The Journal of Experimental Biology 210, 3601-3606
Published by The Company of Biologists 2007
doi:10.1242/jeb.009035

Male accessory glands of *Drosophila melanogaster* make a secreted angiotensin I-converting enzyme (ANCE), suggesting a role for the peptide-processing enzyme in seminal fluid

Caroline M. Rylett[1,*], Michael J. Walker[1,*], Gareth J. Howell[2], Alan D. Shirras[3] and R. Elwyn Isaac[1,†]

[1]*Institute of Integrative and Comparative Biology and* [2]*Institute of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK and* [3]*Department of Biological Sciences, Lancaster University, Lancaster, LA1 4YQ, UK*

*These authors contributed equally to this work

†Author for correspondence (e-mail: r.e.isaac@leeds.ac.uk)

Accepted 26 July 2007

Summary

Angiotensin I-converting enzyme (ACE) expressed on the surface of endothelial cells is responsible for the last step in the synthesis of circulating angiotensin II and the inactivation of bradykinin. Mammalian ACE is also expressed in the prostate with other components of the renin–angiotensin system, and in developing spermatids, where the peptidase activity is known to be critical for normal sperm function. The importance of an ACE gene to male fertility has also been demonstrated in *Drosophila melanogaster*, where *Ance* is expressed in spermatids, and hypomorphic alleles of *Ance* cause a defect in spermiogenesis. Here we show that ANCE, which shares many enzymatic properties with mammalian ACE, is also a product of the male accessory gland of *D. melanogaster*. It is expressed in the secondary cells and is associated with the electron dense granule within the large vesicles of these cells. ACE proteolytic activity is lost from the accessory glands during mating, consistent with transfer to the mated female in the seminal fluid. The accessory gland ACE-like activity might have an evolutionarily conserved function processing biologically active peptides with a role in male fertility.

Key words: *Drosophila,* male accessory gland, angiotensin I-converting enzyme, dicarboxypeptidase, ANCE.

Introduction

Peptidyl dipeptidase A (EC 3.4.15.1) is best known for its role in the mammalian renin–angiotensin system (RAS) as the enzyme responsible for the removal of a C-terminal dipeptide (His-Leu) from the decapeptide angiotensin I to generate the vasopressor octopeptide angiotensin II; hence its common name angiotensin I-converting enzyme (ACE) (Corvol et al., 2004). The enzyme also cleaves a dipeptide from the C terminus of the circulating vasodilator bradykinin, which inactivates this peptide hormone (Erdos, 1979). In mammals, there are two isoforms of ACE; a two-domain somatic protein and a smaller single domain germinal isoform that is found exclusively in male germ cells (Corvol et al., 2004). Although somatic ACE is mainly found on the surface of endothelial cells, soluble enzyme is found in body fluids, including blood, cerebrospinal fluid and seminal fluid (Corvol et al., 2004). The precise function of germinal ACE is not known, but mice deficient in germinal ACE are infertile (Hagaman et al., 1998). Somatic ACE and other components of the RAS are also found in male reproductive tissues (Leydig cells, seminiferous tubules, epididymis, testis and prostate gland) with the prostate being a likely source of the angiotensin II that has been found in human seminal fluid (Leung and Sernia, 2003; O'Mahony et al., 2005; O'Mahony et al., 2000). Angiotensin II might affect fertility in the human male because of its ability to stimulate sperm motility, induce the acrosome reaction and increase oocyte penetration (Kohn et al., 1997; Kohn et al., 1998; Vinson et al., 1996; Vinson et al., 1995). By contrast, the pronounced male infertility of mice lacking germinal ACE is independent of the RAS and probably results from failure to cleave a peptide distinct from angiotensin I (Fuchs et al., 2005).

ACE is an evolutionarily ancient dicarboxypeptidase found in a range of invertebrates (e.g. arthropods and annelids) and even in some bacteria (Rawlings et al., 2006). In insects, ACE is a soluble secreted enzyme that is strongly expressed in the male reproductive tissues (Isaac et al., 2007). In the testes of *Drosophila melanogaster*, germ cells are the major site of ACE (known as ANCE) biosynthesis and male flies homozygous for hypomorphic alleles of *Ance* are infertile (Tatei et al., 1995). This infertility results from a failure in spermiogenesis, suggesting that one of the functions of germinal ANCE in *D. melanogaster* is the processing of a regulatory peptide required for spermatid differentiation (Hurst et al., 2003). Another possible role for ANCE is in the processing of peptides in

seminal fluid, as has been proposed for human prostate ACE. In *D. melanogaster*, the male accessory glands (AG) are responsible for the production and secretion of a large number of proteins into the seminal fluid that mix with sperm on ejaculation (Ram and Wolfner, 2007). These include several peptide and protein hormones as well as enzymes, stress response proteins and immune defence proteins. The peptide/protein hormones are responsible for a variety of physiological and behavioural responses in the post-mated female, including increased rate of ovulation, loss of receptivity to males, improved sperm storage and increased appetite (Carvalho et al., 2006; Chapman and Davies, 2004).

We now show that in addition to expression in the testes, ANCE is also produced in the AG of adult male *D. melanogaster* and that the peptidase is localised to giant vesicles of the secondary cells. ACE-like peptidase activity of the AGs is reduced in the post-mated male, presumably as a result of transfer to the mated female of the AG products as part of the seminal fluid. We speculate that the AG ANCE functions to cleave the C terminus of peptides with a role in reproduction to alter their biological activity.

Materials and methods

Chemicals

Unless otherwise stated chemicals and immunological reagents were purchased from Sigma-Aldrich Co. Poole, Dorset, UK. The Vectastain ABC Kit and Vectashield were obtained from Vector Laboratories Ltd., Peterborough, UK. The Enhanced Chemiluminescence (ECL) Detection Kit was from Amersham Pharmacia Biotech Ltd., UK. The Gemini 5 μ C18 (150 mm×4.6 mm) high-performance liquid chromatography (HPLC) column was purchased from Phenomenex (Macclesfield, Cheshire, UK).

Insects

Wild-type *D. melanogaster* Meigen (Oregon R) were maintained on oatmeal–molasses–agar medium at 25°C (Ashburner and Thompson, 1978).

Immunohistochemistry and in situ hybridisation

Antibodies were raised against recombinant ANCE expressed in *Pichia pastoris* as described elsewhere (Houard et al., 1998). For immunohistochemistry, AGs were dissected in 10 mmol l^{-1} phosphate-buffered saline (PBS; Na_2PO_4–$NaHPO_4$ buffer, pH 7.4, 150 mmol l^{-1} NaCl) and treated with 2% (v/v) hydrogen peroxide in methanol for 5 min, then washed three times in PBS prior to fixation for 20 min in 4% (w/v) paraformaldehyde in PBS. Tissues were blocked in 1% (w/v) bovine serum albumin, 10% (v/v) normal goat serum in PBST [PBS, 0.03% (v/v) Triton X-100] for 1 h at 25°C before incubation in a 1:2000 dilution of primary antibody (either immune or pre-immune serum) in PBST overnight at 4°C. AGs were washed three times in PBST before treatment with the Vectastain ABC Kit according to the manufacturer's instructions. For immunofluorescence assays, AGs were dissected, fixed and incubated with primary antibody as described above. AGs were then incubated in a 1:2000 dilution of FITC-conjugated goat anti-rat IgG in PBST for 1 h at 25°C. After three washes in PBST, tissues were mounted in Vectashield and images were recorded using a Zeiss LSM510 META upright confocal microscope. Samples were excited with the 488 nm laser line of an argon laser running at 5% power output; and emission was collected with a long pass LP505 nm filter. 3D reconstruction and iso-surface rendering were performed in Imaris version 4.0.6 (Bitplane, Zurich, Switzerland) using contours within the Surpass module of the software.

In situ hybridisation experiments were carried out using *Ance* RNA probes according to the method described previously (Siviter et al., 2000); sense probes were used as a control.

Measurement of ACE activity with the substrate hippuryl-L-histidyl-L-leucine (Hip-His-Leu)

ACE activity was determined by incubating AG tissue or AG secretions that had been collected in 5 μl of PBS with the substrate solution (5 mmol l^{-1} Hip-His-Leu in 0.1 mol l^{-1} Tris–HCl, pH 8.3, 0.3 mol l^{-1} NaCl, 10 μmol l^{-1} $ZnSO_4$; total volume 20 μl). After 4 h at 35°C, the enzyme reaction was terminated by reducing the pH to 2.0 with the addition of 8% (v/v) trifluoracetic acid. The final volume was made up to 260 μl with 0.1% (v/v) trifluoracetic acid and the released hippuric acid was quantified by HPLC using a 5 μ C18 (150 mm×4.6 mm) column, as described previously (Lamango et al., 1996).

Immunoelectrophoresis

AG proteins from both Oregon R and *tudor* (*tud*) flies were extracted and separated on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) gel, transferred to a PVDF membrane, and then incubated with anti-ANCE antibody at a 1:5000 dilution in PBST, 5% (w/v) non-fat dried milk powder as described previously (Houard et al., 1998; Hurst et al., 2003). Bound anti-ANCE antibody was detected by using a horseradish peroxidase-conjugated sheep anti-rabbit Fc antibody and the Enhanced Chemiluminescence Detection Kit (Amersham Pharmacia Biotech Ltd., UK) as described in the manufacturer's instructions. *tud* mutant flies were used in addition to wild-type Oregon R because the *tud* males possess much lower levels of testicular ANCE, which might contaminate the protein preparation. Similar levels of ANCE were detected in AG extracts from the mutant and wild-type males, but only blots of the *tud* AGs are presented.

Electron microscopy

Male AGs were dissected from virgin *D. melanogaster* males of between 3 and 5 days old and fixed in 2.5% (v/v) gluteraldehyde in 0.1 mol l^{-1} Na_2PO_4–$NaHPO_4$ buffer, pH 6.9, for 3 h. Specimens were then washed twice in the buffer before post-fixation for 1 h in 1% (w/v) osmium tetroxide in 0.1 mol l^{-1} Na_2PO_4–$NaHPO_4$, pH 6.9. After washing in two changes of buffer, the specimens were dehydrated by using an ascending ethanol series (five concentrations ranging from 20–100%), each step taking 20 min. After one additional change of 100% ethanol, the specimens were embedded in Araldite (Luft, 1961). Sections (80–90 nm) were cut from the Araldite blocks using an Ultramicrotome (Reichert-Jung Ultracut-E, Leica, Milton Keynes, UK) and were stained with uranyl acetate and Reynolds' lead citrate solution (Reynolds, 1963). Sections were examined using a Jeol 1200EX transmission electron

microscope (Jeol UK Ltd, Welwyn Garden City, UK) at an 80 kV accelerating voltage.

Results

ACE activity in the male accessory gland

AGs were found to hydrolysed the ACE substrate (Hip-His-Leu) to release hippuric acid. The specificity of the enzyme assay was confirmed by the complete inhibition of the activity by captopril, a potent inhibitor of insect ACE (Cornell et al., 1995; Lamango et al., 1996). The distribution of the AG ACE was determined by gently compressing AGs from unmated males with forceps to discharge the luminal contents into PBS. A comparison of the ACE activity present in these secretions with the activity remaining in the compressed AGs showed that around 80% of the active enzyme was in the AG lumen (Table 1). When males were allowed to mate with ten females over a 3-day period, the ACE activity of the AGs was reduced by 70%, consistent with the enzyme being transferred during copulation from the AG to the female in the seminal fluid (Table 1).

Immunological confirmation of the presence of ANCE in male accessory glands

To establish the identity of the ACE-like activity found in the male AGs, antibodies that specifically recognise ANCE were employed in western blot analysis of AG proteins (Fig. 1). A single protein band (M_r, 72×10^3) of the expected size was detected with the ANCE antiserum, establishing ANCE as an AG product and the enzyme most likely to be responsible for the ACE-like activity. A second *D. melanogaster* ACE-like enzyme called ACER (ACE-related) can also cleave Hip-His-Leu in a captopril-sensitive manner and therefore ACER might also contribute towards the dicarboxypeptidase activity of the male AGs. However, ACER was not detected in western blots using antibodies raised to recombinant *D. melanogaster* ACER. We therefore conclude that ANCE, rather than ACER, is responsible for the ACE-like activity detected in the male AGs.

Immunohistochemistry revealed strong foci of ANCE protein within the secondary cells that are located in the distal third of the AG (Fig. 2). Confocal microscopy of AGs stained with FITC-labelled secondary antibody clearly identified large vesicles of the secondary cells as the subcellular site of the strong ANCE staining (Fig. 3A). The secondary cells generally contained five or six of these vesicles, some of which appeared to be devoid of ANCE. Electronmicroscopy of AG tissue sections showed that these large membrane-bound vesicles

Table 1. *Dicarboxypeptidase activity in the male Drosophila accessory gland*

Source of enzyme activity	Dicarboxypeptidase activity (pmol Hip formed h^{-1} AG^{-1})
AG minus contents	4.7±1.2
AG contents	17.9±3.9
Whole AG of sex-starved male	16.7±2.7
Whole AG of mated male	5.0±0.5

AG, accessory gland; Hip, hippuric acid.
Values are mean ± s.e.m. (*N*=3).

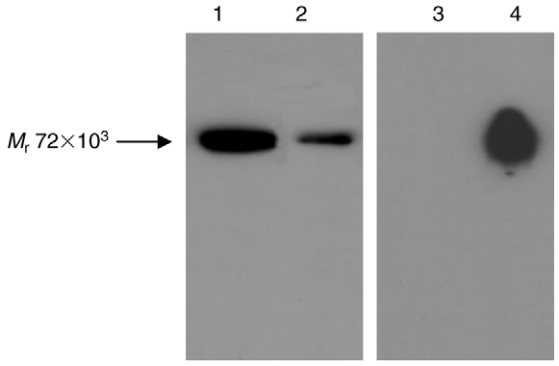

Fig. 1. Western blot analysis shows the presence of ANCE, but not ACER in male accessory glands (AGs). Western blots of male AGs using anti-ANCE antibodies (lanes 1 and 2) and anti-ACER antibodies (lanes 3 and 4). Lane 1, protein from 10 AGs; lane 2, 0.5 ng of recombinant ANCE; lane 3, protein from 10 AGs; lane 4, recombinant ACER, 2 ng.

contain a dense granule surrounded by a filamentous material (Fig. 3C), as noted elsewhere (Bairati, 1968). Comparison of the micrographs with the fluorescent immunocytochemistry images show clearly that ANCE is localised to the dense large granule of the secondary cell. Three dimensional reconstruction of the secondary cells using confocal microscopy found the volume of the vesicles to be on average 103±14 μm^3. Around half of this volume (54±15 μm^3) was taken up by the dense core granule (Fig. 3B).

In situ hybridisation confirms ANCE expression in the secondary cells

In situ hybridisation using digoxigenin-labelled antisense RNA probes for *Ance* revealed strong expression in the secondary cells of the gland and strong staining in the AG lumen

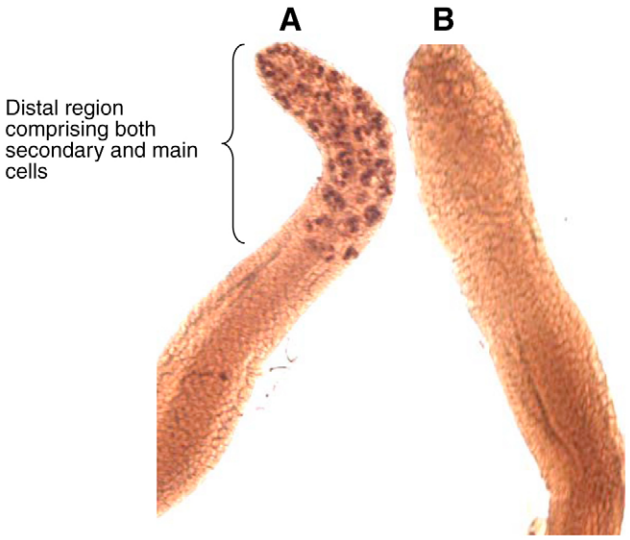

Fig. 2. (A) Immunohistochemical staining of ANCE in the secondary cells of the distal region of the male accessory gland using ANCE-specific primary antiserum and the ABC detection kit. (B) Control experiment using pre-immune serum.

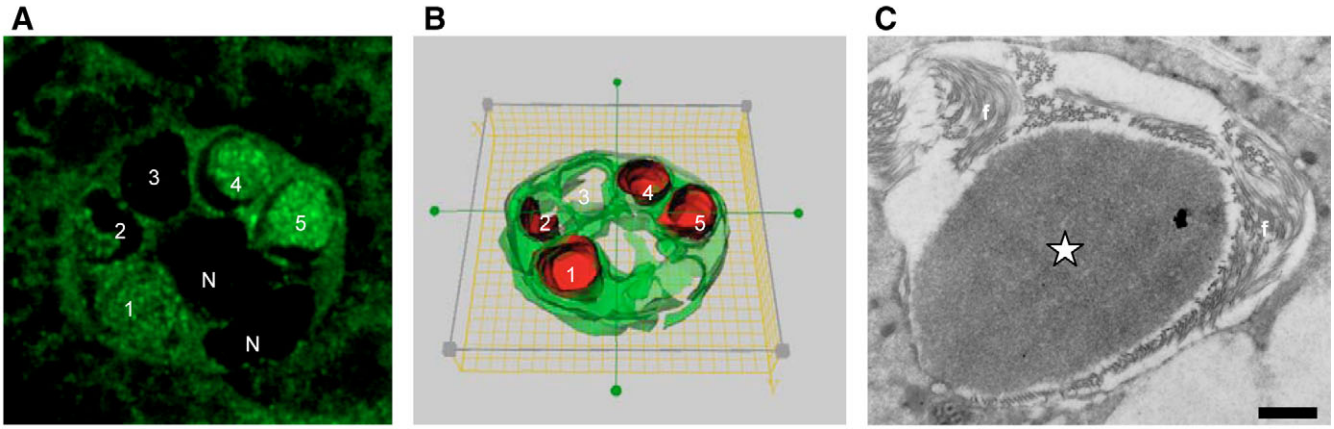

Fig. 3. ANCE is localised to the giant vesicles of the secondary cells of the male accessory gland (AG). (A) Immunofluorescence staining of ANCE in the secondary cells of the distal region of the male AG using ANCE-specific primary antiserum and FITC-conjugated secondary antibodies. Giant vesicles are numbered clockwise and the two nuclei (N) are labelled. (B) Three-dimensional representation of the vesicles (red) in the secondary cell (green) of the AG. Serial confocal sections were collected through the sample, with an optical depth of 1 μm. One of these sections is shown. (C) Electron micrograph of a giant vesicle of an AG secondary cell showing the dense core material (star) and filaments (f). Scale bar, 2 μm.

(Fig. 4). In contrast, the level of staining in the main cells did not appear to be significantly stronger than that obtained using control sense probes.

Discussion

In this study we have shown that a soluble ACE-like peptidase is synthesised by the AGs of male *D. melanogaster* and that the enzyme activity is lost after mating, presumably by transfer to the female in the seminal fluid. A similar transfer of AG ACE occurs in the tomato moth, *Lacanobia oleracea*, where the enzyme ends up in the female spermatheca and bursa copulatrix (Ekbote et al., 2003). It was not possible to follow the fate of the *D. melanogaster* AG ANCE, since the enzyme was also expressed in the female reproductive tissues (R. E. Isaac, unpublished). There are six ACE-like genes in the *D. melanogaster* genome, but only *Ance* and *Acer* products are considered to be enzymatically active; the other gene products are predicted to be proteolytically inactive as they are missing key active site residues (Coates et al., 2000). Using antibodies that differentiate between ANCE and ACER, we have shown that the AG dicarboxypeptidase activity can be attributed to ANCE and not ACER. ANCE is expressed in the secondary cells of the AG and becomes incorporated into the dense granular mass that half-fills the giant vesicles of these cells. This is the first identification of a stored component of this unusual organelle. *In situ* hybridisation using an *Ance* riboprobe was consistent with relatively strong expression in the secondary cells. It has been suggested previously that the secondary cells of *D. melanogaster* AGs discharge their contents by a holocrine secretory mechanism (Chen, 1984). This mode of secretion might explain our observation of *Ance* RNA in the AG lumen.

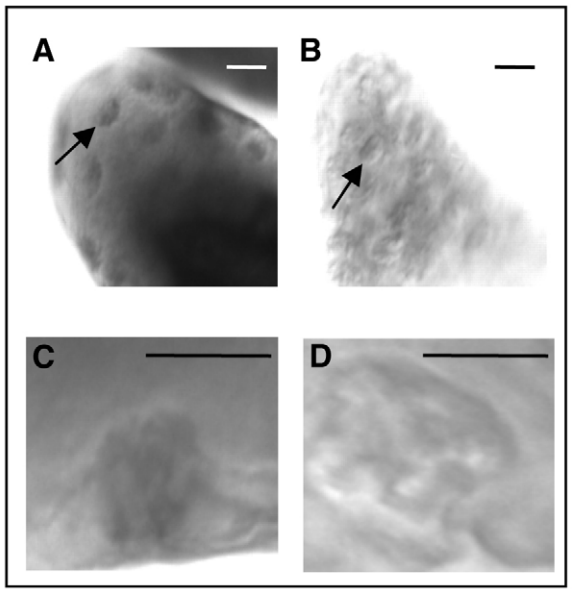

Fig. 4. *Ance* is transcribed in the secondary cells of the male accessory gland (AG). *In situ* hybridisation using digoxigenin-labelled *Ance* antisense riboprobe (A,C) and sense strand control (B,D) reveals higher levels of mRNA in the large secondary cells (arrow points to one of several secondary cells in the field of view). C and D are higher magnification images of secondary cells and AG lumen. Powerpoint was used to manipulate all the images equally to increase contrast. Scale bars, 10 μm.

ANCE is just one of several secreted peptidases produced by the AGs of *D. melanogaster*. In total, eleven peptidases including aminopeptidases, endopeptidases and a γ-glutamyl transpeptidase have already been identified as AG products in *D. melanogaster* (Mueller et al., 2004; Walker et al., 2006). One of these peptidases, an astacin-like endopeptidase, is involved in the cleavage of the male AG ovulin in the female reproductive tract to produce four products, two of which stimulate ovulation in the first 24 h post-mating (Ravi Ram et al., 2006). ANCE might work in concert with other peptidases to process prohormone polypeptides to biologically active peptides in a similar manner to the local RAS of the mammalian prostate (Leung and Sernia, 2003; O'Mahony et al., 2005). The best known biologically active peptide of the male reproductive

tissues is the sex peptide, which stimulates egg-laying and reduces receptivity to males in the mated female (Kubli, 2003). However, ANCE is unlikely to be involved in its biosynthesis because the C terminus of the proprotein does not undergo proteolytic processing. In contrast, Dup99B, a peptide made by the male ejaculatory duct, is a potential substrate for ANCE. The mature Dup99B, the C-terminal amino acid sequence of which is almost identical to the sex peptide, elicits the same responses in the post-mated female as the sex peptide when the peptide is injected into virgin female *D. melanogaster* (Rexhepaj et al., 2003; Saudan et al., 2002). Unlike the sex peptide, the DUP99B proprotein has a basic dipeptide (Arg-Lys) at the C terminus, which must be proteolytically removed to expose the C-terminal cysteine. We have shown previously that peptides with pairs of basic residues at the C terminus make excellent ANCE substrates (Isaac et al., 1998) and it is possible, therefore, that ANCE has a role in cleaving the Arg-Lys dipeptide from the C terminus of a partially processed DUP99B precursor as the AG products mix with the material secreted by the ejaculatory.

We cannot, at present, reach any conclusions regarding the relative importance of the AG and germinal ANCE for male fertility in *D. melanogaster*. This level of understanding will require tissue-specific knock-down of *Ance* expression.

List of abbreviations

ANCE	*Drosophila* Angiotensin converting enzyme
ACER	*Drosophila* Angiotensin-converting enzyme-related
ACE	Angiotensin 1 converting enzyme
AG	accessory gland
RAS	renin-angiotensin system

We thank Adrian Hick, University of Leeds, for assistance with electron microscopy, Pam Gaunt and Carol Sowden for technical support and the laboratory of Pierre Corvol, College de France, for recombinant ANCE and ACER. We also acknowledge the Biotechnology and Biological Research Council for providing studentships to M.W. and C.R. and for supporting the Leeds Bioimaging Facility.

References

Ashburner, M. and Thompson, J. N. (1978). The laboratory culture of *Drosophila*. In *The Genetics and Biology of Drosophila*. Vol. 2a (ed. M. Ashburner and T. R. F. Wright), pp. 1-109. London: Academic Press.

Bairati, A. (1968). Structure and ultrastructure of the male reproductive system in *Drosophila melanogaster* Meig. 2. The genital duct and accessory glands. *Monit. Zool. Ital.* **2**, 105-182.

Carvalho, G. B., Kapahi, P., Anderson, D. J. and Benzer, S. (2006). Allocrine modulation of feeding behavior by the sex peptide of *Drosophila*. *Curr. Biol.* **16**, 692-696.

Chapman, T. and Davies, S. J. (2004). Functions and analysis of the seminal fluid proteins of male *Drosophila melanogaster* fruit flies. *Peptides* **25**, 1477-1490.

Chen, P. S. (1984). The functional morphology and biochemistry of insect male accessory glands and their secretions. *Annu. Rev. Entomol.* **29**, 233-255.

Coates, D., Isaac, R. E., Cotton, J., Siviter, R., Williams, T. A., Shirras, A., Corvol, P. and Dive, V. (2000). Functional conservation of the active sites of human and *Drosophila* angiotensin I-converting enzyme. *Biochemistry* **39**, 8963-8969.

Cornell, M. J., Williams, T. A., Lamango, N. S., Coates, D., Corvol, P., Soubrier, F., Hoheisel, J., Lehrach, H. and Isaac, R. E. (1995). Cloning and expression of an evolutionary conserved single-domain angiotensin-converting enzyme from *Drosophila melanogaster*. *J. Biol. Chem.* **270**, 13613-13619.

Corvol, P., Eyries, M. and Soubrier, F. (2004). Peptidyl-dipeptidase A/angiotensin I-converting enzyme. In *Handbook of Proteolytic Enzymes*. Vol. 1 (ed. A. J. Barrett, N. D. Rawlings and J. F. Woessner), pp. 332-346. London: Elsevier Academic Press.

Ekbote, U. V., Weaver, R. J. and Isaac, R. E. (2003). Angiotensin I-converting enzyme (ACE) activity of the tomato moth, Lacanobia oleracea: changes in levels of activity during development and after copulation suggest roles during metamorphosis and reproduction. *Insect Biochem. Mol. Biol.* **33**, 989-998.

Erdos, E. G. (1979). Kininases. In *Bradykinin, Kallidin and Kallikrein*. Vol. 25 (ed. E. G. Erdos), pp. 428-487. Berlin, Heidelberg, New York: Springer-Verlag.

Fuchs, S., Frenzel, K., Hubert, C., Lyng, R., Muller, L., Michaud, A., Xiao, H. D., Adams, J. W., Capecchi, M. R., Corvol, P. et al. (2005). Male fertility is dependent on dipeptidase activity of testis ACE. *Nat. Med.* **11**, 1140-1142; author reply 1142-1143.

Hagaman, J. R., Moyer, J. S., Bachman, E. S., Sibony, M., Magyar, P. L., Welch, J. E., Smithies, O., Krege, J. H. and Obrien, D. A. (1998). Angiotensin-converting enzyme and male fertility. *Proc. Natl. Acad. Sci. USA* **95**, 2552-2557.

Houard, X., Williams, T. A., Michaud, A., Dani, P., Isaac, R. E., Shirras, A. D., Coates, D. and Corvol, P. (1998). The *Drosophila melanogaster*-related angiotensin-I-converting enzymes Acer and Ance – distinct enzymic characteristics and alternative expression during pupal development. *Eur. J. Biochem.* **257**, 599-606.

Hurst, D., Rylett, C. M., Isaac, R. E. and Shirras, A. D. (2003). The *Drosophila* angiotensin-converting enzyme homologue Ance is required for spermiogenesis. *Dev. Biol.* **254**, 238-247.

Isaac, R. E., Schoofs, L., Williams, T. A., Veelaert, D., Sajid, M., Corvol, P. and Coates, D. (1998). A novel peptide-processing activity of insect peptidyl-dipeptidase A (angiotensin I-converting enzyme): the hydrolysis of lysyl-arginine and arginyl-arginine from the C-terminus of an insect prohormone peptide [Full text delivery]. *Biochem. J.* **330**, 61-65.

Isaac, R. E., Lamango, N. S., Ekbote, U., Taylor, C. A., Hurst, D., Weaver, R. J., Carhan, A., Burnham, S. and Shirras, A. D. (2007). Angiotensin-converting enzyme as a target for the development of novel insect growth regulators. *Peptides* **28**, 153-162.

Kohn, F. M., Henkel, R., Eberl, M. and Schill, W. B. (1997). Inhibition of angiotensin converting enzyme (ACE) by captopril reduces human sperm binding to the plasma membrane of zona-free hamster oocytes. *Reprod. Domest. Anim.* **32**, 11.

Kohn, F. M., Muller, C., Drescher, D., Neukamm, C., el Mulla, K. F., Henkel, R., Hagele, W., Hinsch, E., Habenicht, U. F. and Schill, W. B. (1998). Effect of angiotensin converting enzyme (ACE) and angiotensins on human sperm functions. *Andrologia* **30**, 207-215.

Kubli, E. (2003). Sex-peptides: seminal peptides of the *Drosophila* male. *Cell. Mol. Life Sci.* **60**, 1689-1704.

Lamango, N. S., Sajid, M. and Isaac, R. E. (1996). Purification and properties of an angiotensin-converting enzyme from the housefly, *Musca domestica*. *Biochem. J.* **314**, 639-646.

Leung, P. S. and Sernia, C. (2003). The renin-angiotensin system and male reproduction: new functions for old hormones. *J. Mol. Endocrinol.* **30**, 263-270.

Luft, J. H. (1961). Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**, 409-414.

Mueller, J. L., Ripoll, D. R., Aquadro, C. F. and Wolfner, M. F. (2004). Comparative structural modeling and inference of conserved protein classes in *Drosophila* seminal fluid. *Proc. Natl. Acad. Sci. USA* **101**, 13542-13547.

O'Mahony, O. A., Djahanbahkch, O., Mahmood, T., Puddefoot, J. R. and Vinson, G. P. (2000). Angiotensin II in human seminal fluid. *Hum. Reprod.* **15**, 1345-1349.

O'Mahony, O. A., Barker, S., Puddefoot, J. R. and Vinson, G. P. (2005). Synthesis and secretion of angiotensin II by the prostate gland *in vitro*. *Endocrinology* **146**, 392-398.

Ram, R. K. and Wolfner, M. F. (2007). Seminal influences: *Drosophila* Acps and the molecular interplay between males and females during reproduction. *Integr. Comp. Biol.* doi:10.1093/icb/icm046.

Ravi Ram, K., Sirot, L. K. and Wolfner, M. F. (2006). Predicted seminal astacin-like protease is required for processing of reproductive proteins in Drosophila melanogaster. *Proc. Natl. Acad. Sci. USA* **103**, 18674-18679.

Rawlings, N. D., Morton, F. R. and Barrett, A. J. (2006). MEROPS: the peptidase database. *Nucleic Acids Res.* **34**, D270-D272.

Rexhepaj, A., Liu, H., Peng, J., Choffat, Y. and Kubli, E. (2003). The sex-peptide DUP99B is expressed in the male ejaculatory duct and in the cardia of both sexes. *Eur. J. Biochem.* **270**, 4306-4314.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208-212.

Saudan, P., Hauck, K., Soller, M., Choffat, Y., Ottiger, M., Sporri, M.,

Ding, Z., Hess, D., Gehrig, P. M., Klauser, S. et al. (2002). Ductus ejaculatorius peptide 99B (DUP99B), a novel *Drosophila melanogaster* sex-peptide pheromone. *Eur. J. Biochem.* **269**, 989-997.

Siviter, R. J., Coast, G. M., Winther, A. M., Nachman, R. J., Taylor, C. A., Shirras, A. D., Coates, D., Isaac, R. E. and Nassel, D. R. (2000). Expression and functional characterization of a *Drosophila* neuropeptide precursor with homology to mammalian preprotachykinin A. *J. Biol. Chem.* **275**, 23273-23280.

Tatei, K., Cai, H., Ip, Y. T. and Levine, M. (1995). Race – a *Drosophila* homolog of the angiotensin-converting enzyme. *Mech. Dev.* **51**, 157-168.

Vinson, G. P., Puddefoot, J. R., Ho, M. M., Barker, S., Mehta, J., Saridogan, E. and Djahanbakhch, O. (1995). Type 1 angiotensin II receptors in rat and human sperm. *J. Endocrinol.* **144**, 369-378.

Vinson, G. P., Mehta, J., Evans, S., Matthews, S., Puddefoot, J. R., Saridogan, E., Holt, W. V. and Djahanbakhch, O. (1996). Angiotensin II stimulates sperm motility. *Regul. Pept.* **67**, 131-135.

Walker, M. J., Rylett, C. M., Keen, J. N., Audsley, N., Sajid, M., Shirras, A. D. and Isaac, R. E. (2006). Proteomic identification of *Drosophila melanogaster* male accessory gland proteins, including a pro-cathepsin and a soluble gamma-glutamyl transpeptidase. *Proteome Sci.* **4**, 9.