Glyn Davies
DARG
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REPORT ON THE CONTINUED DRONE-LAYING QUEEN PROJECT 2016-2018

Introduction

This work was undertaken to expand the study undertaken jointly by Devon Apicultural Research Group (DARG) and Devon Beekeepers Association (DBKA) in 2012 which was subsequently awarded the international Vita Award in 2016.

The original hypothesis was based on recent observations by DARG members and others that the frequency of queens becoming partly or wholly drone layers was increasing and that they were doing so at a young age, often within months of starting to lay or after their first winter. Understanding of their multiple mating behaviour and the increasing viral load of honeybee colonies following the endemic establishment of *Varroa destructor* suggested possible links. In addition, DARG had established close collaboration with Dr Declan Schroeder, Senior researcher and virologist at Marine Biological Association Laboratory, Plymouth (MBA). Dr Schroeder 's survey of virus loads in Devon bee colonies in 2012 revealed an unsuspected and concerning 98% of colonies infected by Deformed Wing Virus (DWV). In addition, Professor Steven Martin, when at NBU, had confirmed that preferentially varroa selected drone brood for their own reproductive cycle. This would inevitably increase the frequency of drones infected by DWV.

A series of international studies and publications confirmed that DWV can be found in the reproductive organs of drones and queens. In addition it was confirmed that DWV can be transmitted from drones to queens during mating. The Log-confirmed promiscuity of queens and the occurrence of drone congregation for effective and rapid multiple mating has resulted in DWV being a certain and inevitable STI (sexually transmitted infection). This is in addition to other established paths of infection via trophyllaxis from an infected bee to a

colony sister, to growing larvae, or to the direct transmission of the virus from infected varroa into the haemolymph of a host bee, adult or larva.

But how could this viral infection be a cause of the increased incidence of excessive or total drone laying in young queens? Some of the many fascinating features of honeybee reproduction is the amazing fact that on a single summer afternoon and in less than 20 minutes of multiple copulations, a queen retains only around 10% of her inseminated sperm. This vital package is then stored in her spermatheca. There the sperm is kept alive and active for four or even occasionally, five years. Truly amazing and we have no detailed information how that is achieved. (But see ref Wolfner 2011) (https://doi.org/10.1371/journal.pbio.1001191) The spermatheca must be an exceptionally sensitive, stable and efficient organ. It is also very delicate and able to feed, oxygenate and keep healthy the millions of stored sperm. The tiny spherical wall is just one cell thick. Its functions and structure are at the same time, simple in appearance and inevitably very complicated in function. (Like a violin in the hands of a virtuoso - a wooden box with four strings which can create organised sound beyond comprehension and for hundreds of years! But the living virtuoso has to change - not a complete analogy.). Could DWV interfere with the spermathecal caring functions if a queen receives contaminated semen? Is there a quantity issue? How many of her multiple partners have to be infected for her to be seriously affected? Is this why many queens are labelled "poor layers" or "partial" drone layers having been infected by just a few of her partners?

During the first DLQ in 2012 study we looked at around 30 queens that were donated as they were problematic to their keeper. Critically, we had no healthy normal laying queens donated. Unsurprisingly. So we had no comparisons or controls. We wanted to see if there was pathology visible in the spermatheca of DLQ s. We saw very little except that in the very delicate cells of the epithelium wall some cell nuclei lacked solidity. Spaces occurred within the nucleus material. The DNA . The proportion various cells affected varied considerably. A very dedicated colleague, Reg Godwin, counted every cell and noted the apparently nuclear damaged ones which he termed having "vacuolated" nuclei. The details of this study are summarised in a poster prepared for the 2015 BBKA Spring Convention. It is available on the DARG website as a downloadable pdf file and included in this report. So the basic hypothesis still untested was that deformed wing virus, transmitted during mating, can later impair the functioning of the queen's spermatheca so that effective fertilisation of her eggs is not possible and she becomes a to a greater or lesser extent a drone laying queen.

With so many unanswered questions and omissions from the first study DARG decided that a second survey of queens should be undertaken. We had some money left from the generous donations given to the first project that would help. In addition we obtained the willing support from Abbey Veterinary Services, Newton Abbot who agreed to prepare slides from around 100 spermatheca from the collected queens. Twenty healthy queens were donated by commercial Ged Marshall. They were over two years old, laying well for their age but were being routinely replaced in Ged's requeening programme. Also in the intervening years, Declan Schroeder's work on DWV had progressed considerably. So we agreed that it would be interesting to compare the virus load of each of our donated and examined bees and so compare this with the apparent damage to the spermathecal wall and the reproductive health of each queen.

It was decided that it would be easiest to have contributed queens fixed and preserved locally and a protocol was circulated to experienced volunteer collectors who would be able to verify the queen's condition and quickly kill and preserve her. A brief history was recorded for each one and the specimen queen allocated an identity number. A glutaraldeyde solution was used as a preservative as the previous study found this safer and more quickly penetrated the queen tissues than traditional formalin. (In fact as will be reported later, maybe this was not a wise decision)

Dr Schroeder asked that the head and thorax of each queen be carefully removed, identified and placed in biological ethanol to be later analysed for viral infection. Glyn Davies carried out the removal of these organs and also the spermatheca which was placed in fresh glutaraldehyde. During this dissection, attempt was made to avoid viral cross contamination from one specimen queen to the next. Each dissection used a new disposable scalpel blade, supplemented by a new disposable cocktail stick (a very useful dissecting tool). The dissection was carried out on a cleaned cedar wood block wrapped in fresh cling film. After each dissection the block and non-disposable equipment were cleaned with alcohol sterilising gel. The dissection of each pinned abdomen was carried out under a binocular microscope. (See photographs of dissection.)

The labelled specimen tubes containing the spermathecae were taken to Abbey Veterinary Services in Newton Abbot to be sectioned, stained and mounted on microscope slides.

The stain used was standard Haematoxylin and Eosin. Sections were cut with a diamond edged microtome. Two sections were mounted on each slide.

There were 8 volunteer microscopists. The slides were coded and packed randomly in boxes of five. In effect this was blind testing as the microscopists were unaware of the nature and origin of the source queen. The slides were each examined by two people on separate occasions. The task was to use x400 magnification and count if possible a minimum of 200 nuclei in the wall of the spermatheca and classify them into two categories, Normal and vacuolated. This proved a difficult task. The classification proved to be rather subjective for many specimens. Staining was not consistent and unfortunately the level of preservation in the delicate wall cells was not good in many cases making counting impossible. This problem is discussed later. The total cell counts and recorded results are attached. Because of varying microscopy skills and operator interpretation it was decided that for each specimen where counting was possible the respective counts should be combined and compared as a percentage.

There were four clear categories of queens submitted:

Normal queens, Drone layers, Virgin queens , Poor or non-layers

The results are shown in the attached Pie-charts.

To date we have no corresponding information on the viral condition of the submitted specimens.

Discussion

The results overall suggest that there is no significant difference in the condition of the spermatheca wall in any of the categories. The control healthy queens show a greater frequency of vacuolated nuclei. The comments by Prof Cheryl Scudamore, Royal Veterinary College, on our 2012 investigation seems even more valid that this condition is a natural ageing phenomenon and probably not related to virus infection. As she says, this senescence has not been investigated in bees and in itself is a topic for further investigation. Certainly this should be done before any further attempt is made to investigate the effect, if any, of DWV infection on the spermatheca.

The poor condition of many of slides caused great difficulty and weakened the overall conclusions considerably. This is not a criticism of our support from AV S. Their technical process was standardised and produced many perfectly acceptable results. Any poor quality was due to the condition of the spermatheca when submitted. At the point of dissection it was clear that many queens were in an advanced stage of decomposition and one thing we discovered early in the first DLQ review was that the spermatheca degenerates very quickly

after death of the queen. It was always easy to find the spermatheca and remove it but in many cases the rest of the tissues and internal organs were poorly preserved.

Had we been in a professional situation, time would have to be spent from the start to develop a speedy, efficient system to preserve the submitted queens in a thoroughly tested solution if they cannot be immediately dissected to remove the spermatheca. Even then that would have to be carefully preserved to be in good condition for histological preparation. I am not at all pleased with glutaraldehyde as a preservative in this case. I take responsibility for its use and of course I'm very grateful to MBA for providing it. However further investigation and trial experimentation were needed before we started to collect the queens.

The formula for the glutaraldehyde solution (2% glutaraldehyde; 0.04% calcium chloride in 0.225M cacodylate buffer) was taken from a published report for the examination of soft tissue in the head of adult worker bees by Brown, S M, Napper, R M et al., 2002. At the University of Otago, Dunedin, New Zealand. This should have been trialled for some time before using it. Whether it was significant in the poor quality of staining in so many cases or contributed to the frequently poor preservation of spermathecal tissue we cannot tell.

Coordinating the willing volunteer microscopists was not easy and DARG is very grateful for their services with a sometimes frustrating and tedious task. We all recognise that this is a common feature of scientific research; hours spent examining , counting, measuring and recording. For those of us not involved in professional research it was a revealing insight.

Using Royal Mail to circulate slides and specimens was generally satisfactory although there were a couple of instances when perhaps machine sorting damaged packaging and contents were lost. If we had all been in the same laboratory that matter would have been easier to manage and there would have been consensus on ambiguous slide interpretation. DARG is however, an amateur group with widely dispersed members

There is no doubt that the investigation is still not settled. We are working in an area that needs professional support and maybe we took a step too far in that direction and too quickly. The issue of DLQ incidence and increasing rates of virus infection, not only DWV, is increasingly important. The professional scientists involved with apicultural projects need the support of practical, well-informed beekeepers and not just with financial contributions so this was a rare cooperative activity we have been fortunate and shows what is possible.

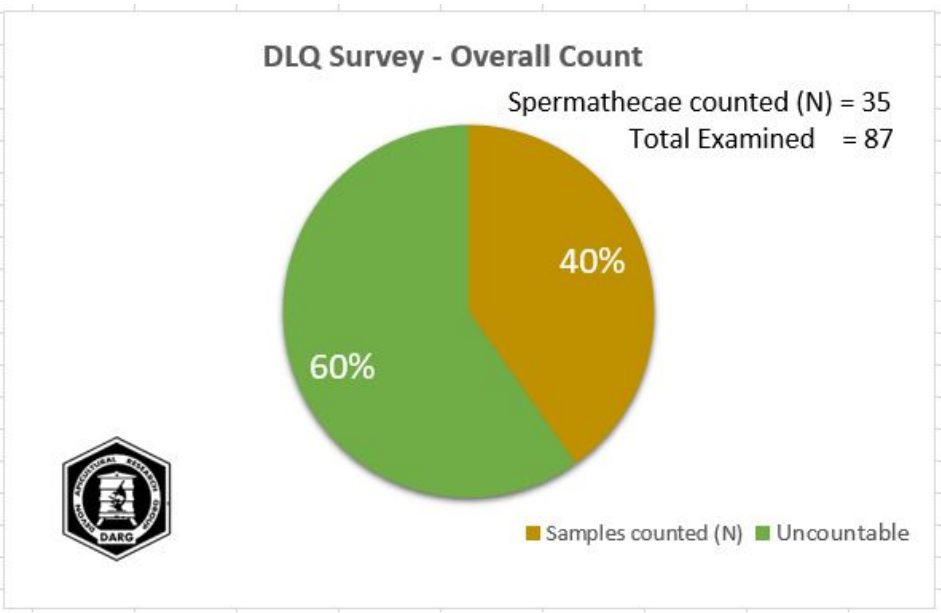
The aim was to get each slide examined twice. They were boxed in standard five slide containers and circulated to allocated microscopists by Royal Mail in padded envelopes, 2nd class large postage.rate. Counts of cell nuclei were recorded identifying two categories normal and vacuolated. The pack of 5 were then either returned or sent to another of the group's microscopists. Comment on each slide was encouraged.

Because the assessment of many nuclei into either category was often a subjective decision, even when they were visible, it raised the issue of how the results should be combined. Microscopists were asked to record where possible 200 nuclei for each specimen. Should there be a mean figure calculated for each specimen? This was rejected as being unreliable as each microscopist would have a different standard. As at the conclusion of the study we were to

compare the relative proportion of healthy and vacuolated nuclei in each of the four categories of queen, it was decided that the absolute number of nuclei counted by any microscopist should be totalled for each specimen. The presence or absence of sperm was also recorded.

The slides in each box were randomly selected from the four categories of queen and renumbered so that microscopists were unaware of the identity and category of the queen supplying each particular spermatheca. The combined counts are shown further on in this report and shows the number of specimens that were impossible to count.

The proportion of submitted queens in each category that gave countable slide specimens was:



Group 1	Healthy Queens	75%	Group 2	Drone Laying Queens	36%
Group 3	Virgin Queens	27%	Group 4	Others inc Non-layers	17%
		Overall 40%			

Also see Chart **Comparison of specimen quality by group**

As only 40% of submitted queens had countable nuclei conclusions cannot be justifiably made regarding differences in the condition of the spermatheca in each group. However, the percentage of "vacuolated" nuclei counted in each of the four groups was :

Group 1	Healthy Queens	8%	Group 2	Drone Laying Queens	3%
Group 3	Virgin Queens	5%	Group 4	Others inc Non-layers	5%

Also see Charts "Condition of spermatheca epithelium wall cells"

Summary Conclusions

1 At dissection the queens were often poorly preserved. We noted in the first (2012) study that the spermatheca wall undergoes very rapid post-mortem lysis.

2 The glutaraldehyde solution was either not appropriate in this situation or the queens were not immersed quickly enough after death/anaesthetisation. The group of control queens (Group 1) were immersed as soon as anaesthetised by refrigeration at 4C and gave the best results in terms of slide quality.

3 The observation that the control queens, all aged around 2 years old, had a greater frequency of "vacuolated " nuclei in the spermatheca wall compared to the other groups is contradictory to our basic hypothesis which predicted maximum frequency in the drone laying queens. However, as there were so few queens satisfactorily tested within each of the other groups, this may not be a valid result.

4 The incidence of viral infection for each queen of the queens in the survey will be an interesting addition to the study. These are currently stored at the MBA laboratory, Plymouth and conversation is taking place regarding their future.

5 The investigation was interesting for those involved and a definite learning experience. It was also an important insight into the professional world of veterinary and apicultural research.

6 DARG is very appreciative of the support from

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