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Functional organization of the macroglomerular complex related to behaviourally expressed olfactory redundancy in male cabbage looper moths

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Abstract. The neurophysiological bases for behaviourally expressed olfactory redundancy in the sex pheromone communication system of the cabbage looper moth, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), were examined by coupling the cut-sensillum extracellular recording technique with a highly specific neuronal marking method for moth peripheral receptors. In seventy-two antennal sensilla, axonal pathways of cobalt-stained neurones could be traced into the male-specific macroglomerular complex in the antennal lobe. In *T. ni* males this comprises five glomeruli, two of which are subdivided into morphologically, and in some instances functionally identifiable, regions. Axonal arborizations of forty-eight neurones (single stainings) showed high fidelity (98%) for containment within a specific glomerulus or glomerular subdivision, and the neuropil targeted seemed to be related to the specificity of a neurone to a particular female-emitted sex pheromone component (Z7–12:Ac, Z7–14:Ac, Z9–14:Ac, 12:Ac, 11–12:Ac, Z5–12:Ac), or to a behavioural antagonist (Z7–12:OH). Double (twenty-one) and multiple stainings (three) showed axons projecting into two or more glomeruli, respectively, with 100% fidelity for the component-specific glomerulus or glomerular subdivision to be targeted. We suggest that the potential for a single minor component to cross-stimulate two or more neurones within a sensillum may enable partial blends to continue to provide sensory input into all of the pheromone-processing glomeruli of the complex. Our interpretation is that redundancy occurs at the receptor level on neighbouring dendrites, and thus allows various four-component partial blends to evoke full pheromone-mediated behaviour.

Key words. Lepidoptera, *Trichoplusia ni*, olfaction, sex pheromone, behaviour, cobalt, sensory neurone, macroglomerular complex.

Introduction

The complete sex pheromone blend released by a female moth is generally more effective than partial blends or than individual pheromone components in evoking the full sequence of mate-finding behaviours by a conspecific male, i.e. upwind flight, source contact, and hairpencil display (Baker *et al.*, 1981; Linn *et al.*, 1986). However, wind-tunnel bioassays conducted by Linn *et al.* (1984) demonstrated that male cabbage looper moths, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), exhibit full expression of pheromone-mediated behaviour in response to partial blends of their six-component pheromone. The complete sex pheromone of this species has been identified as a blend of six acetates. The major component is (Z)-7-dodecenyl acetate (Z7–12:Ac) (Berger, 1966) and there are five minor components: dodecyl acetate (12:Ac), (Z)-5-dodecenyl acetate (Z5–12:Ac), 11-dodecenyl acetate (11–12:Ac), (Z)-7-tetradecenyl acetate (Z7–14:Ac), and (Z)-9-tetradecenyl acetate (Z9–14:Ac) (Bjostad *et al.*, 1984).

Linn *et al.* (1984) showed that as many as two minor components could be removed from the complete blend without diminishing behaviour, and that a behaviourally active partial blend could be composed of different combinations of four components. In each of six behaviourally active partial blends the major component, Z7–12:Ac, was irreplaceable, but certain combinations of three minor components with Z7–12:Ac could compensate for the two missing minor components as if the latter were superfluous. Linn *et al.* (1984) characterized the substitution of specific minor components for one another in a blend as a form of redundancy in the chemical communication channel.

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Todd *et al.* (1992) tried to understand the underlying reasons for the behavioural activity of various four-component partial blends by recording from sex-pheromone-specific neurones within the male's antennal sensilla. They recorded from separate sensilla that contained a neurone specific for Z7–12:Ac and for Z7–14:Ac. A third sensillar type, which had not been found in previous electrophysiological studies on this species (O'Connell *et al.*, 1983; Grant *et al.*, 1988; Mayer, 1993), contained neurones that were tuned to the remaining four minor components in the sex pheromone blend. Either Z9–14:Ac or 12:Ac evoked a response from a small-spiking neurone. These two minor components had been shown to be behaviourally interchangeable in that only one of these two components was necessary in a four-component blend (Linn *et al.*, 1984). The methodology of Todd *et al.* (1992) could not discern if each component was stimulating the same small-spiking neurone, or if each component was exciting a different neurone, both of which produced the same small spike amplitude. A similar dilemma existed for the behaviourally interchangeable 11–12:Ac/Z5–12:Ac-component pair (Linn *et al.*, 1984), which evoked a larger spike of the same amplitude (Todd *et al.*, 1992). Cross-adaptation studies (Kaissling *et al.*, 1987) were attempted, but neurones could only be partially adapted to either component of a stimulus pair (Todd and Baker, unpublished data). The sensory recordings of Todd *et al.* (1992) provided a starting point for understanding how behaviourally necessary receptor neurones could continue to be stimulated by partial blends of components, but also clearly pointed to the need for examining peripheral input in light of central nervous system (CNS) processing of sex pheromone information.

The antennal lobes of male moths contain two 'complexes' of glomeruli, or spherical knots of dense neuropil that are involved in odour processing. A dorsally-situated complex, the macroglomerular complex, consists of only a few glomeruli, and is devoted to first-order synaptic processing of sex pheromone input from antennal receptors (cf. Matsumoto & Hildebrand, 1981). A ventrally-located complex, the ordinary glomeruli, consists of many more glomeruli, and is likely to be involved in processing all of the other odours the male encounters. The morphological and functional organization of the macroglomerular complex has received close scrutiny in only a few moth species (Matsumoto & Hildebrand, 1981; Hansson *et al.*, 1991, 1992, 1995; Christensen *et al.*, 1995a; Ochieng' *et al.*, 1995). A more complete picture of pheromone olfaction and of how specific lepidopteran sex pheromone blends evolve (Löfstedt, 1993) might be gained by studying antennal neuronal pathways leading into the male-specific MGC (Hansson *et al.*, 1992, 1995; Ochieng' *et al.*, 1995), thus filling the gap stemming from the exciting jump to neurophysiological and neuroanatomical studies or higher-order interneurones that integrate odour signals within, and project farther downstream out of, the macroglomerular complex (Matsumoto & Hildebrand, 1981; Christensen & Hildebrand, 1987a, b; 1988; Christensen *et al.*, 1989a, b, 1991, 1995a, b; Kanzaki *et al.*, 1991a, b; Hansson *et al.*, 1991, 1994). Furthermore, the relevance of both sensory and CNS neurophysiological findings to the evolution of pheromone systems can be best understood in the context of behavioural knowledge (cf. Linn *et al.*, 1984).

In the present study we have examined further the neurophysiological bases for behavioural activity of partial sex pheromone blends in male *T.ni* by coupling the cut-sensillum extracellular recording technique with cobalt staining of physiologically identified antennal neurones, and tracing the axons of these neurones into the macroglomerular complex by sectioning and reconstructing the antennal lobe.

Materials and Methods

Moths. Male *T.ni* were obtained from a laboratory colony started from individuals collected in 1980 from traps in Riverside, California. Wild males were periodically added to the colony over the years. Larvae were reared on a pinto bean diet (Shorey & Hale, 1965). Male pupae and the adults that were used for electrophysiological recording/cobalt staining of receptor neurones were kept in an environmental chamber with a LD 14:10 h light cycle and a temperature of 21°C. All adults were used 1–7 days post-emergence.

Chemicals. The sex pheromone components of *T.ni* were purchased from the pheromone library at the Institute for Pesticide Research, Wageningen, The Netherlands. Although it is not considered to be part of the sex pheromone, (Z)-7-dodecenol (Z7–12:OH) was also purchased from this library because male *T.ni* have a receptor neurone specific for this compound (O'Connell *et al.*, 1983; Grant & O'Connell, 1986; Todd *et al.*, 1992). This alcohol has been referred to as a behavioural antagonist because it reduces trap catch of males (McLaughlin *et al.*, 1974; Tumlinson *et al.*, 1972) or affects in-flight manoeuvres (Liu & Haynes, 1993) when it is added to the pheromone blend. Serial dilutions of each pheromone component and of Z7–12:OH were prepared in HPLC grade hexane, and checked by GC to ensure purity (>99%). Solutions were stored in 4 ml glass vials at –4°C.

Morphology. To determine the morphology of the macroglomerular complex nineteen brains were fixed in alcoholic Bouin's, embedded in paraffin, and sectioned frontally at 10 μm. The sections were silver stained according to Rowell (1963) to highlight the glomeruli. The anterior-to-posterior (longitudinal) dimensions of each glomerulus were estimated from 10 μm serial sections of twelve antennal lobes.

Cobalt lysine solution. A solution of cobalt lysine (0.5 M) was prepared by combining cobalt chloride hexahydrate (Sigma Chemical Co.) with L-lysine (Sigma Chemical Co.) in distilled water (Lázár, 1978). The lysine is suggested to enhance cobalt uptake (Gallyas *et al.*, 1978). The solution was stirred for 12–24 h at room temperature, and then the pH was adjusted to 7.2–7.4 using concentrated HCl. The intensity of the stain was highest when the solution was between 3 and 5 weeks old.

Stimulation and staining of receptor neurones. A male *T.ni* was restrained inside a 1 ml disposable pipette tip, with the tapered end cut to allow passage of the head and antennae. The head was encased in wax, and the antennae were immobilized at their bases with wax. A Ag/AgCl ground electrode was inserted into the moth's abdomen, and secured with a piece of moistened tissue placed behind the moth's body.

The moth was placed into a holder of a micromanipulator, and manoeuvred until the antennae were flushed with a stream of purified, humidified air (0.5 ml/s) that passed through a 20-cm-long glass tube (7 mm i.d.) that ended 1 cm from the antennae. A stimulus cartridge was prepared for each of the six sex pheromone components and for Z7–12:OH by pipetting 10 μl of a 1 μg/μl solution

onto a filter-paper strip that was held in a 15-cm-long glass Pasteur pipette; odour puffs from stimulus cartridges that were prepared from more dilute solutions did not elicit action potentials. The tip of the stimulus cartridge was introduced into a hole in the glass tube, and this hole was positioned 17 cm from the antenna. New cartridges were prepared every 3–4 days and stored at –4°C.

The cut-sensillum technique (Kaissling, 1974) was used to record from antennal receptor neurones, and access was gained to neurones by cutting the tip off of a sensillum with glass micro-knives (Van der Pers & Den Otter, 1978). Three physiological types of trichoid hairs could be identified by receptor neurone specificity and sensitivity to sex pheromone components (Todd *et al.*, 1992), and sampling began within 1–2 min of the moth having been secured within the pipette tip. Neurones were manually exposed to a 1 ml puff of each odourant (Roelofs & Comeau, 1969), and an odourant that elicited action potentials was chosen as the stimulus during staining. A physiologically identified neurone was stained with cobalt by coupling the cut-sensillum technique with a highly specific neuronal marking method developed for moth peripheral receptors (Hansson *et al.*, 1992). Cobalt lysine was contained inside of a glass micropipette that was fitted over a Ag/AgCl recording electrode that was attached to a holder, and manoeuvred with a micromanipulator. We attempted to stain a neurone by maintaining contact of the recording electrode with the cut hair tip for 1 h while simultaneously puffing 20–200 ms of odour-bearing air (5–20 ml/s) over the antenna at 0.5 Hz by using a pulse generating machine (Syntech, Inc., Hilversum, The Netherlands). Only one sensillum was used on each antenna. Both the AC (action) and DC (receptor) potentials of neurones were monitored during an odour stimulation on a Philips PM 3335 oscilloscope.

Post-stimulation. After the 1 h stimulation, the hair was disconnected from the recording electrode, and the moth was placed in the refrigerator (4°C) for 2 days to allow the dye to travel the entire length of the neurone. The moth was then decapitated and the brain, still within the head capsule, was treated with ammonium sulfide (Sigma Chemical Co.) before being fixed in alcohol:acetic acid:formaldehyde (8:1:1) for 24 h at room temperature. The brain was dissected, hydrated, and subjected to a standard silver intensification procedure for cobalt-stained neurones (Bacon & Altman, 1977). Brains were subsequently dehydrated, cleared in methyl salicylate, and viewed in wholemount for stained neurones under light microscopy. Because some cobalt-stained axons and the glomeruli were not visible in every wholemount, all brains were infiltrated with propylene oxide before being embedded in Durcupan resin (Sigma Chemical Co.) and sectioned frontally at 10 μm. Sections were counterstained with methylene blue, and the axonal pathways of receptor neurones into the macroglomerular complex were reconstructed using a light microscope equipped with a camera lucida.

Results

Morphology

The macroglomerular complex of *T.ni* males comprises five morphologically distinct glomeruli, as determined by the presence of a clear border around each structure. Subdivisions within two of these glomeruli were observed. We have named each of four of the glomeruli with the letters *a*, *c*, *d* and *g*, respectively, and the glomerular subdivisions with the letters *b*, *e* and *f*, respectively. The fifth glomerulus comprises subdivisions *e* and *f* (Figs 1A–C), and subdivision *b* is one of four morphologically identifiable subdivisions within the large glomerulus we call *a* (Fig. 1B). The status of each glomerulus or glomerular subdivision as part of the pheromone-processing complex was confirmed when it consistently contained axonal arborizations of a component-specific receptor neurone, as indicated by single stains (Table 1). When frontal sections containing the antennal lobes were viewed in sequence from posterior-to-anterior, the five glomeruli became visible in the following order: *a* = *b*, *c*, *g*, *d* and *e* = *f*, respectively. The positions of all of the glomeruli are presented below in reference to glomerulus *a*.

Glomerulus *a* is the most elongated longitudinally of the five glomeruli (93.3 ± 7.9 μm long, mean ± SD), and is also generally the widest in diameter (Fig. 1A). In some sections the four dorso-ventral subdivisions of glomerulus *a* can be seen (Fig. 1B), each of which appears to receive separate input from axons of antennal receptor neurones (Figs 1B, C). The medial-most subdivision (*b*) receives input from functionally different axon types than the other three subdivisions. When the whole MGC is in view, glomerular subdivision *b* is flanked by the remaining portion of glomerulus *a* laterally and by glomerulus *c* medially (Fig. 1A).

Glomerulus *c* appears 14.2 ± 7.9 μm anterior to glomerulus *a*, and is located medial to glomerulus *a* (Fig. 1A). It measures 66.7 ± 15.6 μm long. Glomerulus *g* comes into view 19.2 ± 9.0 μm anterior to glomerulus *a*, and is lateral to glomerulus *a* (Fig. 1A). Glomerulus *g* measures 61.7 ± 11.1 μm long. Both glomeruli *c* and *g* can be nearly as wide in diameter as glomerulus *a* (Fig. 1A). One glomerulus, *d*, is situated ventral to glomerulus *c*, and one glomerulus, comprising subdivisions *e* and *f*, is situated ventral to glomerulus *g* (Fig. 1A). Glomerulus *d* appears 27.5 ± 4.5 μm anterior to glomerulus *a*, and measures 47.5 ± 8.7 μm long. Glomerular subdivisions *e* and *f* appear 30 ± 6.0 μm anterior to glomerulus *a*, and measure 58.3 ± 10.3 μm long when taken as a whole. Incoming axons from the antennal nerve appear to arrive at each glomerulus or glomerular subdivision already organized into discrete bundles of axons that have peeled away from larger major tracts located farther inside of the antenna (Figs 1B, C).

Coarse interneuronal fibres, which seem to originate from the lateral and medial cell body clusters, can usually be seen connecting the five glomeruli within a central area ventral to glomerulus *a*, and between glomeruli *d* and glomerular subdivision *e* (Figs 1A–C). The ordinary glomeruli, themselves arranged in a spherical fashion around coarse central neuropil, fill the remainder of the antennal lobe ventral to the MGC glomeruli (Figs 1A–C).

Sensillar types

We screened 949 long trichoid sensilla on male *T.ni* antennae for their receptor neurones' specificity and sensitivity to the six sex pheromone components and to Z7–12:OH. Only 382 of these sensilla were used for neuronal marking; the rest (567) represent attempts to find the rarer trichoid sensilla on each antenna. One trichoid hair type was abundant (84% of the sensilla sampled), and contained a large-spiking receptor neurone tuned to Z7–12:Ac, and a small-spiking neurone tuned to Z7–12:OH (Fig. 2A) with

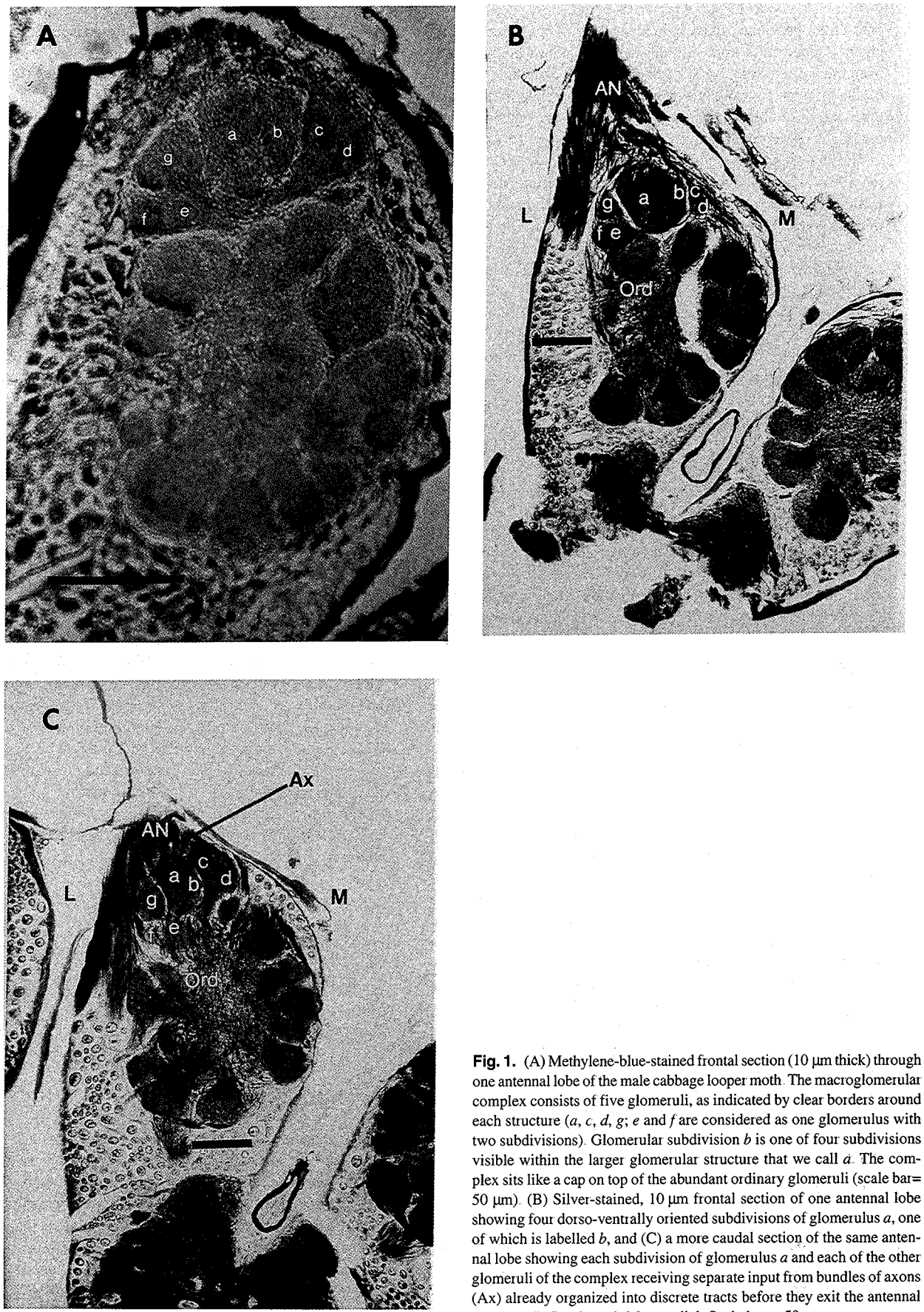

Fig. 1. (A) Methylene-blue-stained frontal section (10 μm thick) through one antennal lobe of the male cabbage looper moth. The macroglomerular complex consists of five glomeruli, as indicated by clear borders around each structure (*a*, *c*, *d*, *g*; *e* and *f* are considered as one glomerulus with two subdivisions). Glomerular subdivision *b* is one of four subdivisions visible within the larger glomerular structure that we call *a*. The complex sits like a cap on top of the abundant ordinary glomeruli (scale bar= 50 μm). (B) Silver-stained, 10 μm frontal section of one antennal lobe showing four dorso-ventrally oriented subdivisions of glomerulus *a*, one of which is labelled *b*, and (C) a more caudal section of the same antennal lobe showing each subdivision of glomerulus *a* and each of the other glomeruli of the complex receiving separate input from bundles of axons (Ax) already organized into discrete tracts before they exit the antennal nerve (AN). L = lateral; M = medial. Scale bar = 50 μm.

Table 1. Arborizations of antennal receptor neurones within glomeruli or glomerular subdivisions of the macroglomerular complex in male *T.ni.*

					Axonal arborizations within MGC glomeruli or glomerular subdivisions																	
					Single						Double							Multiple				
Sensillar type	Stimulus	Stained	Empty	Total attempts	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a+c*	*a+d*	*b+e*	*b+g*	*d+g*	*a–c*	*b–d*	*a–g*
Major component	Z7–12:Ac	26	140	166	15	0	0	0	0	0	0	11	0	0	0	0	0	0	0
	Z7–12:OH	12	49	61	1	0	9	0	0	0	0	2	0	0	0	0	0	0	0
Minor component	Z7–14:Ac	7	12	19	0	0	0	4	0	0	0	0	3	0	0	0	0	0	0
Minor component	Z9–14:Ac	7	55	62	0	0	0	0	5	0	0	0	0	1	0	0	0	0	1
	12:Ac	9	14	23	0	7	0	0	0	0	0	0	0	0	0	0	1	1	0
	11–12:Ac	5	23	28	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0
	Z5–12:Ac	6	17	23	0	0	0	0	0	0	2	0	0	0	2	2	0	0	0
	Total stained	72																	
	Total empty		310																
	Total attempts			382															

Neither neurone was excited by exposure to the other five minor components. Also, our recordings showed no evidence that the small-spiking neurone tuned to Z7–12:OH fired during test puffs of Z7–12:Ac; this major component evoked action potentials only from the large-spiking neurone (Fig. 2A). We refer to this type of sensillum as 'major component sensilla' (Table 1), although in earlier neurophysiological studies they were called 'high-spontaneous-activity' sensilla (O'Connell *et al.*, 1983; Grant & O'Connell, 1986).

The other two trichoid hair types were rarely encountered, and are categorized as 'minor component sensilla' (Table 1). One type of minor component sensillum (2% of the sensilla sampled) contained a receptor neurone that was tuned to Z7–14:Ac, with no action potentials elicited by exposure to the other components or to Z7–12:OH (Fig. 2B) (possibly the 'low-spontaneous-activity' sensilla of Grant *et al.*, 1988). The other type of minor component sensillum (14% of the sensilla sampled) contained at least four receptor neurones, each of which was tuned to a specific minor pheromone component: one neurone is tuned to Z9–14:Ac and a second neurone to 12:Ac, with both producing a small spike of the same amplitude; neurones 3 and 4 produce larger spikes of equivalent amplitude, with one neurone tuned to 11–12:Ac and the other to Z5–12:Ac (Fig. 2C). Within this sensillar type, hyperpolarizations were observed following exposure to Z7–12:Ac, Z7–14:Ac, and Z7–12:OH (see DC traces in Fig. 2C), as was first documented in Todd *et al.* (1992). Ultrastructural studies performed previously on male *T.ni* antennae indicate that most of the long trichoid sensilla contain either two or three nuerones (R. Y. Zacharuk, unpublished data).

Cobalt staining of neurones. For 382 long trichoid sensilla we attempted to mark a specific receptor neurone with cobalt, and in seventy-two of these sensilla we were successful: the axonal arborizations of cobalt-stained neurones could be traced into glomeruli of the MGC (Table 1) (Fig. 3). In forty-eight of the seventy-two sensilla a single receptor neurone was stained (single stain), and in twenty-one sensilla, two neurones were stained (double stain) (Table 1). Multiple staining (three or more neurones) was obtained in three sensilla (Table 1). During the screening of these three sensilla to determine receptor neurone specificity, action potentials were only elicited by exposure to Z9–14:Ac, 12:Ac, 11–12:Ac and Z5–12:Ac. Hyperpolarizations were elicited following exposure to Z7–12:Ac, Z7–14:Ac and Z7–12:OH.

For all neurones within each of the three physiological types of trichoid hairs, the ability to generate action potentials in response to each odourant pulse generally lasted between 1 and 5 min. However, action potentials were observed to only the first five to ten pulses in many preparations that later exhibited one or more stained neurones. The receptor potential continued to be evoked by pulses throughout the 1 h stimulation. Cobalt appears to suppress the mechanisms involved in generating action potentials in response to individual puffs of odour within a few minutes after initiation of odour pulsing.

Major component sensilla. We sampled 227 major component sensilla. For 166 of these sensilla we used Z7–12:Ac as the stimulus and twenty-six stainings were obtained (Table 1). In each of the fifteen single stains an axon arborized within glomerulus *a* (Fig. 4). In each of the eleven double stains one axon arborized within glomerulus *a* and a second axon arborized within glomerulus *c* (Fig. 4). Within 1–5 min after initiation of stimulation

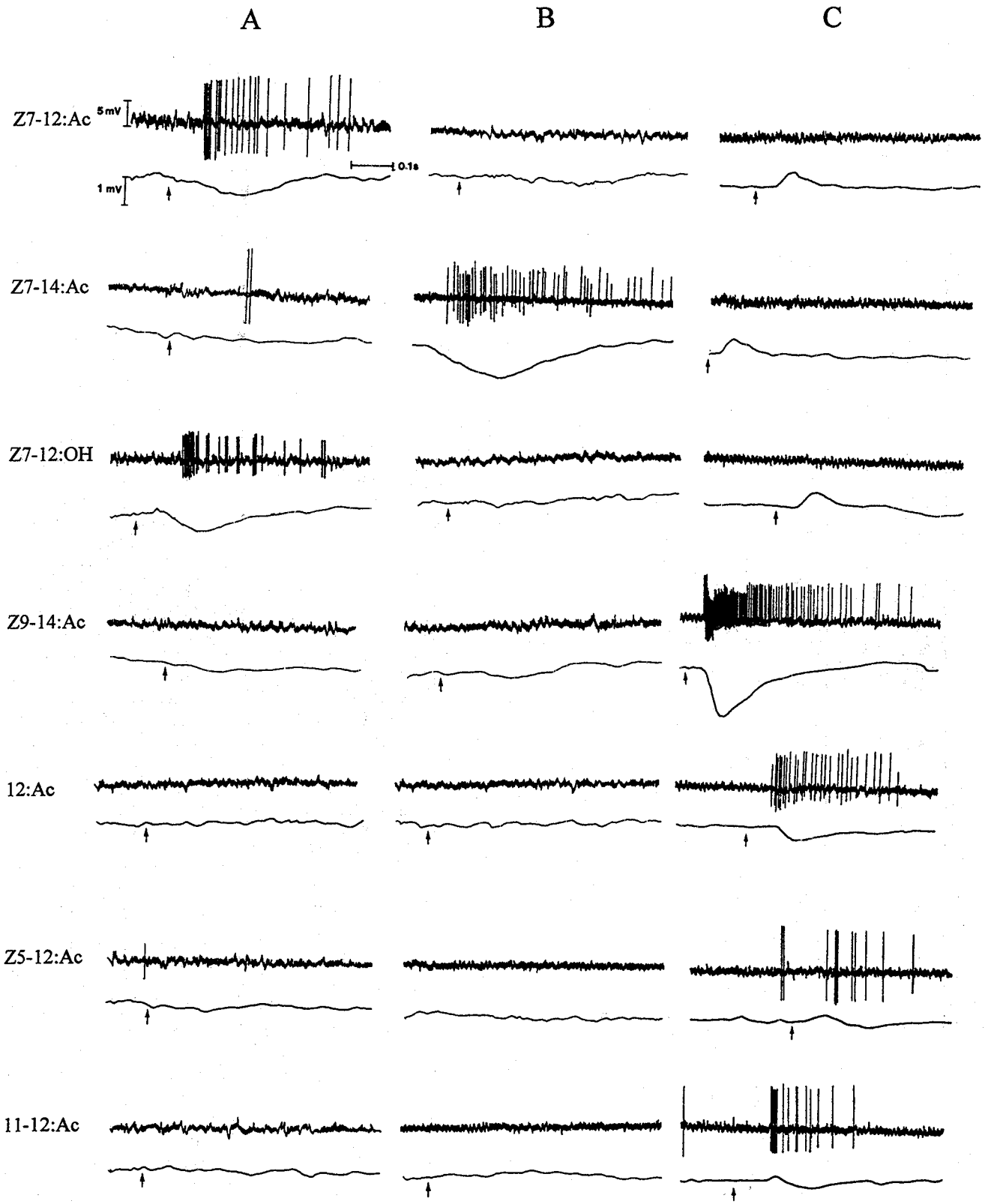

Fig. 2. Typical electrophysiological responses (AC = top trace; DC = bottom trace) of neurones within three types of trichoid sensilla on the antennae of male *T. ni*. Odour stimuli were the six sex pheromone components of this species, and a behavioural antagonist to upwind flight (Z7–12:OH). A = major component of sensilla; B = Z7–14:Ac-minor-component sensilla; C = four-component minor-component sensilla (see *Sensillar types* in Results for details). Arrows represent stimulus presentation.

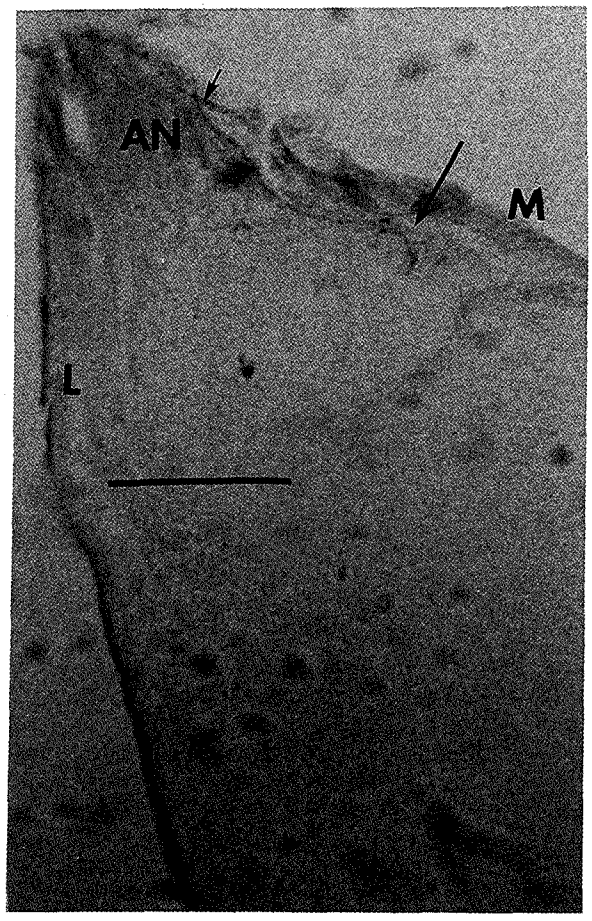

Fig. 3. Methylene blue-stained 10 μm section through the antennal lobe of a male *T.ni* showing axonal arborizations of a Z7–12:OH-specific neurone within glomerulus *c* of the macroglomerular complex (long arrow). The fibre also can be seen travelling down the antennal nerve and entering the antennal lobe (small arrow). Glomerular organization is only lightly highlighted with methylene blue stain. AN = antennal nerve; L = lateral; M = medial. Scale bar = 50 μm.

with Z7–12:Ac the small-spiking Z7-12:OH neurone in this sensillum began firing. Of the sixty-one attempts with Z7–12:OH as the stimulus, ten single stains were obtained: axons from nine neurones arborized within glomerulus *c* (Figs 3 and 4), and the axon of the tenth neurone arborized within glomerulus *a* (Table 1). Both of the double stainings with the Z7–12:OH stimulus showed one axon arborizing within glomerulus *c*, and a second axon arborizing within glomerulus *a* (Table 1).

Minor component sensilla. A receptor neurone was excited only by exposure to Z7–14:Ac in nineteen sensilla, and the axons of seven of these neurones were stained with cobalt (Table 1). All Z7–14:Ac stainings showed one axon arborizing within glomerulus *d*; the second axon in three double stainings arborized within glomerulus *a* (Fig. 5), but confinement to a particular subdivision was not evident. In 136 sensilla, action potentials were elicited by exposure to Z9–14:Ac, 12:Ac, 11–12:Ac and Z5–12:Ac. Out of sixty-two attempts, seven neurones stained with cobalt when the stimulus was Z9–14:Ac, of which five stains were single, one was double, and one was multiple (Table 1). All Z9–14:Ac stainings had one axon arborizing within glomerular subdivision *e* (Figs 6 and 7); a second axon in the one double staining arborized within glomerular subdivision *b* (Fig. 7). In the antennal lobe with multiple staining, each of the five MGC glomeruli appeared to receive an axon originating from one of seven neurones (Fig. 7). Twenty-three attempts at staining were made with 12:Ac as the stimulus; seven single stains and two triple stains were obtained (Table 1). All 12:Ac stains had one axon arborizing within glomerular subdivision *b* (Figs 6 and 7), and in the triple stainings two additional axons arborized within glomeruli *a* and *c*, and within glomeruli *c* and *d*, respectively (Fig. 7). The stimulus was 11–12:Ac for twenty-eight sensilla, and in five of these sensilla only a single receptor neurone took up cobalt (Table 1), and sent its axon into glomerular subdivision *f* (Fig. 6). Twenty-three attempts were made to mark a neurone by stimulation with Z5–12:Ac, and six were successful; two single stains and four double stains (Table 1). All Z5–12:Ac stains showed one axon projecting into glomerulus *g* (Fig. 6). A second axon from the double stainings projected either into glomerular subdivision *b* or into glomerulus *d* (Fig. 7).

The termination regions of axons from antennal receptor neurones in the macroglomerular complex of male *T.ni* were thus consistent to which stimulus was used (Table 1). In single stainings resulting from stimulation by any one of the six pheromone components (thirty-eight), there was 100% fidelity of arborization to a particular component-specific glomerulus or glomerular subdivision. Single (ten) and double (two) stainings resulting from stimulation with Z7–12:OH showed 92% fidelity of arborization to glomerulus *c*; the axon from a neurone in one of the ten single stainings arborized within glomerulus *a*, not within glomerulus *c*, as did the second axon in the two double stainings (Table 1). In the nineteen double stainings with a specific sex pheromone component, nineteen axons arborized within the correct (as indicated by single stainings) component-specific glomerulus or glomerular subdivision (Table 1). The order of glomeruli targeted by the second axons in double stainings, and third axons in the two triple stainings (Table 1), may be related to receptors site affinities for particular components.

Discussion

There is a growing body of evidence that the morphologically distinct glomeruli composing the macroglomerular complex of male moths also represent functionally distinct processing centres for incoming information about individual sex pheromone components (Hansson *et al.*, 1992, 1995; Ochieng' *et al.*, 1995). We have shown that male *T.ni* also exhibit this glomerular partitioning of sex pheromone components with axons of some component-specific neurones projecting into glomeruli that appear homogenous internally, and others projecting into specific subdivisions of larger glomerular structures. Todd *et al.* (1992) proposed that the number of glomeruli composing this complex, and the axonal arborization patterns of receptor neurones within it might aid in understanding olfactory redundancy in the sex pheromone communication system of *T.ni*.

Our present results have improved our knowledge of odour-quality processing in *T.ni*. For instance, Todd *et al.* (1992) hypothesized that the relationship of the macroglomerular complex to

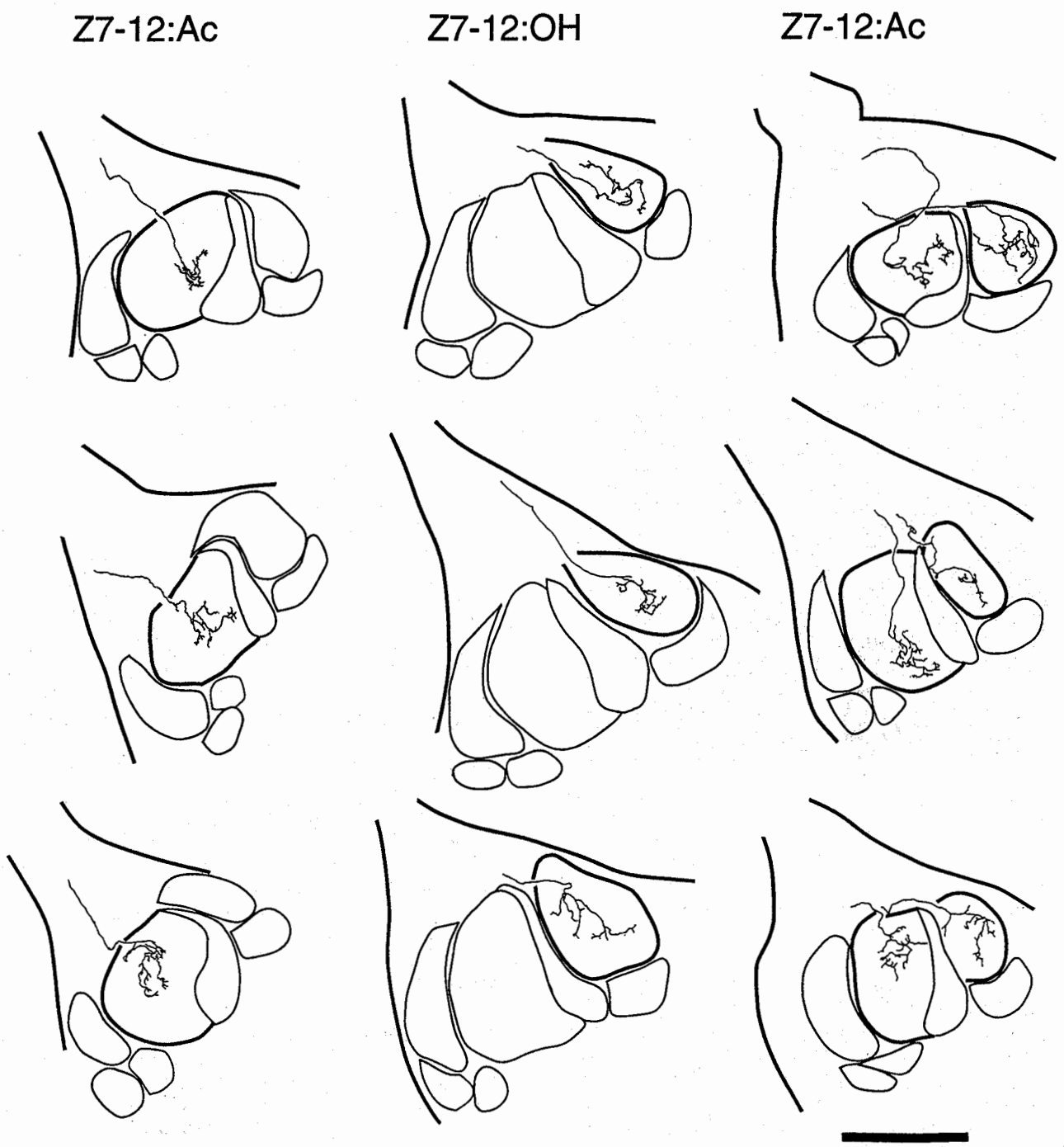

Fig. 4. Reconstructions of axons from receptor neurones within major component sensilla (see *Sensillar types* in Results) on the antennae of male *T.ni*, showing their pathways into glomeruli of the macroglomerular complex. The stimulus was either Z7–12:Ac or Z7–12:OH. Each antennal lobe is oriented such that lateral is to the left and medial is to the right of the antennal nerve entrance. Scale bar = 50 μm.

behaviourally expressed olfactory redundancy would involve only four glomeruli that receive sex pheromone component input. However, our morphological studies of male *T.ni* antennal lobes show that five glomeruli compose the complex, some of which have subdivisions into which component-specific information flows. The single-cell recordings of Todd *et al.* (1992) showed that the neurones specific for the major component Z7–12:Ac and the minor component Z7–14:Ac were located within separate trichoid hairs on the antennae, and they speculated that the axons of these component-specific neurones would project into two different glomeruli. Our cobalt stains have confirmed that Z7–12:Ac and Z7–14:Ac-specific neurones do target two different glomeruli of

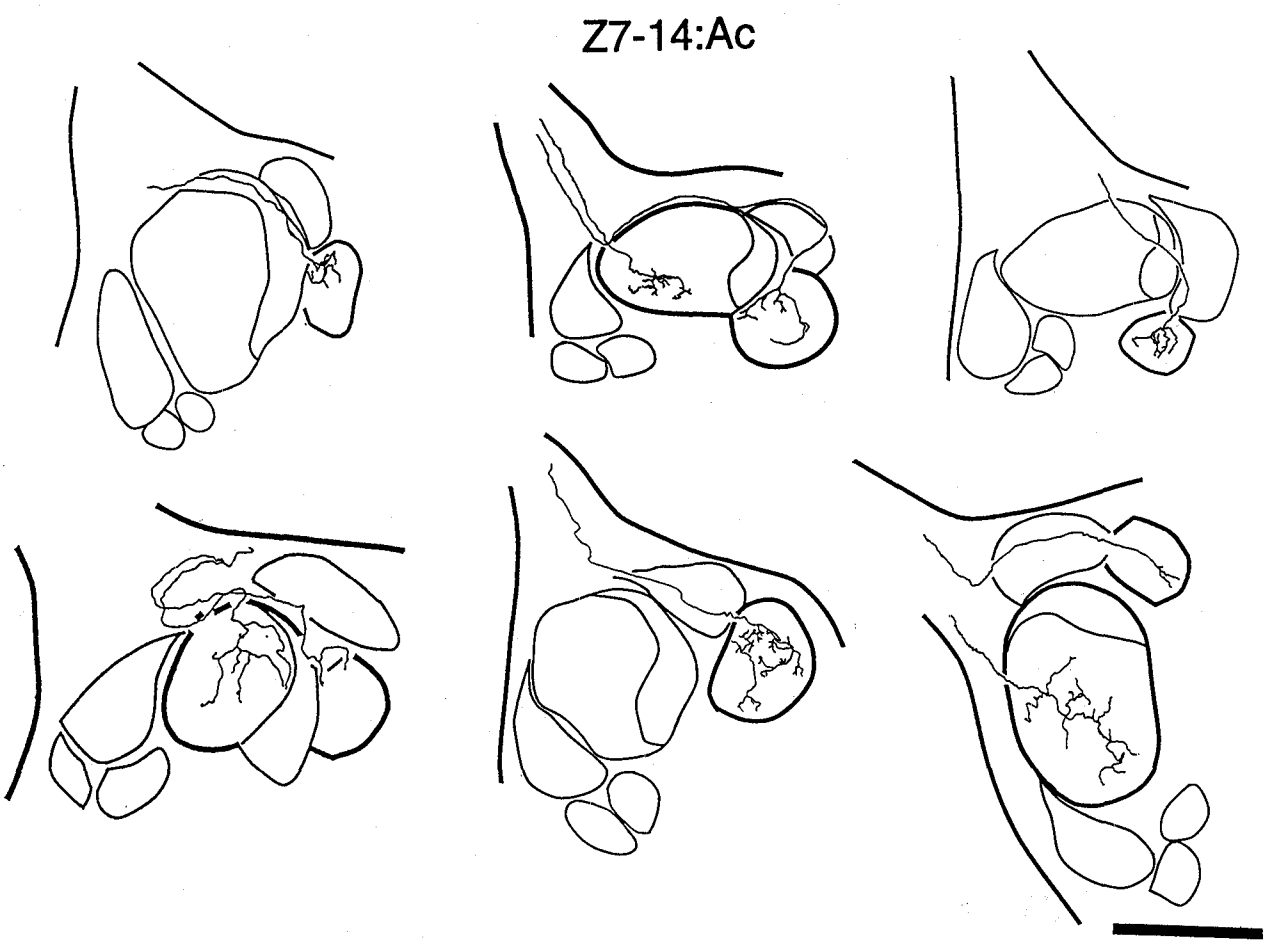

Fig. 5. Reconstructions of axons from receptor neurones within Z7–14:Ac minor-component-specific sensilla (see *Sensillar types* in Results) on the antennae of male *T.ni*, showing their pathways into glomeruli of the macroglomerular complex. The stimulus was Z7–14:Ac. Each antennal lobe is oriented such that lateral is to the left and medial is to the right of the antennal nerve entrance. Scale bar = 50 μm.

the complex. Todd *et al.* (1992) also hypothesized that the neurones located within a third type of trichoid hair that were tuned to behaviourally interchangeable pairs of minor components [Z9–14:Ac/12:Ac (small-spiking neurone) and 11–12:Ac/Z5-12:Ac (larger-spiking neurone)] might share a glomerulus either because both components in a pair stimulated the same neurone but with each component filling a functionally different receptor-site type, or because each component of the pair excited its own specific neurone, with the two neurones projecting into the same glomerulus. Our current results support neither of these alternatives. Rather, they indicate that there are at least four neurones in the minor-component sensilla that respond to behaviourally redundant pairs of components because the axons of Z9–14:Ac-, 12:Ac-, 11–12:Ac- and Z5–12:Ac-sensitive neurones, respectively, project consistently into different glomeruli or glomerular subdivisions of the macroglomerular complex (Table 1, Fig. 6). The few double stainings in response to one component that we obtained from this sensillar type (Fig. 7) imply that behaviourally expressed redundancy occurs when two or more neurones each have the potential of being stimulated by the same minor component in a blend. We suggest that the degree of cobalt uptake should mirror the relative affinities of the stained neurones' dendritic receptor sites for the stimulus component. However, currently, the cross-'stimulation' by one minor component is only indicated at the dendritic level by the propensity of a second or third neurone to stain in addition to the primary-component-targeted neurone. For this cross-stimulation to translate into behaviour, it must ultimately manifest itself in the relative frequency of action potentials generated by the neurones involved. We are in the process of determining the degree to which action potentials are evoked in these different minor-component-tuned neurones when a single minor component to which they are not tuned is used as a stimulus. This task will not be easy considering the similar spike amplitudes in these neurones.

Only the double and multiple stainings in the four-minor-component type of sensilla have potential relevance to redundancy, and that is why we have focused on them, even though they represent only 29% of all the stainings in this sensillar type. In our experiments five out of eight stainings were double and affecting the input from redundant pairs of components when a single component of the pair was used for stimulation. In the other three cases (multiple stainings) the cross-affinities of the neurones according to staining do not pertain to redundant pairs,

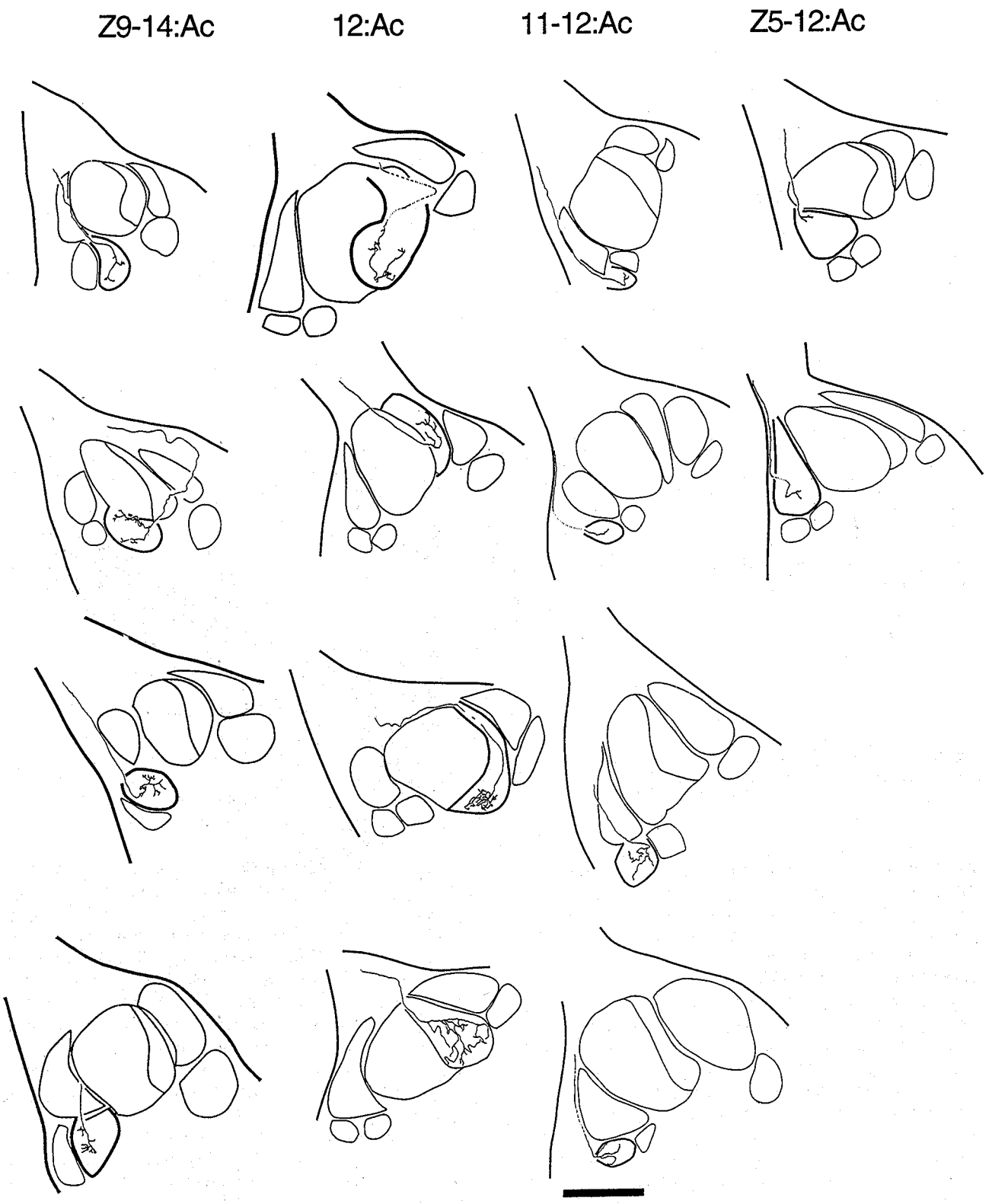

Fig. 6. Reconstructions of axons from receptor neurones tuned to Z9–14:Ac, 12:Ac, 11–12:Ac or Z5–12:Ac within minor-component-specific sensilla (see *Sensillar types* in Results) on the antennae of male *T. ni*, showing their pathways into glomeruli of the macroglomerular complex. Single stainings by stimulation with each minor component show axonal arborizations within specific glomeruli or glomerular subdivisions of the macroglomerular complex. Each antennal lobe is oriented such that lateral is to the left and medial is to the right of the antennal nerve entrance. Scale bar = 50 μm.

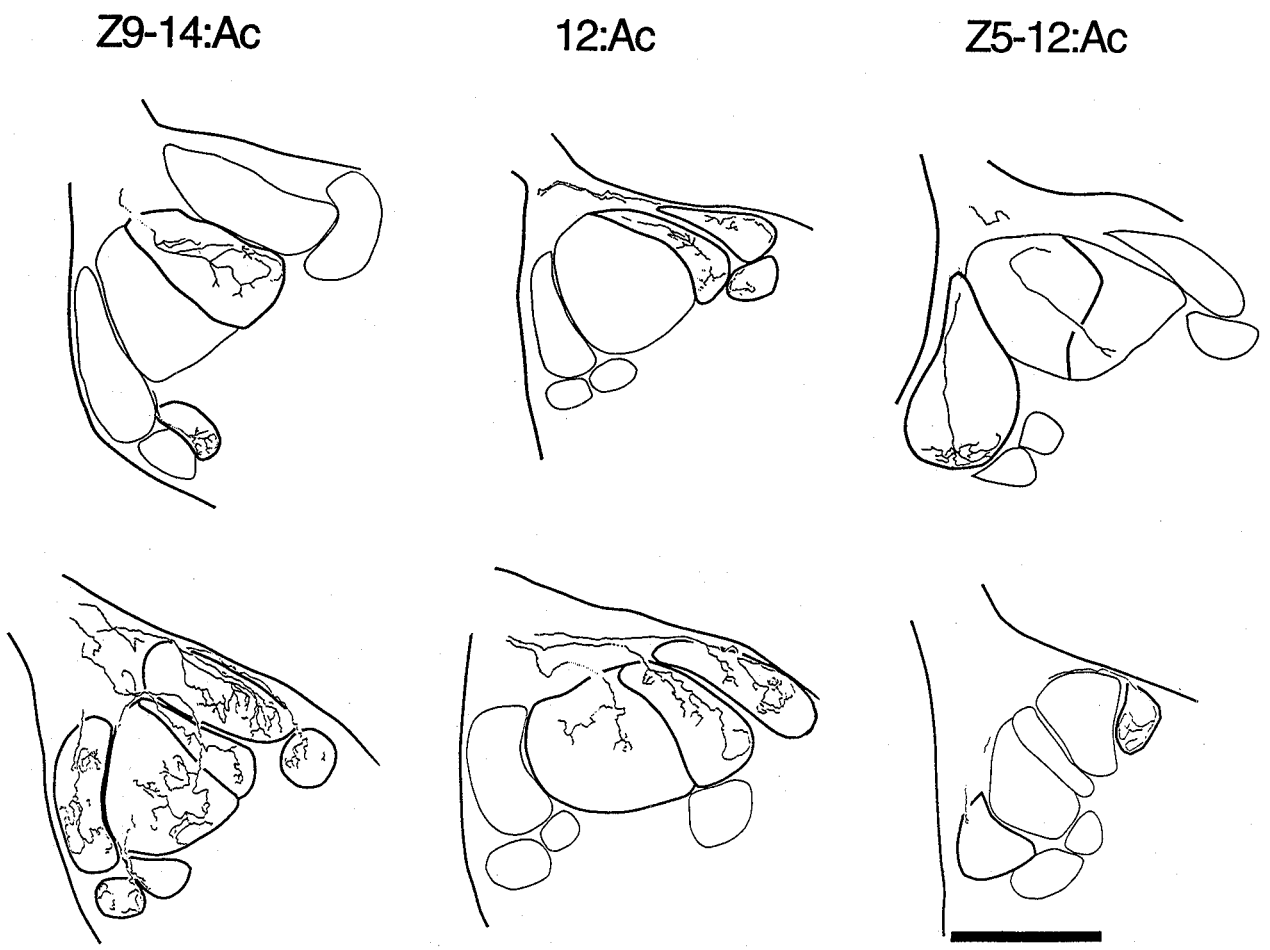

Fig. 7. Double and multiple stainings within minor-component-specific sensilla (see *Sensillar types* in Results) on the antennae of male *T.ni* that contain at least four receptor neurones, each of which is tuned to a specific minor sex pheromone component. Two or more axons are shown projecting into glomeruli or glomerular subdivisions of the macroglomerular complex when only one minor component was used as the stimulus. Each antennal lobe is oriented such that lateral is to the left and medial is to the right of the antennal nerve entrance. Scale bar = 50 μm.

nor would these cross-affinities be able to be translated into action potentials affecting behaviour because the cross-stained neurones (projecting into glomeruli *a*, *c* and *d*, targeted by Z7–12:Ac, Z7–12:OH and Z7–14:Ac-tuned neurones, respectively) were neurones that hyperpolarized when stimulated by these three compounds to which they are tuned.

One value of these multiple stainings, and two of the four double stainings with the Z5–12:Ac stimulus, is that they provide evidence that more than four neurones may be present in this sensillar type (Table 1). We draw such a conclusion because each of these stainings involves at least one glomerulus (*a*, *c* or *d*) not specific to the minor component neurones known to be in this hair type, and because the possibility of a simultaneous connection of the cobalt-filled recording electrode with each of the three types of trichoid hairs, which could also result in unexpected combinations of glomeruli in the complex being targeted, is remote. Also, if a multiple connection was made accidentally, we would see excitation to Z7–12:Ac and to Z7–12:OH, not hyperpolarizations as were seen here. In the specific case of the multiple stain, all four minor-component neurones known from single and double stainings to be in this hair type are already accounted for in their respective glomeruli (*g*) or glomerular subdivisions (*b*, *e* and *f*). The extra three neurons thus target the remaining three glomeruli (*a*, *c* and *d*).

Double stainings in the other two sensillar types are not relevant to olfactory redundancy in this species because none of these stainings affected neurones or glomeruli having to do with redundant pairs of components. However, they do provide evidence for receptor-affinity-correlated uptake of dye that is linked to the generation of action potentials and to behaviour. Behavioural antagonism, not redundancy that positively affects behaviour, would be the result of cross-reactivity of the two neurones known to be within the major component sensilla. We suspect that most (84%) of the double stainings (Table 1) in this sensillar type are related to stimulation caused by the breakdown of Z7–12:Ac into Z7–12:OH by sensillar esterases. Repeated pulsing of Z7–12:Ac may cause a build-up of Z7–12:OH in the sensillar lymph. We observed that within 1–5 min after initiation of stimulation with Z7–12:Ac, the small-spiking neurone in these sensilla did become excited; thus cobalt could conceivably enter this neurone as if Z7–12:OH were the stimulus, even though the stimulus applied to the outside of the hair is Z7–12:Ac (Fig. 4).

Cross-reactivity indicated by the remaining double stainings when Z7–12:OH was the stimulus in major component sensilla would not translate into behaviour. First of all, Z7–12:OH is not emitted by female *T.ni*. Secondly, any action potential activity in Z7–12:Ac neurones caused by Z7–12:OH in a blend that contains Z7–12:Ac would be lost in the large number of Z7–12:Ac action potentials coming into glomerulus *a*.

A glomerulus devoted to input from Z7–12:OH-specific neurones may be located with the sex-pheromone-processing glomeruli of the MGC because of its role in receiving input about the hydrolysis of Z7–12:Ac, thus providing information about excessive amounts of the major component. The turnip moth, *Agrotis segetum*, uses (Z)-5-decenyl acetate (Z5–10:Ac) as its major sex pheromone component, and the macroglomerular complex of the male may contain its Z5–10:OH-specific glomerulus (Hansson *et al.*, 1992) for the same reason. We suggest that the primary role of the alcohol-specific neurone as a hydrolysis detector in both of these species is supported by the fact that these alcohol-tuned neurones are found only within sensilla that also contain a second neurone that is tuned to the major pheromone component (Hansson *et al.*, 1992; Todd *et al.*, 1992).

Double stainings within Z7–14:Ac-minor-component sensilla also do not relate to behaviourally expressed olfactory redundancy because the cross-staining-related stimulation of the Z7–12:Ac neurones by Z7–14:Ac in this type of hair would probably be lost in all of the action potential activity coming into glomerulus *a* from the Z7–12:Ac-tuned neurones when the entire pheromone blend in the plume contacts the major component sensilla. The double staining is again instructive in that it is consistent with neurones within this type of sensillum being known to be highly sensitive to Z7–14:Ac and much less sensitive to Z7–12:Ac (Grant & O'Connell, 1986; Grant *et al.*, 1988). Grant & O'Connell (1986) and Grant *et al.* (1988) attributed these sensitivities to the same neurone, but our cobalt stainings demonstrate that two neurones are actually involved.

Based on our results, the complete six-component sex pheromone released by a female *T.ni* can be expected to result in sensory input into six different regions of the neuropil in the male's macroglomerular complex, and this is the blend that evokes optimal mate-finding behaviour at all phases of the response, from initiation of flight to hairpencil display (Linn *et al.*, 1984). However, our results indicate that we might expect various partial blends to be as good behaviourally as the complete blend because they can continue to provide stimulatory input into the populations of antennal lobe interneurones arborizing within these regions, notwithstanding that such blends will be deficient in one or more components. With further removal of various components from a blend, action potential input from the periphery will become unbalanced, with some glomeruli or subdivisions eventually not receiving sensory input. This lack of sensory input into specific glomerular regions in the ensemble could be enhanced by local interneurones, many of which in another species, *Manduca sexta*, seem to arborize within all of the ordinary glomeruli, all of the glomeruli of the macroglomerular complex or both (Hildebrand *et al.*, 1992; Christensen *et al.*, 1993). In *M.sexta* the local interneurones are GABAergic, receive excitatory input form the antennal receptors, and in one olfactory pathway of this species inhibit both other local interneurones as well as projection interneurones sending information to higher brain centres.

We suggest that the 'across-fibre pattern' of integration of odour quality from partially labelled lines that has been hypothesized for years (cf. Dethier, 1972) actually might now be viewed in *T.ni* as an *across-glomerular* pattern, because the thousands of lines representing but a handful of specific types of pheromone-detecting antennal neurones are organized into six functionally distinct glomerular regions. We therefore suggest that in moths such as *T.ni* and *A.segetum* perhaps one reason for the orderly functional compartmentalization of glomeruli into synaptic centres handling distinct lines of neuronal activity is to spatially facilitate the sampling of the across-fibre pattern of activity over the entire antenna by local- and projection-interneurone-coupled systems using the simple arrangement of the antennal lobe glomeruli. Of course, other pathways transferring information about other features of the pheromone blend and the fine structure of the pheromone plume should also exist, and not all species exhibit the distinct macroglomerular complex compartmentalization (Hansson *et al.*, 1995) found in *T.ni*, *A.segetum* and *M.sexta* (Hansson *et al.*, 1991, 1992).

Because of the behavioural and olfactory flexibility in the sex pheromone communication system of *T.ni*, this species is ideal for studying behavioural (Linn *et al.*, 1984), sensory physiological (O'Connell *et al.*, 1983; Grant & O'Connell, 1986, Grant *et al.*, 1988; Mayer, 1993; Todd *et al.*, 1992), and neuroanatomical aspects of olfaction. Specifically with this moth species, we believe that we are making progress toward understanding how partial odour blends can apparently smell complete, and how different partial blends can apparently smell similar.

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