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Original article

Antennal glands in male bees: structures for sexual communication by pheromones?

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Abstract – Morphological investigations were conducted on male antennae of three Apidae species, *Apis mellifera*, *Bombus pascuorum* and *Xylocopa violacea*. Male antennal glands were present in all species, with some differences regarding both external morphological characters and internal cytological features. There are externally obvious sites of pheromonal release (provided with evident pores) in *B. pascuorum* and *X. violacea* males, while pores are lacking in *A. mellifera* males. Internally *A. mellifera* presents a glandular complex composed of class 1 secretory cells, while the two other species possess two types of glands (with class 1 and class 3 secretory cells) associated with the same release sites. The functional hypothesis for the secretion of these glands is that they may act as a sex pheromone during courtship behaviour.

***Apis* / *Bombus* / *Xylocopa* / gland / ultrastructure / sex recognition**

1. INTRODUCTION

Chemical communication in the Hymenoptera plays a fundamental role in mediating the main behaviours of both solitary and social species (Gary, 1974; Ayasse et al., 2001). In social Hymenoptera numerous glands have been reported in different parts of the body, all of them producing a specific secretion which could be volatile (either of low or high volatility) or perceived through contact (Billen and Morgan, 1998). Among sensory appendages, the antennae are the most important for perceiving such signals, in that they are characterised by the highest concentration of sensillae, compared to the other body parts (Miller, 1972; Zacharuk, 1985). Several morphological and electrophysiological studies have been carried out in different Apoidea species, describing the antennae sensillar patterns in both males and females (Gupta, 1992; Wcislo, 1995; Ågren and Hallberg, 1996). In *Apis mellifera* L. several morphological studies have focused on the antennal sensilla of the different colony members (Richards, 1952; Slifer and Sekhon, 1961; Dietz and Humphreys, 1971), leading to mapping of the different number and type of sensilla in the three forms (Chauvin, 1968): the workers have about 6500 sensilla per antenna (Esslen and Kaissling, 1976), the queen, 2–3000 (Dade, 1962) while the drones have the highest number, about 20 000. This enormous difference among castes and sexes is mostly due to sensilla placodea which make up about 18 600 in drones (Brockmann and Brückner, 2001). Therefore all the male antennomeres, except for scape and pedicel, are almost completely covered by sensilla placodea which have an olfactory function. Despite these detailed

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ultrastructural investigations, no antennal structures other than sensilla have as yet been reported.

The discovery that apparent or inconspicuous structures in male antennae are the release sites of exocrine glands in several groups of Hymenoptera (Dahms, 1984; Bin and Vinson, 1986; Isidoro et al., 1996; Romani et al., 1999) poses the question whether the drones of honeybees are also equipped with such secretory structures. Here we investigated males of *A. mellifera* and two other Apoidea species, one social (*Bombus pascuorum* Scopoli) and one solitary (*Xylocopa violacea* L.) to determine the presence of antennal secretory structures.

2. MATERIALS AND METHODS

Male insects were obtained from local beekeepers (for *A. mellifera*) or were directly collected in the field in the vicinity of Perugia, Italy (*B. pascuorum* and *X. violacea*).

2.1. Light microscopy

For light microscopy observations, 10 males of the three species (*A. mellifera*, *B. pascuorum* and *X. violacea*) were anaesthetised in CO_2 and immediately immersed in 2.5% glutaraldehyde in 0.1M cacodylate buffer + 5% glucose, pH 7.2–7.3. Then each antennomere (ranging from A3 to A12) was detached, to help fixative penetration, and left at 4 °C for about 2 hours. After rinsing overnight in cacodylate buffer, the specimens were post-fixed in 1% OsO_4 at 4 °C for about 1 hour and rinsed in the same buffer. Dehydration in a graded ethanol series was then followed by embedding in an Epon-Araldite mixture, with propylene oxide used as bridging solvent. Semithin (1.5 µm) sections were taken with glass knives on a L.K.B.® "Nova" ultramicrotome, stained with 1% methylene blue and viewed using a phase-contrast Leitz® Dialux 20 EB photomicroscope.

2.2. Scanning electron microscopy

For SEM observations, the head of 10 males of each species were removed (after CO_2 anaesthesia) and immediately immersed in 50% ethanol-water solution. After dehydration in a graded ethanol series, the heads complete with antennae were critical point dried in a Balzers Union® CPD 020 unit, gold coated in a Balzers Union® SCD 040 unit, and then examined through SEM Philips® XL 30.

2.3. Transmission electron microscopy

For TEM observations, 10 males of each species were processed as already described for light microscopy. Thin (60–150 nm) sections were taken with a Diatome® diamond knife on a L.K.B.® "Nova" ultramicrotome and mounted on collodium-coated 50 mesh grids. The sections were investigated with a Philips EM 400 T after staining with 1.8% uranyl acetate distilled water solution (15 min, room temperature) and 6% lead citrate distilled water solution (5 min, room temperature).

3. RESULTS

In the three species observed, we found evident sexual dimorphism between the males' and females' antennae. In these species male antennae are of the geniculate type and made-up of 13 antennomeres, while the female antennae have 12 segments. The antennomeres were progressively numbered from the scape (A1), pedicel (A2) and so on. Because of the differences we found both on the external and internal morphological features of male antennae, the three species will be treated separately.

3.1. Apis mellifera

In this species, male antennae are longer than those of females and appear to be curved slightly outwards. SEM observations show indeed that the antennomeres ranging from A5 to A9 are characterized by a slight curvature and have a small area bearing scattered, non-innervated hairs and deprived of sensilla placodea (on the ventral side) (Fig. 1A, B). On A5 this area is much bigger (length about 400 µm, width about 50 µm, surface area about 20 000 μm^2) than in the remaining antennomeres, where it appears as a small triangular area, distally located (surface area about 4000 μm^2).

On A5, at the level of the area deprived of plate sensilla, the epithelium is thicker and consists of about 400 secretory units with a cuboidal form (Fig. 1C). On the apical part of each glandular cell, in direct contact with the cuticle, the plasma membrane forms many tight and rather deep microvilli (Fig. 1D), while on the basal part the cell is bounded by a

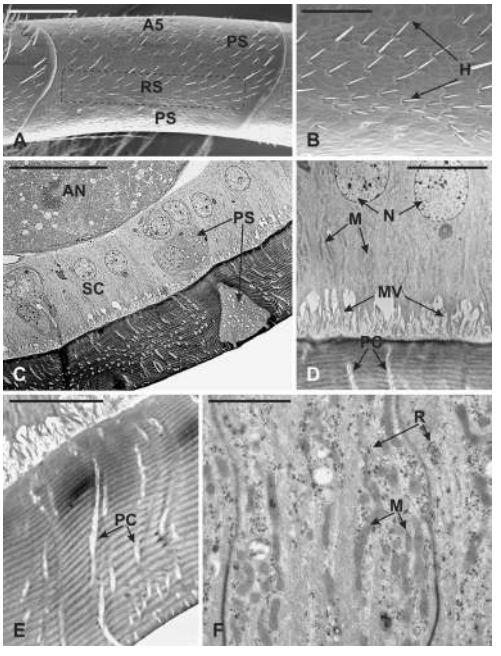

Figure 1. SEM micrographs of *Apis mellifera* drone 5th antennomere (A5) showing (in A) the release site of the glands (as shown inside the dashed rectangular box) surrounded by the plate sensilla and (in B) detail of the cuticle with evidence of the non-innervated hairs. TEM micrographs of A5 cross sections showing the thick glandular epithelium appressed to the cuticle (in C), details of the secretory cells (in D), particulars of the cuticle with the pore canals (in E) and cytological features of the secretory cells (in F). AN, antennal nerve; H, non-innervated hairs; M, mitochondria; MV, microvilli; N, nuclei; PC, pore canals; PS, plate sensilla; R, ribosomes; RS, release site; SC, secretory cells. Bar scale: A = 100 µm, B = 50 µm, C = 20 µm, D = 5 µm, E = 10 µm, F = 1 µm.

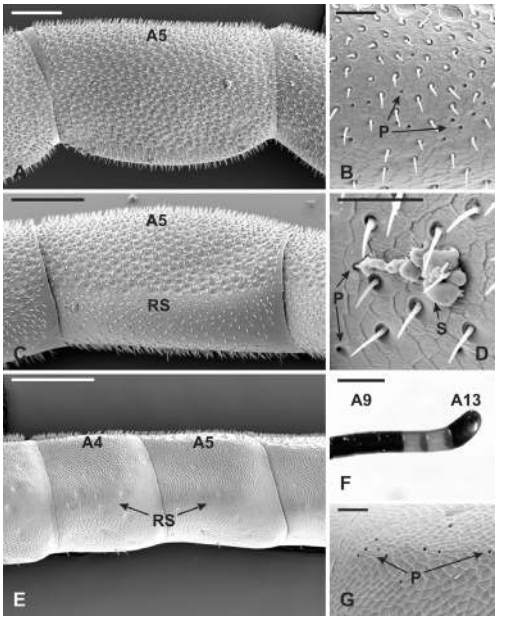

Figure 2. (A–D) SEM micrographs of *Bombus pascuorum* male A5 showing the release site in lateral (A) and ventral (C) view, the pores (P) characterising this area (B) and the secretion (S) produced by the glands (D). (E) SEM micrographs of male antenna in *Xylocopa violacea* showing the release site (RS) of glands; (F) stereomicroscope picture showing the distal half of the antenna, in which A11 and A12 are differently coloured; (G) SEM detail of the release site pores. Bar scale: A and C = 100 µm, B, D and G = 20 µm, E = 200 µm, F = 0.5 mm.

basement membrane. The cytoplasm contains a large ovoid nucleus, basally located, and numerous small elongated mitochondria dispersed throughout the cell (Fig. 1D, F). Golgi complexes are few and not well developed, while clusters of free ribosomes are abundant. The cuticle covering the glandular epithelium is traversed by distended pore canals which open in very small pores (about 35 nm in diameter) at the level of the external epicuticle allowing the passage of secretions (Fig. 1E).

3.2. Bombus pascuorum

In this species male antennae are curved, with antennomeres which appear to be subelliptical. The situation is different in the females where the antennae are straight with sub-cylindrical antennomeres. SEM observations show how male antennae are characterised, in their ventro-lateral side, by the presence of multiporous areas, resembling tyloids, which can be found from A3 to A13 (Fig. 2A, C). These areas are typically elevated with respect to the remaining antennomeres, and show the presence of numerous cuticular pores (about 0.5 µm in diameter) associated with relatively few hairs, which could be mechanoreceptors (Fig. 2B). Evidence of secretory activity is provided by curls

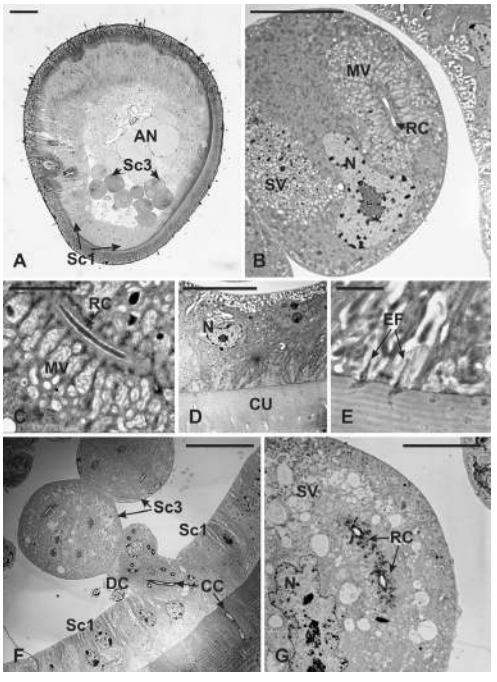

Figure 3. (A) Light microscopy cross section of *B. pascuorum* male antenna showing the arrangement of the two types of glands. (B–E) TEM of *B. pascuorum* male antenna highlighting the class 3 secretory cells (B–C) and those of class 1 (D–E). F–G: TEM of *X. violacea* male antenna with evidence of the two types of gland associated with the same release site and cytological features. AN, antennal nerve; CC, conducting canal; CU, cuticle; DC, duct cells; EF, epicuticular filaments; MV, microvilli; N, nuclei; RC, receiving canal; Sc1, class 1 secretory cells; Sc3, class 3 secretory cells; SV, secretory vesicles . Bar scale: A=0.1 mm, B, D and G = 10 µm, C and E = 2 µm, F = 25 µm.

of paste-like secretion oozing from the pores (Fig. 2D). Serial longitudinal and cross semithin and thin sections taken at the multiporous area level revealed the presence of two types of glands associated with the porous cuticle (Fig. 3A). The first gland type consists of several, isolated bicellular glandular units composed of an innermost secretory cell (producing the secretion) and an outermost duct cell (which produces the cuticular duct); hence, each bicellular unit is connected externally to a single pore. The secretory cells, which appear to have a globular shape, float in the antennal lumen and are provided with large nuclei generally located near the receiving canal (Fig. 3A, B). The cytoplasm typically presents organelles such as stacks of smooth endoplasmic reticulum and numerous ribosomes. In addition to numerous mitochondria, the cell has well developed Golgi complexes that produce large numbers of coated secretory vesicles containing dark secretory material, which in some cases completely fills the vesicles (Fig. 3B). The secretion gathers in the apical extracellular space before entering the elongated and convoluted receiving canal (Fig. 3B, C). The canal cell is characterised by reduced cytoplasm in which the conducting canal is embedded throughout its entire length. The elongated conducting canal forms many convolutions until it reaches the external cuticular pores.

The second gland type is made up of a single layer of cuboid cells, which are sandwiched between the cuticle and the secretory cells of the above described bicellular glands (Fig. 3A). This layer (about 0.1 mm thick), is composed of numerous adjacent secretory cells which are connected to the outside via very thin pore canals, through which the secretion is released by means of epicuticular filaments (Fig. 3D, E). These filaments extend from the inner cuticular wall (at the pore canal level) towards the secretory cells. In conclusion, in *B. pascuorum* the two types of glands share the same release site.

3.3. Xylocopa violacea

In males of this solitary species, the antennae appear quite different from the female's, being flattened dorso-ventrally in their distal half, whereas female antennae are cylindrical. The main external difference between sexes is the different colour shown by the antennomeres A11 and A12, which are reddish-brown compared with the rest of the antenna which are black (Fig. 2F). Furthermore, the apical antennomere (A13) is bent onward and shows an evident rounded outer margin (Fig. 2F). The antennomeres A3 to A10 are characterised on their ventro-lateral side by the lack of setae that cover most of the rest of the antennal surface (Fig. 2E). This glabrous region presents an elevated area perforated by several cuticular pores (Fig. 2G). The antennomeres differ in their pore distribution, with pores

being more concentrated (with a decreasing trend) on A3-A8, very few on A9-A10 and not present at all on A1-A2 and A11-A12. Ultrastructural investigations revealed a situation analogous to what we found in *B. pascuorum*; i.e., the presence of two types of glands sharing the same release site (Fig. 3F). The first one is composed of clusters of bicellular secretory units, the ultrastructural features of which are quite similar to those described in *B. pascuorum*, although the secretory vesicles (which accumulate in the vicinity of the receiving canal) appear to be electron lucid (Fig. 3G). The canal cells, which have a more evident cytoplasm, wrap around the conducting canal up to the thick cuticle (Fig. 3F). The other gland type completely covers the inner cuticular wall below the release site, and is composed of several unicellular secretory units arranged in a single layer (Fig. 3F).

4. DISCUSSION

Male antennal glands have been found and ultrastructurally characterised in several groups of Hymenoptera. With the exclusion of the Symphyta which have not yet been investigated, males of many species of the major hymenopteran groups, such as Terebrantia (Bin and Vinson, 1986; Bin et al., 1999a) and Aculeata (Felicioli et al., 1998; Isidoro et al., 2000; Romani et al., 2002; Scaramozzino et al., 1996), have glands on the antennomeres. A detailed list would include numerous superfamilies comprising parasitoids, gall makers, solitary and social wasps, and pollinators such as bees. In the three species we investigated we also found male antennal glands, with different external and internal morphology. Those of *A. mellifera* have secretory features that correspond to the class 1 gland type (Noirot and Quennedey, 1974, 1991; Quennedey, 1998), which can be easily overlooked during external observations due to the fact that the tiny pores of the release site cannot be seen through SEM investigation, a situation which has been observed in other hymenopterans (Romani et al., 1999; Pedata et al., 1995). In contrast, both *B. pascuorum* and *X. violacea* have apparent release sites easy detectable through SEM external observations, with antennal secretory units belonging both to class 1 and 3 (Noirot and Quennedey, 1974, 1991; Quennedey, 1998) and associated with the same release site. This evidence is reported here for the first time in the Apoidea. This new discovery, also of the honey bee drone, confirms that the presence of antennal glands in males is a trait common to diverse hymenopterans.

Mapping the secretory structures in hymenopteran antennae revealed that the number of glanded antennomeres can vary from one up to 17 (Isidoro et al., 1996). The glanded antennomeres themselves, or a portion of them, can be slightly or remarkably modified so that the shape can be indented, notched, incrassate, etc. The release sites can also vary remarkably in shape, size, location, texture and pilosity. For example, they can be located dorsally, ventrally or laterally, and have the form of a flattened area, a raised plate, a fluted peg, a sharp carina, shallow depression or a deep pit (Isidoro et al., 1996, 1999; Bin et al., 1999a). Some of these structures have been known for a long time and used as taxonomic characters without defining their function or misinterpreting it. At present, they constitute valuable biotaxonomic tools to help understand sex recognition and reproductive isolation on a physical and chemical basis. Regarding the function of the secretion, it has been shown in several cases of Terebrantia (Bin et al., 1988; Isidoro et al., 1999; Romani et al., 1997; Bin et al.,1999b) and in one case of Apoidea (Felicioli et al., 1998) that it mediates sex recognition, or possibly female receptivity or sedation. Intense or prolonged antennation between partners has been described during the precopulatory phase of many Hymenoptera and has been defined in various ways, such as stroking, whipping, etc. (Quicke et al., 1997). These various forms of antennation are associated with the release of a paste-like secretion which must be spread onto female gustatory receptors (Isidoro et al., 1996). Another form of antennation, occurring in *Osmia cornuta* Latr. which undertakes antennal fanning, i.e. without contact between the antennae, is likely associated with a volatile pheromone (Felicioli et al., 1998). In addition, antennation types would make it possible to predict the number and location of glanded antennomeres and vice versa. The

same functional hypothesis for the role of antennal sex pheromones could apply also to *B. pascuorum* and *X. violacea*, in light of existing behavioural studies on mating behaviour in *B. terrestris* which report the importance of male antennal contact with the female to induce receptivity (Djegham et al., 1994).

Our next step will be to undertake morphological investigations of other *Apis* species and other Apinae to have a comparative view of these antennal structures. These studies, possibly associated with improved behavioural observations on honey bee and/or other more easily recordable species, could confirm the existence of a courtship pheromone.

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Résumé – Les glandes antennaires chez les abeilles : des structures pour la communication sexuelle par les phéromones ? Le but de cette étude était de déterminer la présence des glandes antennaires chez les mâles de trois espèces d'Apidae, l'abeille domestique (*Apis mellifera* L.), le bourdon *Bombus pascuorum* Scopoli et l'abeille charpentière *Xylocopa violacea* L. À ce jour les glandes antennaires des mâles ont été décrites chez plusieurs groupes d'Hyménoptères (Cynipidae, Ichneumonidae, Scelionidae, Aphelinidae) et ont montré une répartition variable du nombre d'antennomères possédant des cellules glandulaires. Les sites d'émission peuvent être apparents et facilement visibles lors d'une observation externe au microscope électronique à balayage (comme par exemple les carina, tyloïdes, plaques élevées, les échancrures dentelées) ou bien discrets et difficiles à détecter. Les trois espèces d'abeilles mentionnées ont été étudiées par les techniques de microscopie optique, à balayage et électronique. Un dimorphisme sexuel est évident chez les trois espèces. Chez *A. mellifera*, les mâles ont des antennes légèrement courbées et une région dépourvue de sensilla placodea, en particulier dans les antennomères A5 à A9 (Fig. 1A, B). Les observations au microscope électronique à transmission ont montré la présence sous cette région d'un épithélium glandulaire composé d'une seule couche de cellules sécrétrices de classe 1, dont la sécrétion est évacuée par des pores cuticulaires (Fig. 1C–F). Chez *B. pascuorum* et *X. violacea* les antennes des mâles sont caractérisées par des sites d'émission manifestes ayant la forme de surface élevées localisées ventrolatéralement, respectivement sur les antennomères A3-A13 (Fig. 2A–D) et A3-A10 (Fig. 2E–G). Ces régions perforées correspondent sur le plan interne à deux types de glandes associées à un même site d'émission : l'un composé de cellules sécrétrices de classe 3 et l'autre de cellules sécrétrices de classe 1 (Fig. 3A–G). La sécrétion de ces glandes est émise sur la région perforée, où l'on peut également observer des sécrétions accumulées en forme de boucles. L'hypothèse fonctionnelle pour les sécrétions produites par les glandes antennaires des mâles chez ces Apidés est qu'elles agiraient comme phéromones de reconnaissance sexuelle ; ce qui a déjà été démontré chez des Hyménoptères Térébrants et chez un Aculéate. Des observations comportementales sont nécessaires pour confirmer cette hypothèse chez ces trois espèces et des recherches morphologiques devraient être menées chez d'autres espèces d'Apidés.

***Apis* / *bombus* / *Xylocopa* / glande / ultratructure / reconnaissance sexuelle**

Zusammenfassung – Antennendrüsen bei männlichen Bienen: Strukturen zur sexuellen Kommunikation mit Pheromonen? Das Vorhandensein von Drüsen auf Antennen von männlichen Bienen wurde bei drei Apisarten untersucht: bei der Honigbiene *Apis mellifera* L., der Hummel *Bombus pascuorum* Scopoli und der Holzbiene *Xylocopa violacea* L. Männliche Antennaldrüsen wurden bisher für mehrere Gruppen der Hymenopteren (Cynipidae, Ichneumonidae, Scelionidae, Aphelinidae) beschrieben, wobei sich eine unterschiedliche Verteilung der Anzahl von Drüsenzellen auf die Antennomere zeigte. Die Bereiche der Abgabe von Sekreten kann deutlich sein und leicht bei externer Betrachtung mit dem SEM erkannt werden (wie zum Beispiel als „carina, tyloid, herausgehobene Platten, Einkerbungen") oder auch unauffällig und schwer zu erkennen. Die oben erwähnten Arten wurden unter dem Licht-, Rasterelektronen- und dem Elektronenmikroskop untersucht. Bei allen drei Arten gab es einen deutlichen sexuellen Dimorphismus. Bei *A. mellifera* Drohnen sind die Antennen leicht gebogen und besonders bei den Antennomeren zwischen A5 bis A9, gibt es einen Bereich völlig ohne Sensilla placoidea (Abb. 1A, B). TEM Untersuchungen zeigten, dass sich unter diesem Bereich ein Drüsengewebe befindet, das sich aus einem einschichtigen Drüsengewebe mit Zellen der

Klasse 1 besteht, deren Sekret durch kutikuläre Poren nach außen abgegeben wird (Abb. 1C–F). Bei *B. pascuorum* und *X. violacea*, sind die männlichen Antennen durch deutliche Bereiche der Sekretabgabe charakterisiert, und zwar liegen sie erhöht im ventro-lateralen Bereich der Antennomere A3-A13 (Abb. 2A–D) und A3-A10 (Abb. 2E–G). Diese perforierten Bereiche sind im Inneren mit zwei unterschiedlichen Typen von Drüsen verbunden, die beide an der selben Stelle ihr Sekret ausscheiden. Ein Typ besteht aus sekretorischen Zellen der Klasse 3 und der andere aus der Klasse 1 (Abb. 3A–G). Das Sekret dieser Drüsen wird auf einen perforierten Bereich abgegeben, wo man es manchmal als wellenförmig akkumuliertes Sekret erkennen kann. Als funktionelle Hypothese für das von den Männchen dieser Bienen erzeugte Sekret wird angenommen, dass es als Sexpheromon wirken könnte, besonders unter Berücksichtigung, dass diese Hypothese bereits für Hymenopteren (Terebrantia und Aculeata) besteht. Verhaltensversuche sind nötig um diese Hypothese bei den hier untersuchten Arten zu testen, und außerdem sollten bei anderen Arten weitere morphologische Untersuchungen durchgeführt werden.

***Apis* / *Bombus* / *Xylocopa* / Drüsen / Ultrastruktur / Sexerkennung**

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