Received: 10 January 2022 | Accepted: 5 May 2022

DOI: 10.1111/imb.12783

COMMENTARY

Insect Molecular Biology

Royal Entomological Society

On how to identify a seminal fluid protein: A commentary on Hurtado et al.

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Seminal fluid proteins (Sfps) are transferred along with sperm to females at mating. Their striking effects on the behaviour and physiology of females in many insects, and their interesting evolutionary dynamics—with some Sfps evolving rapidly (Haerty et al., 2007; Patlar et al., 2021; Swanson et al., 2001; Wagstaff & Begun, 2007) and others being more conserved (Findlay et al., 2014; Kelleher et al., 2009; McGeary & Findlay, 2020; Wigby et al., 2020)—have attracted considerable attention from biologists (Avila et al., 2011). However, the study of Sfps across insects is hampered by the small size of many species, which means that ejaculates often cannot be collected externally in the same way as they can in many vertebrate—or some larger invertebrate—systems.

In the model insect *Drosophila melanogaster*, Sfps have been identified through several methods (reviewed in Avila et al., 2011). Recently, Hurtado et al. published an updated review of the methods, and proposed modified criteria for Sfps that led them to generate a list of such proteins (Hurtado et al., 2021). This list differs in substantial respects from a comprehensive Sfp list that we previously published (Wigby et al., 2020). The list used can greatly influence the conclusion one makes in evolutionary analyses and future functional genetic studies of Sfps in *D. melanogaster* and other species. Therefore, the field needs an unbiased and thorough Sfp database. As such, we think it important to discuss the criteria proposed by Hurtado et al.

We agree with Hurtado et al. that there is a core set of especially well-established Sfps (termed 'Known Seminal Genes', KSGs, in Hurtado et al.) for which there is near unequivocal evidence of transfer from males to females at mating, eg, from studies using antibodies (eg, Bertram et al., 1996; Findlay et al., 2014; LaFlamme et al., 2012, 2014; Lung et al., 2002; Lung & Wolfner, 1999; Monsma et al., 1990; Ravi Ram et al., 2005; Sepil et al., 2020; Singh et al., 2018; Sirot et al., 2009; Sirot, Wolfner, & Wigby, 2011; Wigby et al., 2009; Wong et al., 2008), isotopic labelling (Findlay et al., 2008; McCullough et al., 2022; McDonough-Goldstein et al., 2022) or semi-quantitative proteomics (Sepil et al., 2019). These protein-focused methods currently represent the 'gold standard' in the field for Sfp identification.

We also agree with Hurtado et al. that there are likely many more Sfps beyond the KSGs, and that currently these candidate Sfps are based on less direct evidence. However, while we recognize that assessing the evidence for candidate Sfps in the absence of direct protein identification is somewhat subjective, we caution against the criteria used by Hurtado et al. (2021). Their additional Sfp candidates are limited to genes that show high and exclusive expression in the male accessory glands in data from FlyAtlas and modENCODE (Brown et al., 2014; Leader et al., 2018). Such genes had previously been referred to as 'Acps', for 'Accessory Gland Proteins' (Wolfner, 1997). Hurtado et al. further applied in silico analysis to predict from nucleic acid sequence data proteins that had signal peptides and extracellular activity. Knowledge of which genes are highly expressed in the accessory glands and, their predicted behaviour, is valuable: eg, it can form part of the toolkit for identifying new Sfps, and it may be useful for functional or evolutionary studies of accessory gland tissues, or, more broadly, sex-biased gene expression. However, many known Sfps do not conform to one or more of the expression or sequence criteria used in Hurtado et al.'s paper. For example, some *D. melanogaster* Sfps are not highly or exclusively expressed in the accessory glands (see figs S2 and S5 in Wigby et al., 2020, and table S1 of Hurtado et al., 2021)—some derive from the ejaculatory duct, ejaculatory bulb and/or the testes (Avila et al., 2015; Bretman et al., 2010; Cavener, 1985; Findlay et al., 2008; Iida & Cavener, 2004; Ludwig et al., 1991; Lung & Wolfner, 2001; Rexhepaj et al., 2003; Richmond et al., 1980; Sepil et al., 2019; Takemori & Yamamoto, 2009), some are additionally expressed in females (Findlay et al., 2008; Sepil et al., 2019), and a recent proteomic study found that 67 Sfps that are expressed at high levels in Sfp-producing glands are additionally expressed in testes (McCullough et al., 2022). Moreover, the reproductive tissue that expresses a particular Sfp gene can change over evolutionary time (Cavener, 1985; Sirot et al., 2014). Almost a third of *D. melanogaster's* KSGs (52/174) would be excluded using the expression cut-offs applied by Hurtado et al. Finally, it is worth noting that in silico signal peptide and extracellular predictions are imperfect in predicting whether or not proteins are secreted, for a variety of mechanistic reasons, including occurrences of unconventional protein secretion (Corrigan et al., 2014; Monteleone et al., 2015; Rabouille, 2017).

By using different criteria, Hurtado et al. (2021) and we (Wigby et al., 2020) arrived at different extended lists of 'high confidence' candidate Sfps. Both lists share the 174 KSGs, but they diverge beyond that, leading to tallies of 220 and 294 Sfps respectively.

Hurtado et al.'s criteria exclude a number of proteins that are arguably good Sfp candidates. For example, although Obp8a and Glaz proteins have not yet been detected in females after mating, both proteins are detected in male accessory glands; they are found in higher abundance in the accessory glands of virgin, as compared to mated, males (consistent with transfer to females); they have predicted signal peptides and extracellular activity, and they are accessory gland biased in expression. However, by failing to reach arbitrary expression cut-offs, they are not included in Hurtado et al.'s list. *D. melanogaster* Sfps have a dynamic range in abundance of $\sim 10^3$ (Findlay et al., 2008) which suggests that a high expression cut-off will exclude many true Sfps. Instead, 46 highly expressed accessory gland genes are included in Hurtado et al.'s extended list, for which there is no additional evidence of protein production or dynamics ('unconfirmed high confidence candidates' [UHCCs]). It is debatable whether these UHCCs represent more robust Sfp candidates than at least some of those listed in Wigby et al. (2020) but excluded by Hurtado et al. (2021).

By making exclusive and high accessory gland gene expression requirements for UHCCs, the Hurtado et al. Sfp list also risks biases in further analyses. Sfps that are additionally expressed in other parts of the male, or the female, and which thus may have additional functions (including non-reproductive), are excluded from being considered as UHCCs. This becomes problematic for evolutionary analyses of Sfps of the type performed by Hurtado et al. Genes with multiple pleiotropic functions may be under evolutionary constraint driven by some but not all of their functions (Hoffmann, 2013; Meisel, 2011; Parsch & Ellegren, 2013). By selectively removing these potentially more constrained genes in favour of accessory gland-specific (and presumably reproduction-specific) genes, Hurtado et al. risk predicting artificially high Sfp gene turnover across species. Biases in Sfp datasets may also compromise the efficiency and precision of future functional studies aimed at understanding the role of Sfps and their composition in fertility, because all such approaches rely on the accuracy of the Sfp list.

In summary, though a valid, and historically important, way to identify some Sfps, the use of high and exclusive accessory gland expression and predicted signal sequences as sole criteria for Sfps, as in Hurtado et al., excludes Sfps that are expressed at lower levels and in locations other than male accessory glands (including expression in both sexes) and Sfps that are secreted by non-standard mechanisms (Avila et al., 2011; Bertram et al., 1996; Boes et al., 2014; Cavener, 1985; Findlay et al., 2008; Lung & Wolfner, 2001; Sepil et al., 2019; Sirot, Hardstone, et al., 2011). We believe that it is important to include all of these categories in comprehensive studies of Sfp function and evolution.

ACKNOWLEDGEMENTS

The authors would like to thank the editors for allowing us to publish this commentary, Geoff Findlay for comments on an early version, Ben Hopkins for discussion, and Steve Dorus and an anonymous referee for reviews that improved the manuscript. SW was funded by a BBSRC grant (BB/V015249/1). IS was funded by a BBSRC fellowship (BB/T008881/1). NCB was supported by NIH/NICHD grant R01-HD059060 (to A.G. Clark and MFW); MFW thanks that grant and NIH/NICHD grant R37-HD038921 for support.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

No new data presented.

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How to cite this article: Wigby, S., Brown, N.C., Sepil, I. & Wolfner, M.F. (2022) On how to identify a seminal fluid protein: A commentary on Hurtado et al. *Insect Molecular Biology*, 1–4. Available from: https://doi.org/10.1111/imb.12783