differences among groups for both intact wings ($F_{[3,74]} = 12.35$, $P < 0.0001$) and wingless ($F_{[2,32]} = 15.70$, $P < 0.0001$). Subsequent planned pairwise comparisons for flies with intact wings (adjusted $\alpha = 0.01$) revealed that both cac^{S} and w^{+} $comt^{ST53}$ exhibited mating durations that were different from WT, whereas for cac^{TS2} they were not. w^{+} $comt^{ST53}$ was statistically different from WT but not from cac^{TS2} mostly because of a very tight distribution of copulation durations for the latter, which overlaps that of WT, compared with a much more spread-out distribution of the former (such that the *comt* distribution did not fully overlap the WT values). Such copulation times for cac^{TS2} were not significantly different from those of w^{+} $comt^{ST53}$ males ($P \geq \alpha = 0.01$), but the former mated for significantly shorter times than did cac^{S} males. For the wingless subset of these tests, pairwise comparisons (adjusted $\alpha = 0.017$) revealed that only w^{+} $comt^{ST53}$ had significantly shorter mating times compared with WT. Wingless cac^{TS2} ones exhibited mating durations (statistically) similar to those wingless w^{+} $comt^{ST53}$ males. To compare copulation times of flies with intact wings *vs.* wingless, a one-way ANOVA was performed, with genotype as the main effect, which showed significant differences among genotypes ($F_{[5,95]} = 11.86$, $P < 0.0001$). Planned comparisons for each genotype (WT, cac^{TS2}, and w^{+} $comt^{ST53}$) showed that both WT and cac^{TS2} males had similar copulation times ($P \geq$ adjusted $\alpha = 0.017$) for either condition (intact or wingless), whereas w^{+} $comt^{ST53}$ males showed significantly shorter copulation times when they were dewinged. A two-way ANOVA on mating durations, with genotype X wing condition as the main effect, revealed genotype and wing condition effects as well as an interaction effect between the two ($F_{[2,96]} = 24.72$, $P < 0.0001$; $F_{[1,96]} = 6.46$, $P = 0.013$; and $F_{[2,96]} = 5.12$, $P = 0.01$, respectively). * P significant when < adjusted $\alpha = 0.009$; ** P significant < $\alpha = 0.013$; *** P significant < $\alpha = 0.01$; and **** P significant < $\alpha = 0.017$.

[a] Different from WT.

[b] Different from cac^{TS2}.

Wingless versions of such males exhibited relatively poor mating performance when tested in parallel with similarly dewinged wild-type males (Schilcher 1977; Kulkarni and Hall 1987).

In the current experiments, the *w comt*^{ST53} males performed worst of all when intact or dewinged: only one mating in 42 trials (which happened to be achieved by a wingless form of this double mutant). This speaks to the latency values presented in Table 2. Times elapsed between pairing the flies and initiation of courtship were low for all male types except *w comt*^{ST53} (latency values for the latter males, in both winged and wingless forms, were significantly longer than those of wild type). With regard to mating initiation, *cac*^{TS2} males performed well with respect to courting pairs that did copulate (latency values for the wingless form of this mutant "look long" in Table 2 but were not significantly different from wingless wild type). The longest mating-initiation latencies recorded (Table 2) were for wingless *comt*^{ST53} males (although, in their w^+ form, they did not perform significantly worse than wild type, and the 55-min value for the *w*-impaired version of this mutant is from an *n* of 1). As expected (Greenspan and Ferveur 2000), winglessness on the part of courting males, whatever their genotype in these experiments (Table 2), led to relatively mediocre mating performances both in terms of percentages of copulations achieved and nominally stretched out latencies (the mating-initation times for wingless wild type or *comt* were significantly longer; that for wingless *cac*^{TS2} was statistically equivalent to the corresponding wild type, notwithstanding the twofold longer value for these mutant males).

Measurements of copulation durations revealed certain mutant peculiarities. *cac*^{TS2} males exhibited ~20% shorter-than-normal durations (Table 2), which are typically in the range of 15–20 min (*e.g.*, Lee *et al.* 2001). However, these mutant *vs.* wild-type values were not significantly different (see legend to Table 2), and the nominal subnormality for *cac*^{TS2} is not a general one for variants involving the locus; *cac*^{S} males exhibited ~30% *longer* mating times compared with matched controls (a significant difference in this case: Table 2). Mutant *vs.* wild-type males were "matched" experimentally (see Table 2 legend), but background genotypic differences may have been partly responsible for the disparities. In this regard, males carrying the *comt*^{ST53}-bearing *X* chromosome in which *cac*^{TS2} was induced also showed shorter-than-normal mating durations (the former mutation alone leading to values that were significantly less than those timed for wild-type male-female pairs). Therefore, it could be that the nominal shortening associated with *cac*^{TS2} is a genetic-background effect or that this *comatose* mutation (located on an *X* from which the new *cacophony* mutation had been crossed away) leads to a copulation phenotype similar to that nominally caused by *cac*^{TS2}. Incidentally, the matings performed by either *cac*^{TS2} or *comt*^{ST53} males were fertile in each case for which fecundity of the relevant female was monitored (n = 12 and 8, respectively).

Courtship song: To ask whether *cac*^{TS2} affects a refined behavioral character, compared with its gross paralysis at high temperature (Dellinger *et al.* 2000) and subnormal features of aggregate courtship performances (this report), outputs from the wing vibrations of courting mutant males were recorded. Such flies were found to exhibit higher-than-normal numbers of intrapulse cycles (CPP) among their song sounds (as exemplified in Figure 2B). This mutant character was temperature dependent, in that *cac*^{TS2} CPPs were ~25% higher at 30° (the warmest condition employed) compared with 20° or 25°; at the two lower temperatures, the mutant's CPPs were ~10–15% higher than those of wild-type males, but at 30° *cac*^{TS2} values were ~35% higher than normal (Figure 3A). Implicitly, the *cac*$^{+}$ CPPs were largely invariant over the 10° range, as expected (Peixoto and Hall 1998). Other such controls came from recordings of *comt*^{ST53} and *comt*^{ST17} males, which yielded CPP values that varied only slightly as a function of temperature; actually, the values for *comt*^{ST53} were ~8% higher at 30° compared with those at 20° (as plotted in Figure 3A), while CPPs for *comt*^{ST17} males changed not at all over this thermal range (see Figure 5). The pulse amplitudes of *cac*^{TS2} males were also heat sensitive (Figure 3B), exhibiting a steady increase (overall, 50%) as the temperature was raised, compared with those of both control types (which varied almost not at all from 20° → 25° and at 30° increased in a less-marked manner, compared with *cac*^{TS2}, for *comt*^{ST53} only). Therefore, in warm temperatures *cac*^{TS2} males show an aberrant song phenotype very similar to the song abnormalities exhibited by the *cac*^{S} mutant at all temperatures that have been applied (Schilcher 1977; Kulkarni and Hall 1987; Peixoto and Hall 1998; Smith *et al.* 1998b). That is, the original *cacophony* mutant gives higher-than-normal CPP and amplitude values in cool, mild, or relatively warm conditions.

Elements of these earlier studies showed that the carrier frequencies for *cac*^{S} pulses (IPF) were neither abnormal nor varied appreciably with temperature (Peixoto and Hall 1998). However, *cac*^{TS2} males tended to give lower-than-normal IPFs; the disparity from wild-type values (~25% lower for *cac*^{TS2} IPFs) was greatest at 30° (Figure 3C). Rates of pulse production speed up as the temperature is raised, which harks back to the first song study performed on *D. melanogaster* (Shorey 1962; also see Peixoto and Hall 1998). Against this background, *cac*^{TS2} males, along with control wild-type and *comt*^{ST53} males, were expected to (and did) exhibit shorter IPIs as the temperature was raised from 20° to 30°. At the latter temperature, only interpulse-interval values differed among genotypes (see legend to Figure 3), but IPIs for the mutants were not dramatically longer than normal.

With regard to the effects of genotype and tempera-

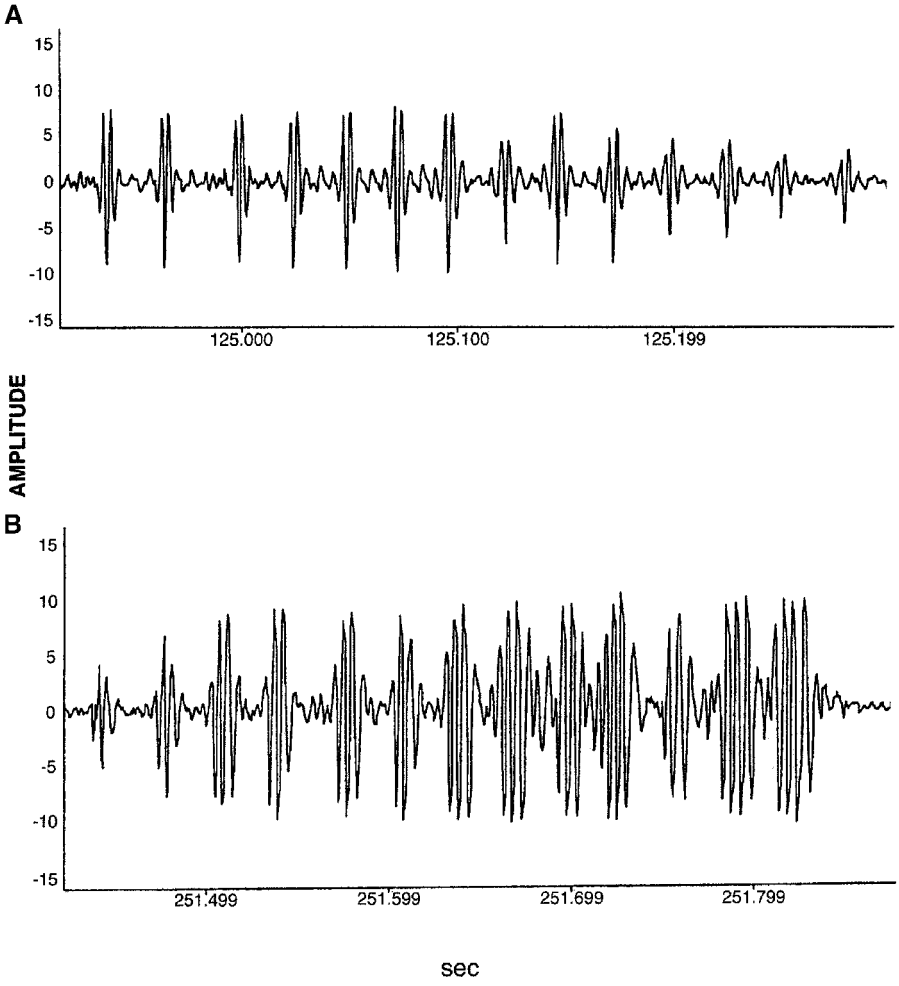

FIGURE 2.—Examples of pulse trains produced by a (A) wild-type (WT) and a (B) cac^{TS2} male. Both flies had their courtship wing vibrations recorded at 30° (*cf.* Figures 3 and 4). These snatches of song were generated by application of subroutines within the LifeSong software (elements of which, as referred to in MATERIALS AND METHODS, digitize the audio record of a given courtship). The abscissas show time values representing the actual recording time (starting at 0 sec for a given male-female pair). The two bouts shown each occurred over ~0.5 sec of time. The ordinate represents arbitrary measurements of pulse loudness (against background/no-fly noise levels that fluctuated between −1 to +1). Average numbers of cycles per pulse for the WT and the mutant train: 2.9 and 5.0, respectively.

ture on cycles per pulse, a two-way ANOVA revealed effects of both variables but no "interaction effect" (see legend to Figure 3). For this reason, and owing to more general concerns about appreciating the effects of *cacophony* mutations on song-pulse qualities, it occurred to us that gross values for the CPP song parameter (normally in the range of 3–4) do not adequately reveal the anomalous pulse polycyclicity caused by *cacophony* mutations (which led to overall average CPPs in the range of 4.5–5.0, referring for cac^{TS2} to courtship at the nonpermissive temperature). In this regard, while logging mutant *vs.* normal song records one perceives dramatically abnormal song bouts for *cacophony* males—the frequent occurrence of pulse *trains* that are largely polycyclic—whereas wild-type records rarely include such trains. Thus it is subjectively crystal clear when one logs a cac^{S} song (recorded at "any" temperature) or a cac^{TS2} one (at 30°) that the visual display of courtship sounds came from recording the song of a mutant as opposed to a wild-type male. Nonetheless, we performed a newly conceived bout-distribution analysis on the songs stemming from effects of certain mutant *vs.* normal genotypes. For this, the computer extracted by objectively preset criteria the various "average CPP" bout types from a given song record, which had been initially digitized merely by marking pulse locations as opposed to indicating anything about their qualities at that stage of the operation (a remark made because of bias that could come into play, as implied above). As a result of these bout analyses, average cycles per pulse per song train were displayed as histograms whose abscissas varied from a mean-CPP category of ≤2 to one representing an average >5. Examples of wild-type and *cac*-mutant songs from individual males displaying *intra-fly* variations in CPP bout types are in Figure 4. The mutant distributions referring to cac^{TS2} at 30° or cac^{S} at that temperature or 20° are substantially skewed to the right compared with the wild-type plots at both temperatures.

Table 3 shows bout-analysis results from all the mutant and normal song records for which proportions of bouts falling into the five CPP categories were averaged among males of a given type. (For example, what proportion of an individual cac^{TS2} male's bouts fell into a given category? Then, what was the average proportion for that category among such mutant males whose songs were recorded at a given temperature?) Highlights of these tabulated findings are that only 7 and 6% of song bouts from the average wild-type male fell into the high-

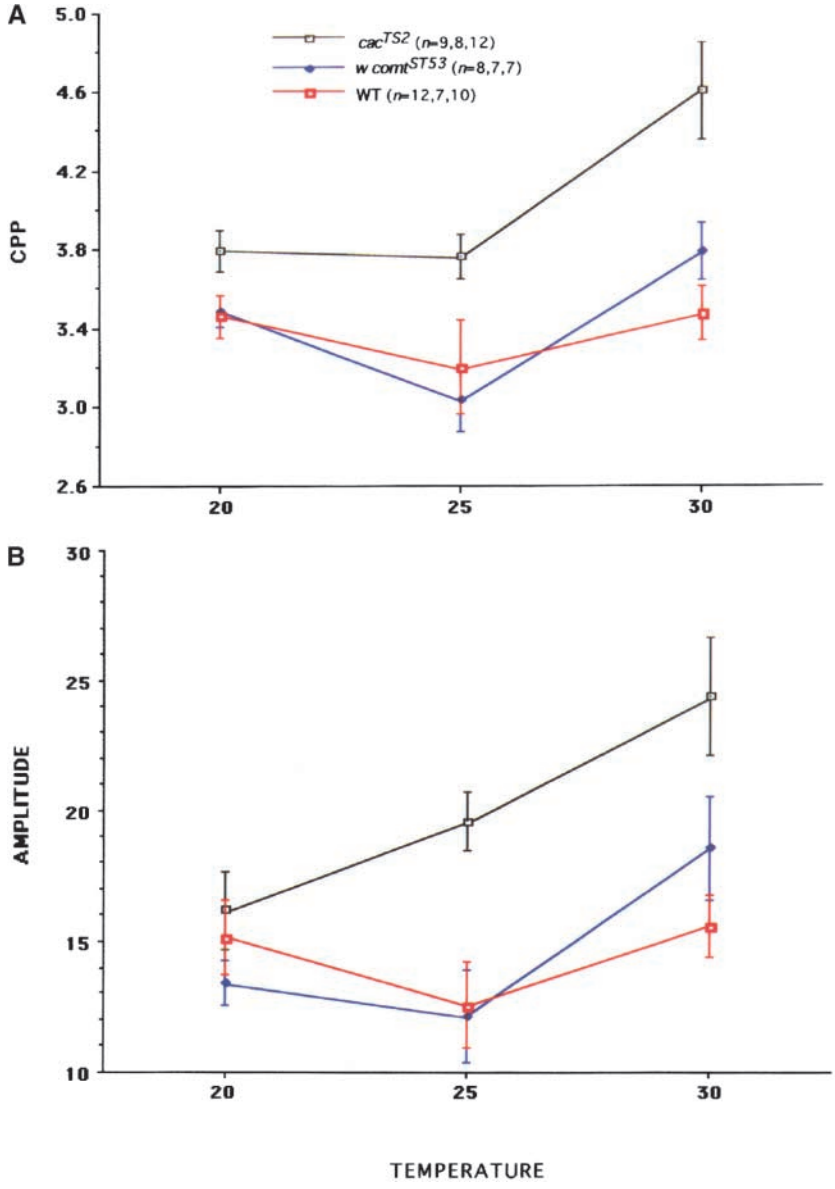

FIGURE 3.—Courtship-song parameters. With respect to the three different recording temperatures (see abscissas), the numbers of courtships recorded are in parentheses accompanying the genotype labels within A and are given in order according to the three recording temperatures. These results revealed the basic differences between cac^{TS2} and normal songs: higher CPP and amplitude of sounds for the mutant (see A and B), and slightly but significantly longer-than-normal IPIs (see D). (A) Cycles per pulse, an appreciation of which can be gleaned from Figure 2. Here, the gross overall average CPPs for songs of a given male were computed and used to generate the means (±SEM) plotted for males of a given genotype singing at a given temperature. A one-way ANOVA was performed on log-transformed CPP values, using the data from all three temperatures with genotype as the main effect, and it showed significant differences among genotypes ($F_{[8,79]} = 8.13$, $P < 0.0001$). Subsequent planned pairwise comparisons revealed no significant differences between CPP at 20° *vs.* 30° for wild-type (WT) males or *w* $comt^{ST53}$ males (all $P \geq 0.05$). However, cac^{TS2} males exhibited significantly greater CPPs at 30° compared with 20° ($P < 0.05$). At 20°, males of all three genotypes yielded the same CPP (per male) averages (all $P < 0.05$). At 25°, cac^{TS2} males were "already" generating higher CPPs compared with males of the cac^{+} "parental" strain (*w* $comt^{ST53}$) or with WT males ($P < 0.05$); however, the values for cac^{TS2} were not different when comparing the 25° with the 20° records. At 30° cac^{TS2} values were significantly different from both the *w* $comt^{ST53}$ and the WT ones (all $P < 0.05$). A two-way ANOVA was performed on the CPP data, with genotype (GENO) and temperature (TEMP) as the main effects; for CPP there were GENO and TEMP effects ($P < 0.0001$ and $P < 0.0001$, respectively), but no interaction effect (GENO × TEMP) between the two ($P = 0.17$). (B) Amplitude, an arbitrarily scaled parameter (as exemplified in Figure 2). A one-way ANOVA was performed on these pulse-loudness indicators, with genotype as the main effect, and it showed significant difference among genotypes ($F_{[8,79]} = 5.95$, $P < 0.0001$). Subsequent comparisons revealed that all genotypes had the same song amplitude at 20° (all $P \geq 0.05$), whereas, at 30°, the pulse amplitudes of cac^{TS2} male songs were significantly higher than those of *w* $comt^{ST53}$ or WT males (all $P < 0.05$). Note that amplitudes monotonically increased with temperature for the two mutant types but did not for WT songs. (C) Intrapulse frequency, representing the average carrier frequencies in Hertz, computed from Fourier analyses of all pulses within a given song record (*cf.* WHEELER *et al.* 1989). A one-way ANOVA was performed on the IPFs, with genotype as the main effect, and it showed significant difference among genotypes ($F_{[8,79]} = 6.70$, $P < 0.0001$). Subsequent comparisons revealed that these carrier frequencies did not change with temperature for any of the three genotypes (all $P \geq 0.05$), although the IPFs were different among the male types at 30° (all $P < 0.05$). (D) Interpulse intervals, representing the brief timespans of relative "silence" between a given pair of pulses within a song-bout train (see Figure 2). A one-way ANOVA was performed on log-transformed IPIs (in milliseconds), with genotype as the main effect, and it showed significant difference among genotypes ($F_{[8,79]} = 52.53$, $P < 0.0001$). Subsequent pairwise comparisons revealed that all genotypes showed the same inverse relationship between IPI and temperature. All three male types sang with IPIs that were not different at 20° (all $P \geq 0.05$) but were significantly different from each other at 30° (all $P < 0.05$). Increased CPPs at 30° contributed to these slightly longer-than-normal IPIs of cac^{TS2} and *w* $comt^{ST53}$ (higher such values reflecting longer pulses); this is because a given IPI was measured between the temporal centers of a pair of successive pulses. [LifeSong produces a pulse "envelope" from each such sound and computes the timespan between the peaks of two adjacent envelopes to specify that IPI (*cf.* VILLELLA *et al.* 1997).]

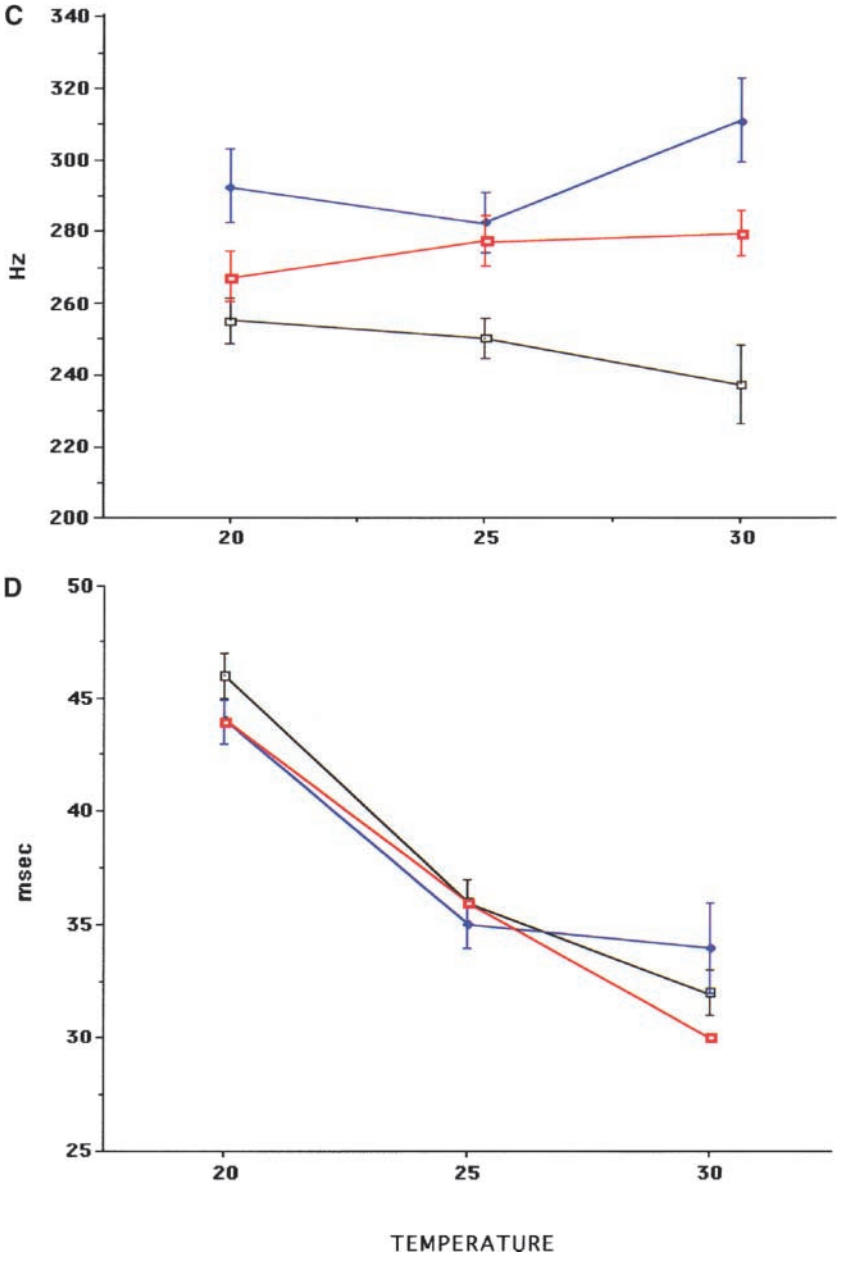

FIGURE 3.—*Continued.*

est category (>5 CPP) from the 20° and the 30° recordings, respectively. In contrast, the corresponding values for cac^{TS2} at these two temperatures were 11 and 36%. A meta-analysis of cac^{S} songs recorded over a wide temperature range (PEIXOTO and HALL 1998), but not then subjected to bout breakdowns, revealed that 53–55% of this mutant's bouts fell into the highest (>5 CPP) category after 20° and 30° recordings were analyzed appropriately (Table 3).

Recall that cac^{TS2} was induced via its interaction with a *comatose* mutation. We therefore tested males whose *X* chromosomes were mutated at each locus. Interestingly, cac^{TS2} $comt^{ST17}$ males generated song pulses whose CPP values were squarely within the normal range (*cf.* Figure 3A) at all temperatures (Figure 5), *i.e.*, a suppressive effect of the latter mutation's effect vis à vis that of the former alone (*cf.* Figure 3A). No interaction could be gleaned between cac^{S} and this *comt* mutation (Figure 5), because CPPs of the doubly mutant males were relatively high and *cacophony*-like at all temperatures (*cf.* PEIXOTO and HALL 1998 and Figure 3A).

Visually mediated responses of flies or parts thereof: Given the visual-response abnormalities exhibited by certain *cacophony* mutants, cac^{TS2} was tested for phototaxis by applying a Y-tube device that had previously been used to uncover the photophobic behavior (alluded to in the Introduction) that is caused by cac^{nbA} mutations (KULKARNI and HALL 1987; SMITH *et al.* 1998b). However, cac^{TS2} was revealed to be photophilic in tests for which the flies either were chronically at low temperature or were returned to that condition after exposure to 29° (Table 4). The latter experiment caused

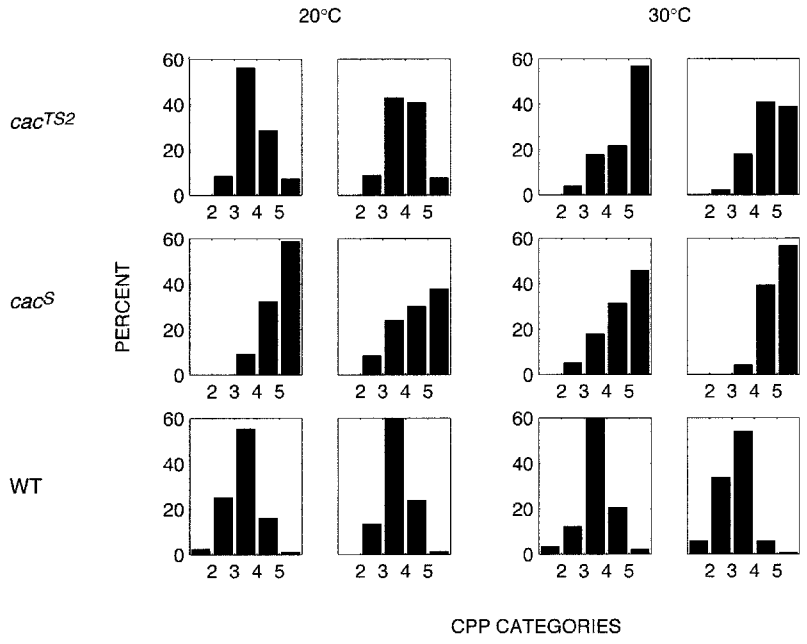

FIGURE 4.—Examples of cycles-per-pulse bout types in songs from individual males. These distributions are based on the percentages of pulses for a given song that fell into the following CPP categories: ≤2, >2–3, >3–4, >4–5, and >5 (see legend to Table 3 for details, and note that the abscissa labeling here gives shorthand indicators for the five CPP categories). Histogram examples are depicted for males of three genotypes, whose songs were recorded at 20° or 30°. For the wild-type (WT) males, the distributions at both temperatures (representing a total of four different song recordings) show that most of the pulses were between 3 and 4 CPP (*cf.* Figure 3A). For the cac^S songs exemplified, the distributions are shifted toward higher CPPs, independent of temperature. For the cac^{TS2} examples, the distributions at 20° are similar to the WT histograms (particularly for the mutant case on the left), but are shifted substantially to the right at the higher temperature.

relatively high numbers of cac^{TS2} flies to remain in the phototaxis "start tube" (see MATERIALS AND METHODS); this would seem to reflect generally poor locomotion caused by the higher temperature (see *General features of locomotion*) as opposed to a cac^{TS2} phototactic defect, let alone a photophobic one. In any case the proportions of cac^{TS2} *vs.* wild-type flies that did not move toward either light or darkness (upon postheating light stimulation) were not significantly different (see legend to Table 4). In contrast, a severe locomotor impairment was observed in a negative-control test performed on the semisick $comt^{ST53}$ mutant (Table 4): ~40–90% of such flies remained at the start at low temperature, and all of them did after exposure to the higher one (however, this *comatose* mutant was not paralyzed after 29° treatment).

With regard to another kind of light-induced response, room-temperature ERG recordings from cac^{TS2} flies ($n = 3$) gave normal-looking tracings (compared to three wild types) in terms of the shapes of light-on transient spikes, light-coincident photoreceptor potentials (LCRPs), light-off transients, and repolarization times (data not shown). Measuring the magnitudes of these three components (interfly averages in millivolts) revealed the mutant on-spike to be half normal and the LCRP and off-spike 60% normal; only the light-on transient was significantly lower ($P < 0.05$). Magnitudes of the three kinds of control values (in millivolts) were squarely in the range of those tabulated for wild-type ERGs in our earlier studies (RENDAHL *et al.* 1992, 1996; SMITH *et al.* 1998b). For the 30°–31° recordings, the corresponding metrics (for cac^{TS2} compared with wild type) were 50, 100, and 80% ($n = 3$ and 5, respectively); none of the mutant values were significantly lower than those of the control ones. Therefore flies expressing this *cacophony* mutation, even when heat stressed, exhibited nowhere near the marked deficits of these spike and LCRP amplitudes that had been observed in recordings of cac^{nbA} mutants (HOMYK and PYE 1989; SMITH *et al.* 1998b). Moreover, we suspect that the reasonably robust light-on and light-off transient spikes reflect visual system functioning that would be sufficient to allow for the fairly solid behavioral response to visual stimuli that was exhibited by cac^{TS2} (see above), which is nothing like the anomalous photophobic behavior that is caused by cac^{nbA} mutations (KULKARNI and HALL 1987). Having said this, the ERGs for cac^{TS2} involved a readily observable anomaly when recorded at high temperature: Instead of a quick repolarization to baseline voltage after the light went off (an average of 0.4 sec in the current controls, which is characteristic of wild-type records), the mutant individuals exhibited relatively prolonged repolarization times (an average of 2.3 sec, which was significantly longer than normal: $P < 0.05$). This ERG anomaly is reminiscent of what was found for flies expressing one of the *nightblind* alleles (*nbAP73*)—the one that causes relatively mild visual-system malfunction compared with the other two cac^{nbA} mutants analyzed in this manner by SMITH *et al.* (1998b and Figure 3 of that report).

General features of locomotion: To determine whether the sluggishness inferred to be associated, if only marginally, with cac^{TS2}'s courtship performances and phototaxis would be exhibited in a situation devoid of specific sensory stimuli, generic locomotor behavior was observed. After exposing flies to 29° and quantifying locomotion at 25° in an arena test, or monitoring such movements of nonheated flies at the latter temperature, cac^{TS2} males were found to be approximately one-quarter to one-third as active as wild type (Table 5). The mutant's

TABLE 3

Cycles per pulse according to bouts of singing

Genotype	Temperature	*n*	Bouts/male	% of pulses within various CPP categories				
				≤2	>2–3	>3–4	>4–5	>5
WT	20°	12	948 ± 94	2 ± 1	16 ± 2	52 ± 3	24 ± 2	7 ± 3
	30°	10	1082 ± 243	2 ± 1	17 ± 4	48 ± 3	27 ± 6	6 ± 2
cac^{TS2}	20°	9	815 ± 104	1 ± 1	9 ± 2	47 ± 3	31 ± 3	11 ± 3
	30°	12	670 ± 81	0 ± 0	6 ± 2	27 ± 3	30 ± 3	36 ± 5
cac^{S}	20°	6	322 ± 36	0 ± 0	2 ± 1	13 ± 4	32 ± 5	53 ± 8
	30°	6	316 ± 69	0 ± 0	2 ± 1	13 ± 4	30 ± 3	55 ± 4

A newly produced feature of LifeSong software (*cf.* Bernstein *et al.* 1992) was applied to extract song bouts (thus tone-pulse trains) of reasonable length (see materials and methods) and then to determine the average numbers of CPP per bout. Bout types were then categorized for a particular male's song as to what proportions fell into the following five categories (ranges of mean CPPs per bout): ≤2, >2–3, >3–4, >4–5, and >5. These results permitted plots of bout-type distributions for a given mutant or wild-type (WT) male to be generated (see Figure 4). The final calculations took the data from all males of a given genotype (numbers in the leftmost data column) and computed mean percentages of bouts (±SEM) that fell into the various categories. The songs from only the two extreme recording temperatures used in this study (*cf.* Figure 3) were subjected to this analysis. The raw data processed in this manner for cac^{S} came from Peixoto and Hall (1998); those investigators dealt with CPPs for this mutant type only in terms of gross, overall average values for all pulses generated during the song performance of a given male (*cf.* Figure 3). Two one-way ANOVAs were performed on the >5 CPP bout-category percentages (log-transformed data) at 20° and 30°, with genotype as the main effect, and both showed significant differences among genotypes ($F_{[2,26]} = 16.20$, $P < 0.0001$ and $F_{[2,27]} = 28.66$, $P < 0.0001$, respectively). Subsequent planned comparisons revealed that cac^{S} tone pulses had significantly more CPP than WT ($P < \alpha = 0.017$) at 20° but cac^{TS2} did not; moreover, cac^{S} was significantly more polycyclic than cac^{TS2} ($P < 0.017$). Subsequent planned comparisons at the higher temperature revealed that cac^{TS2} and cac^{S} songs had significantly higher CPP values compared with WT ($P < 0.017$) and that both were not different from each other at 30°. A third one-way ANOVA was performed to compare the >5 CPP values at 20° *vs.* 30°, with genotype as the main effect, and revealed significant differences among groups ($F_{[5,54]} = 18.81$, $P < 0.0001$). Subsequent pairwise comparisons revealed that only cac^{TS2} had significantly different ($P < \alpha = 0.017$) percentages of pulses that were in the >5 CPP category when comparing values from the two temperatures. cac^{S} and WT had the same percentage of bouts within this CPP category, and this did not change with temperature ($P \geq 0.017$).

activity improved somewhat (giving approximately half-normal counts) when tested at 22°–23°. *comt* males (in the two genetic backgrounds noted in Table 5) tended to be more sluggish than cac^{TS2} flies, especially in the 25° or 29° → 25° tests.

After exposing mutant and normal flies to mechanical shock (Table 6), cac^{TS2} males exhibited longer-than-normal recovery times in various iterations of these experiments (*e.g.*, low-temperature rearing → test at intermediate temperature or rearing in the latter condition → high-temperature exposure). This stress test was the one kind for which *comt* males were less severely impaired than those carrying cac^{TS2} (except in one respect, because $w\ comt^{ST53}$ animals were killed during development at 29°, as implied in the third subsection of Table 6).

DISCUSSION

Correlations between Dmca1A-encoding genotypes and *cacophony*-mutant phenotypes: The newest *cacophony* mutant (Dellinger *et al.* 2000) is a courtship variant, as was (and is) the original *cac* mutant (Schilcher 1977). Thus, cac^{TS2} males are somewhat impaired in their overall courtship performance, including mating ability. However, cac^{TS2} males courted more vigorously and effectively (Table 2) than one might expect from monitoring their generic locomotor activity (Tables 5 and 6). One component of the courtship performance of cac^{TS2} males implies a behavioral problem that goes beyond the nature of the sounds they communicate to females. They performed worse than wingless wild-type males did (Table 2), which indicates that this mutant is more pleiotropically defective than a "song only" variant.

Nevertheless, the most sharply defined courtship defect exhibited by a cac^{TS2} male is its heat-sensitive anomalies of tone pulses that emanate from the wing vibrations it directs at a female (Figures 2–4, Table 3). These abnormalities of cycles per pulse and pulse amplitude were found to be similar to the nonconditional courtship-song peculiarities exhibited by the original cac^{S} mutant (*e.g.*, Peixoto and Hall 1998). That the respective mutant phenotypes are alike is important, because cac^{TS2} males did not have to exhibit any kind of singing eccentricity: Inasmuch as the isolation of this mutant involved behavioral criteria that had nothing to do with courtship (Dellinger *et al.* 2000), the outcome of song-testing cac^{TS2} could have left cac^{S} as the only singing variant associated with this gene. But both the original and the

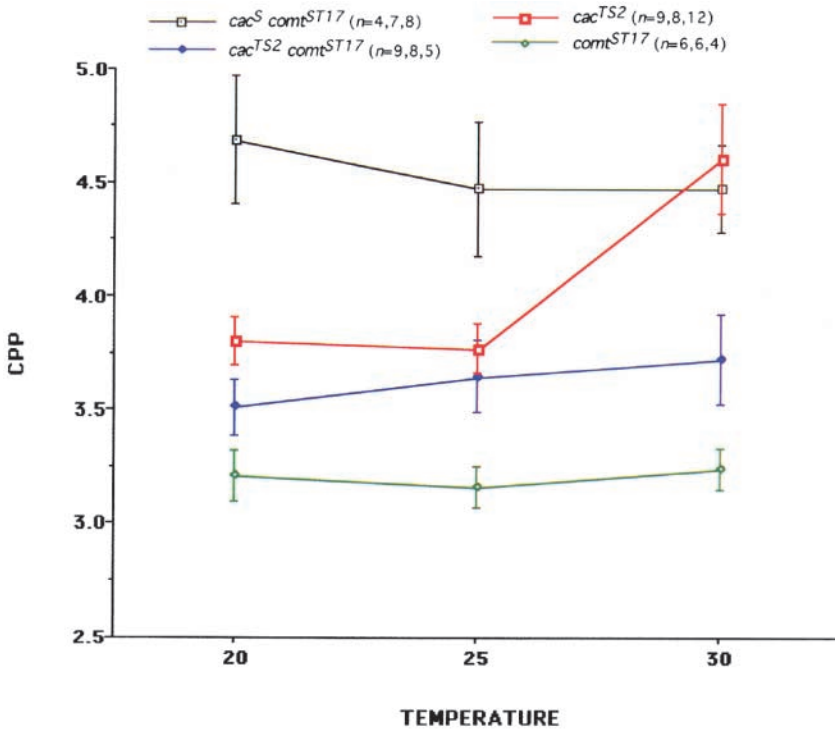

FIGURE 5.—Cycles-per-pulse values from songs of single and double mutants. These songs were analyzed for the four song parameters, but only the CPP values are presented (*cf.* Figure 3A, from which the cac^{TS2} data are replotted here). CPPs of the cac^{S} com^{ST17} and cac^{TS2} com^{ST17} double mutants were compared to those within songs of com^{ST17} and cac^{TS2} males and recorded at the different temperatures shown on the abscissa. A one-way ANOVA was performed on log-transformed CPP values (using the data from all three temperatures) and revealed significant differences among genotypes ($F_{[11,85]} = 9.11$, $P < 0.0001$). Subsequent pairwise comparisons, with genotype as the main effect, showed that CPPs at 20° *vs.* 30° remained the same for all genotypes (all $P \geq 0.05$) except for the increase exhibited by cac^{TS2} at that elevated temperature ($P < 0.05$). Songs of cac^{S} com^{ST17} males had higher CPP values across all three temperatures, compared with com^{ST17} (all $P < 0.05$). In contrast, the cac^{TS2} com^{ST17} doubly mutant type generated CPPs higher than those of com^{ST17} at 30° but not significantly different at 20°; yet the songs of these two male types did not approach the polycyclicity exhibited by cac^{S} com^{ST17} ($P < 0.05$ for all temperatures). The cac^{TS2} males gave CPPs at 30° that were similar to those of the cac^{S} com^{ST17} double mutant ($P \geq 0.05$).

newest *cacophony* mutations cause courtship-song peculiarities, and it is interesting that the anomalously loud and polycyclic pulses produced by both cac^{S} and cac^{TS2} males do not involve an appreciable derangement of such sounds: Each mutant type remains nicely patterned with respect to the qualities of individual "clicks" and their rate of production (as exemplified in Figure 2). Once again, if cac^{TS2} turned out to be song defective it was not a foregone conclusion that such males would produce these sounds in a manner more salutary than that of other singing variants, such as those expressing *slowpoke* (*slo*) mutations. In this regard, *slo* potassium-channel mutants were identified using generic behavioral criteria (as was cac^{TS2}) and were found later to sing aberrantly (rare among ion-channel variants: KULKARNI and HALL 1987; PEIXOTO and HALL 1998) and to exhibit erratic patterns of anomalous tone pulses (PEIXOTO and HALL 1998).

This brings us to the question of why it might be that the songs of cac^{S} and of cac^{TS2} (30°) males are not only song defective, but also similarly so in their tone-pulse qualities. As was introduced in conjunction with documenting cac^{TS2}'s intragenic site change (Figure 1, Table 1), this amino-acid substitution is very near the EF hand within Dmca1A, directly C-terminal to the aforementioned IVS6 transmembrane domain (Figure 1B). The highly conserved EF hand and adjacent residues among calcium-channel α1 subunits of various species are involved in channel inactivation mediated by Ca^{2+} binding (*e.g.*, ZHANG *et al.* 1994; ZHOU *et al.* 1997; DOUGHTY *et al.* 1998). Thus, this form of inactivation involves a calcium-influenced conformational change that occurs via cation binding within the EF hand's helix-loop-helix (PETERSON *et al.* 2000). Given the P-to-S substitution in cac^{TS2} immediately C-terminal to the EF hand (Figure 1)—where this evolutionarily conserved proline (*e.g.*, Figure 1A) is changed to a polar serine that has more conformational freedom—one imagines that the local three-dimensional structure in which the EF hand finds itself is altered in the mutant. The function of this domain would be altered accordingly but not ruined at permissive temperatures. Thus, the amino-acid substitution in cac^{TS2} near the EF hand suggests that this protein change could cause the Dmca1A calcium channel to exhibit altered inactivation kinetics. Whereas inactivation features of the α1 subunit encoded by *cac* are unknown, it is reasonable to speculate that that process becomes less robust than normal in the cac^{TS2} mutant as the flies are heated from 20° to 30°. Why the dynamics of inactivation may be subtly heat sensitive over the temperature range just stated is difficult to surmise, although perhaps it is the case that this process can barely occur at all at 37°, accounting for the grossly subnormal synaptic neurotransmission that occurs at that extreme temperature (KAWASAKI *et al.* 2000).

This hypothesis, as it relates to cac^{TS2}'s behavioral phenotype within a "physiological" range of temperatures, goes on to suggest that anomalously polycyclic pulses in the songs of males expressing this mutation smack of a channel-inactivation change that would alter the

TABLE 4

Phototaxis

Genotype	*n*	% moving to light (mean ± SEM)	% moving to dark (Mean ± SEM)	% remaining at start (mean ± SEM)
		Tested at room temperature (22–23°)		
cac^{TS2}	16	75.2 ± 6.8	2.5 ± 1.7	22.3 ± 6.8
w $comt^{ST53}$	13	9.3 ± 3.8[a]	2.9 ± 1.6	87.8 ± 4.4[a]
w^+ $comt^{ST53}$	25	55.7 ± 8.0[a]	1.3 ± 1.3	43.0 ± 8.1[b]
WT	19	82.7 ± 4.5	6.4 ± 2.7	10.9 ± 4.4
		Tested at room temperature after exposure to 29°		
cac^{TS2}	7	72.7 ± 11.0	0 ± 0	27.3 ± 11.0
w $comt^{ST53}$	4	0 ± 0[a]	0 ± 0	100 ± 0[a]
w^+ $comt^{ST53}$	5	0 ± 0[a,b]	0 ± 0	100 ± 0[a,b]
WT	7	72.2 ± 13.0	24.2 ± 13.6	3.6 ± 3.6

n is the number of trials, in each of which a handful of flies of a given genotype (see MATERIALS AND METHODS) was given the opportunity to walk toward light, into darkness, or remain in the intra-apparatus container within which the group was initially placed. The three different kinds of percentages were calculated from each trial and then averaged. The rearing/testing conditions indicated are explained in MATERIALS AND METHODS. One-way ANOVAs for each parameter (arcsine transformed)—percentages of flies going toward light, toward darkness *vs.* remaining in the start tube—were performed with genotype as the main effect. ANOVAs on percentages of flies at light and at start showed significant differences among groups ($F_{[3,72]} = 15.64$, $P < 0.0001$ and $F_{[3,72]} = 16.63$, $P < 0.0001$, respectively), whereas the results for flies at dark indicated no significant differences among genotypes ($F_{[3,72]} = 1.54$, $P = 0.21$). Subsequent planned pairwise comparisons revealed that percentages at light were significantly different for w^- and w^+ $comt^{ST53}$ compared with WT ($P < \alpha = 0.013$), but not for cac^{TS2} ($P \geq 0.013$). Moreover, there was no difference in the number of flies that moved toward the light when comparing cac^{TS2} to w^+ $comt^{ST53}$ ($P \geq 0.013$). Subsequent comparisons for the number of flies at start showed that significantly more flies remained there for *w* $comt^{ST53}$ and w^+ $comt^{ST53}$ ($P < \alpha = 0.013$), whereas cac^{TS2} were not distinguishable from WT ($P \geq 0.013$). In addition, the percentages of flies remaining at the start for w^+ $comt^{ST53}$ were not different from cac^{TS2} ($P \geq 0.013$). ANOVAs on percentage at light and on percentage at start after exposure to 29° (arcsine-transformed data as above) revealed significant differences among genotypes ($F_{[3,22]} = 14.98$, $P < 0.0001$ and $F_{[3,22]} = 46.63$, $P < 0.0001$, respectively), whereas percentages at dark were not different among genotypes ($F_{[3,22]} = 3.07$, $P = 0.053$). Subsequent planned comparisons revealed that the percentages at light were significantly different for *w* $comt^{ST53}$ and w^+ $comt^{ST53}$ compared with WT ($P < \alpha = 0.013$) as was the case for flies that had not been exposed to the higher temperature (see above). Contrary to the results in the top half of the table, cac^{TS2} behaved in a significantly different manner ($P < 0.013$) from w^+ $comt^{ST53}$ with respect to the percentage at light. Hence, this *comatose* mutant was affected by the higher temperature (again, compare percentages at start in top *vs.* bottom halves of table). Subsequent planned comparisons on percentages at start showed that percentages of *w* $comt^{ST53}$ and w^+ $comt^{ST53}$ flies remaining there (after high-temperature exposure) were significantly higher than WT (*P*s < 0.013), but cac^{TS2} behaved similarly to the latter (control) type ($P \geq 0.013$). Moreover, cac^{TS2} behaved differently from w^+ $comt^{ST53}$ in terms of the "at start" values ($P < 0.013$).

[a] Different from WT ($P <$ adjusted $\alpha = 0.013$).

[b] Different from cac^{TS2} ($P < \alpha = 0.013$).

contribution of calcium currents to the overall behavioral process in question. Thus, the repetitive-pattern phenotype, which is a reasonable descriptor for trains of Drosophila song pulses, would not have the intrapulse cycles inactivated as "tightly" as in wild type.

What about the songs of cac^S males, whose pulses are similarly polycyclic (albeit without the temperature sensitivity that accompanies the cac^{TS2} phenotype)? The cac^S mutant is accounted for by an amino-acid substitution within the sixth membrane-embedded region of the penultimate intra-Dmca1A repeat (SMITH *et al.* 1998b), a.k.a. IIIS6 (Figure 1B). Certain types of calcium channels prevent excessive influx of calcium when the channel opens by voltage-mediated inactivation (HERING *et al.* 2000). Pore-forming S6 transmembrane domains play a role in modulating voltage-dependent calcium-channel inactivation (HERING *et al.* 2000). This has been revealed (1) by creating chimeric α1-subunit polypeptides in which portions of IIIS6 from fast-inactivating channels replaced those of a slow-inactivating one, leading to inactivation kinetics characteristic of the donor calcium-channel type (TANG *et al.* 1993) and (2) by physiological disruptions of channel functions that are pointed to by the etiology of certain patho-physiological mutants in humans (reviewed by JEN 1999); certain such S6 mutations slow and others accelerate the development of inactivation (HERING *et al.* 2000). Therefore, a mnemonic device for apprehending the song abnormality exhibited by cac^S mutant males is, again, subnormal inactivation of intratone-pulse sounds, owing to their

TABLE 5

General locomotor activity

Genotype	No. of line crossings: *n*	Mean ± SEM
Tested at room temperature (22–23°)		
cac^{TS2}	20	14.0 ± 2.0[a,*]
w $comt^{ST53}$	17	11.5 ± 2.9[a,*]
w^+ $comt^{ST53}$	16	19.3 ± 2.6
WT	19	28.2 ± 4.1
Tested at 25°		
cac^{TS2}	20	5.5 ± 2.0[a,*]
w $comt^{ST53}$	20	4.0 ± 1.1[a,*]
w^+ $comt^{ST53}$	20	6.8 ± 2.1[a,*]
WT	20	23.2 ± 2.5
Tested at 25° after exposure to 29°		
cac^{TS2}	17	6.5 ± 2.4[a,*]
w $comt^{ST53}$	18	0.0 ± 0.0[a,*]
w^+ $comt^{ST53}$	20	5.2 ± 1.1[a,*]
WT	17	18.4 ± 2.7

Males of the four genotypes indicated were tested as described in MATERIALS AND METHODS for general locomotion in the various conditions (or postheating situation). One-way ANOVAs were performed on the results of these line-crossing counts (arcsine-transformed) for flies tested at room temperature, at 25°, or after exposure to 29°. All three ANOVAs revealed significant differences among genotypes ($F_{[3,71]} = 6.94$, $P = 0.0004$; $F_{[3,79]} = 18.47$, $P < 0.0001$; and $F_{[3,71]} = 25.49$, $P < 0.0001$, respectively). Subsequent planned pairwise comparisons revealed that all genotypes tested under the three different conditions were different from WT (all $P < \alpha = 0.013$), except for $w^+ comt^{ST53}$ tested at room temperature (P value ≥ 0.013). Moreover, cac^{TS2} did not behave differently from parental strain $w^+ comt^{ST53}$ with respect to locomotor activity under the three different conditions (all $P \geq 0.013$). (Note: cac^{TS2} was compared only to the similarly eye-pigmented *comatose* type just indicated.) * P significant when < adjusted $\alpha = 0.013$.

[a] Different from WT.

inappropriate polycyclicity (SCHILCHER 1977; KULKARNI and HALL 1987; PEIXOTO and HALL 1998). However, in this case the putative inactivation defect would have a different mechanistic etiology compared with that hypothesized for the cac^{TS2}-mutated polypeptide.

That channel inactivation can be mutationally altered in more than one way makes it nonmiraculous that the two different sites and kinds of amino-acid alterations in the two song-defective *cacophony* mutants are similarly non-wild type. But what if any change within the *cac*-encoded Dmca1A polypeptide would lead to the same kind of altered channel function insofar as song regulation is concerned? The cac^S and cac^{TS2} tone-pulse phenotypes could represent some sort of default mutant phenotype. This possibility (in its extreme form) will not wash, however, because the gene has been mutated to a variety of different phenotypes. Some *cac* mutations are embryonic lethals (*e.g.*, KULKARNI and HALL 1987; SMITH *et al.* 1998b), as are mutations at another locus in this species that encodes a similar function (EBERL *et al.* 1998), although it is not obvious *a priori* that they would have this property: Certain ion-channel-encoding genes in this species are nonvital (*e.g.*, TANOUYE *et al.* 1981; ZHOU *et al.* 1999), and there is not only more than one (*cf.* EBERL *et al.* 1998) but probably four calcium-channel genes in *D. melanogaster* (ZHENG *et al.* 1995; LITTLETON and GANETZKY 2000), allowing for the possibility that they might have been found to be mutually redundant in terms of gross viability. Other *cac* mutants—identified initially by the visual-response defects that prompted some of the current phenotypic assessments (Table 4)—could have proved to be song-abnormal variants. The flip-side of this coin is that cac^S exhibits a very mild and abstruse ERG defect (SMITH *et al.* 1998b), and cac^{TS2} has now been found to be subtly impaired in this manner at a relatively high temperature (see RESULTS). With regard to the possibility of song defects being caused by the *nightblind-A* alleles of *cacophony*, only the faintest CPP variations from the norm could be teased out of one cac^{nbA} male type (SMITH *et al.* 1998b). In retrospect, song normality for such mutants seems to fit with the absence of heat-sensitive locomotor abnormalities in cac^{nbA} mutants (PEIXOTO and HALL 1998), compared with the fouled-up locomotion that is induced in cac^S or cac^{TS2} flies as the temperature approaches or exceeds 40° (PEIXOTO and HALL 1998; DELLINGER *et al.* 2000). Therefore, the essentially normal song pulses exhibited by cac^{nbA} males may not be informative with respect to the "default hypothesis." However, one further kind of *cacophony* mutant seems on point: a newly isolated "enhancer-of-cac^{TS2}" mutation. It causes heat-sensitive locomotor and synaptic-physiological defects to be accentuated, owing to a recently induced alteration that maps to the *cac* locus (R. W. ORDWAY, personal communication). Once again, the singing phenotype of the enhancer-of-cac^{TS2} males could be anything, including "automatically" polycyclic and loud in terms of pulse qualities. Yet, males expressing this putatively doubly mutated form of the gene yielded CPP values identical to those of wild type in song recordings made at 25°, and such values increased by only ~6% at 30° (A. VILLELLA and J. C. HALL, unpublished observations; *cf.* Figure 3A). Pulse amplitudes in the new mutant's song were like those of wild-type males (at 25°) and increased not at all as the temperature was raised (*cf.* Figure 3B). Therefore, a *cacophony* mutant type that can be thought of as substantially worse off than wild type (in terms of its generic isolation phenotype) yielded neither courtship-sound characteristics that might have been expected (*i.e.*, worse than cac^{TS2}, perhaps severely nonpatterned *slo*-like songs) nor ones that would support the default hypothesis about relationships between *cac* genotypes and song phenotypes.

We now consider interactions between *cacophony* and

TABLE 6

Recovery times after mechanical shock

			Recovery times (sec; mean ± SEM)			
		Rearing:	18°	25°	29°	25°
Genotype	*n*	Testing:	25°	25°	25°	25° after exposure to 29°
cac^{TS2}	10		8.5 ± 1.1[a,**]	5.6 ± 1.2[a,**]	5.8 ± 0.8[a]	16.8 ± 6.0[a,**]
w $comt^{ST53}$	10		3.0 ± 0.3[b,**]	1.4 ± 0.1[b,**]	Dead	4.9 ± 0.6[a,b,**]
WT	10		3.1 ± 0.3	1.3 ± 0.1	2.6 ± 0.3	1.6 ± 0.3

Times of recovery from vortexing were determined as described in MATERIALS AND METHODS, which also gives details about the rearing and testing conditions noted in the data-column headers. One-way ANOVAs were performed on recovery times (log-transformed data) after mechanically shocking flies that had been reared at the three different temperatures. All ANOVAs revealed significant differences among genotypes ($F_{[2,29]} = 26.50$, $P < 0.0001$; $F_{[2,29]} = 45.08$, $P < 0.0001$; $F_{[1,19]} = 25.15$, $P < 0.0001$, respectively). A further ANOVA was performed on recovery times after exposure to the higher temperature, with genotype as the main effect, and revealed significant differences among groups ($F_{[2,29]} = 29.36$, $P < 0.0001$). Subsequent planned comparisons showed that cac^{TS2} took significantly longer (all $P < 0.05$) to recover compared with WT or *w* $comt^{ST53}$, independent of rearing or testing conditions, whereas *w* $comt^{ST53}$ was different from WT only after the higher temperature exposure (P value < 0.017). ** P significant when $< \alpha = 0.017$, except for flies that were reared at 29° and tested at 25°; the P value for cac^{TS2} was significant per $\alpha = 0.05$ (since *w* $comt^{ST53}$ were not testable).

[a] Different from WT.

[b] Different from cac^{TS2}.

another synaptic function, the NSF protein encoded by comatose. The deleterious interactions of cac^{TS2} with *comt* mutations, which are not allele specific in terms of heat-induced paralysis (DELLINGER *et al.* 2000), make the amelioration of cac^{TS2}'s song defect by a *comt* mutation (Figure 5) counterintuitive but not unprecedented in general terms (*cf.* GANETZKY and WU 1982, who reported non-allele-specific suppression of channel defects in Drosophila doubly mutant for excitability variants). Whereas the *cac*-with-*comt* interactions (in different conditions and behavioral circumstances) are difficult to explain, the current results (Figure 5) reinforce the importance of testing ion-channel mutants for "microbehaviors" as well as for their grossly appreciable locomotion. Again, cac^{TS2} may or may not have turned out to be a song mutant, and combining with a *comt* mutation would not necessarily lead to a singing phenotype analogous to the effects of the double mutation on the fly's overall ability to move about. Perhaps this is because courtship song is regulated by synaptic functions occurring locally within certain central nervous system ganglia; these are likely to be located in the ventral nerve cord, as inferred from song analyses of cac^{S}//cac^{+} mosaics (HALL *et al.* 1990; *cf.* SCHILCHER and HALL 1979). However, it is also possible that excitable-cell etiology of *cac*-induced song defects resides more peripherally, at neuromuscular junctions (*cf.* KAWASAKI *et al.* 2000).

"Kind and gentle" *cacophony*-related behavioral variations: We end this discussion by refocusing specifically on calcium channels and their courtship-behavior roles (leaving aside the cac^{TS2} isolation phenotype, or the physiological consequences of this mutation, and the miniconundrum involving certain *cac*-with-*comt* interactions). Recall that the tone-pulse phenotypes of cac^{S} and cac^{TS2} males (at 30°) were, in the course of this DISCUSSION, termed "non-wild type" as opposed to defective. Indeed, the mutant characteristics do not appear to involve song pathologies of the sort that can occur in other Drosophila variants such as the *slo* mutants mentioned above and in *D. melanogaster* males expressing several (but not all) types of genetic changes involving the *no-on-transient-A* (*nonA*) gene (RENDAHL *et al.* 1992, 1996; STANEWSKY *et al.* 1993, 1996). Instead, the two *cacophony* mutants seem to cause more of a modulation of song quality than an aberration, such that these particular ion-channel mutants (compared with the *slo* ones or those in other species cited in the Introduction) come under the heading of "kinder and gentler" neurobiological variants (GREENSPAN 1997), that is, those that cause altered neuronal or neuromuscular functions without really deranging them.

Nevertheless, the differences between cac^{+}-controlled and *cac*-mutant singing are clearly appreciable (most readily apprehended here in Figure 4 and Table 3). It is as if these genetically variant males are generating songs that are not of their species. Indeed, the relevant genotypic changes are of the kind that can occur microevolutionarily: single amino-acid substitutions both in cac^{S} (SMITH *et al.* 1998b) and in cac^{TS2} (Figure 1 and Table 1; R. W. ORDWAY, personal communication). Such subtle alterations—whose kind and gentle attributes are far from those attributable to loss-of-function *cac* mutants—are not marked enough from the Dro-

sophila female's perspective to make her "process" a *cacophony* song as different from that of a cac^+ male (unlike what happens when she is exposed to interspecific singing): Kulkarni and Hall (1987) showed that recombining away a mutation apparently coinduced with cac^S led to normal mating receptivity. This, by the way, accounted for the mating-initation decrement exhibited by wingless cac^S males in the original strain (Schilcher 1977), which was doubly defective for genetic reasons, as opposed to what we infer for cac^{TS2}, which is pleiotropically (albeit mildly) defective for certain courtship-related (Table 2) and other locomotor actions (Tables 4–6) under the influence of that mutation alone.

If the *cacophony* gene can diverge evolutionarily such that a female-perceivable difference in tone-pulse quality were to result (see, for example, Cowling and Burnet 1981, Campesan *et al.* 2001b), the Dmca1A sequence may have to accumulate more than single amino-acid substitutions. In this regard, a partial comparison of the *cac* sequences in *D. simulans* to that of *D. melanogaster* uncovered no such interspecific differences (Peixoto *et al.* 2000), and these two species are similar in terms of intrapulse cycle numbers (Bernstein *et al.* 1992). Yet it seems warranted to sequence Dmca1A-encoding genes in more distant evolutionary relatives. The latter comparisons are likely to reveal interspecific *cac* differences, by analogy to what has been found for the *nonA* gene (Campesan *et al.* 2001a) and owing to the intriguing lead about the *X*-linked, "*cac* region" etiology of a song disparity between a certain pair of Drosophila species (Paallysaho *et al.* 2001). Neither of them is *D. melanogaster*, but one of these species (*D. virilis*) happens to be the same as that studied by Campesan *et al.* (2001a,b) with respect to *nonA*. Would such anticipated molecular divergences among *cacophony* genes have functional meaning for song control, both descriptively (*cf.* Figures 2–4) and in terms of courtship bioassays (*cf.* Table 2)? For this, *nonA* has shown the way by virtue of interspecific gene-transfer experiments that revealed this gene to convey species-specific behavioral characteristics (Campesan *et al.* 2001b).

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Note added in proof: The unpublished molecular results involving the $cac^{\wedge TS2}$ mutant—obtained by Ordway and co-workers and mentioned in the current article—were recently documented (F. Kawasaki, S. C. Collins and R. W. Ordway, 2002, Synaptic calcium-channel function in *Drosophila*: analysis and transformation rescue of temperature-sensitive paralytic and lethal mutations of *cacophony*. J. Neurosci. **22:** 5856–5864).

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