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Parasite Immunology Invited Review

The immunobiology of myiasis infections

– whatever happened to vaccination?

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20 SUMMARY

21 The current state of myiasis vaccine technologies are reviewed mainly in the primary research
22 genera of *Lucilia* and *Hypoderma*. The importance of myiasis flies as primary causes of
23 morbidity and mortality in agricultural species and man has not diminished despite the
24 existence of good control strategies. However, the development of vaccines against myiasis
25 infections has been relatively quiescent for more than ten years despite the rapid development
26 of genomic and proteomic analysis and of skills in data interpretation. The value of vaccine
27 research in an era of chemical primacy is analysed. In fact, recent findings of drug resistance
28 and the impact of animal welfare concerns should mean a renewed interest in alternative
29 controls. The reasons that this has not been true to date are explored and new possibilities
30 discussed.

31

32

33 **Keywords.** Myiasis, Vaccines, Immunology, Sheep Blowfly, *Lucilia*, *Hypoderma*, Warble
34 Flies, Bot Flies, Mulesing, Vaccine antigens.

35

INTRODUCTION

Myiasis infections are caused by the larvae (maggots) of a range of blowfly species with various consequences for the host. Generally the species can be divided into three groups based on their pathology including those causing skin infections to various depths (cutaneous myiasis) those infecting body orifices and the gastrointestinal tract (bot flies) and those which infect and often migrate subcutaneously (the warbles). The bots and warbles are members of the Oestridae while the skin infecting flies are largely Calliphoridae though a few Sarcophagidae are also active. A large range of species engage in parasitism though only a few are obligate parasites. Genetic analysis suggests that obligate parasitism has evolved on a number of occasions among the Calliphoridae while the Oestridae are a monophyletic group of obligate parasites¹. The most important species with respect to human and domestic animal health include Calliphoridae such as *Lucilia* spp - sheep blowflies; the screwworms - *Cochliomyia* and *Chrysomya* spp and the Sarcophagid, *Wohlfahrtia magnifica*; while the Oestridae include *Oestrus ovis* – nasal flies; *Gasterophilus* spp– horse bot flies; *Dermatobia hominus* - human warble fly and *Hypoderma* spp – warble flies². A range of other flies can infect the skin or body orifices of debilitated or moribund animals and humans or cause secondary infections but many of these are opportunistic carrion flies with less economic but significant individual effect.

The two major infections that have been the subjects of most research efforts for novel controls and a better understanding of pathology and immune reactivities, are the sheep blowflies, *Lucilia cuprina* and *L. sericata* and the warble flies, *Hypoderma bovis* and *H. lineatum*. These species mainly infect sheep (*Lucilia*) and cattle (*Hypoderma*) respectively and cause significant pathology and mortality in the case of *Lucilia*³, while *Hypoderma* causes morbidity and losses including damage to hides⁴. Other species can cause significant disease in various hosts in specific areas including the screwworms especially in South

America; though the success of the sterile insect release in the USA and Central America has limited the range of *Cochliomyia* ⁵. The old world screwworm is *Chrysomya bezziana* and some vaccine work has been undertaken, mainly as a result of this species' threat of invasion of the Australian mainland from the Indonesian archipelago leading to vaccine funding in the early 2000s. Much more widespread but similarly under utilized in research efforts are the common bot flies such as *Oestrus* and *Gasterophilus* and a range of other species that effect livestock and wild animals worldwide. Work on these species is limited but has included some analysis of pathology and basic vaccine responses ^{6,7,8}. Finally work on *Dermatobia* has been very limited but promising while the wonderfully named *Wohlfahrtia magnifica*, which may be expanding its range in Europe, Africa and parts of Asia ⁹ and causes significant losses in a wide range of livestock including camels ¹⁰ is relatively untouched.

Control of all these infections has been and still is, reliant mainly on chemical insecticides though management strategies are also important for the sheep blowfly to allow control in extensive grazing situations ¹¹. The reliance on insecticides and the inexorable rise of drug resistance to most classes of insecticide, has driven the search for alternative control mechanisms including the use of genetic, biological and immunological technologies ¹². The other more recent driver particularly for sheep blowfly control has been animal welfare concerns around the mulesing operation and to a lesser extent tail docking ^{13, 14}. Mulesing is the surgical removal of the wool bearing skin on either side of the vulva which regrows as bare skin and is thus much less subject to urine and faecal staining ¹⁵. This can reduce the chance of blowfly strike around the tail by over 60% ¹⁵ but it is undertaken without anaesthetic and has thus been targeted by PETA (People for the Ethical Treatment of Animals) and others as a major animal welfare concern ¹⁶. As a result the wool industry in Australia is keen to phase out the use of this procedure ¹⁶.

A recent and perhaps surprising direction for pathological and immunological analyses especially of the blowfly *Lucilia sericata* is the use of the maggots for wound debridement and infection control, particularly in Europe¹⁷. This work has been driven by the appearance of multiple drug resistant strains of various bacteria and the need to treat a range of wounds that are refractory to the more usual treatments¹⁸. The maggots are placed in the wound either directly or more usually contained in fine net bags and their secretions clean out the eschar and reduce the bacterial load to encourage healing¹⁹. The success of this approach has resulted in a more detailed analysis of the larval secretions and an analysis of the site of application on the host to better understand the cleaning and healing response. There is also an effort to isolate particular enzyme and anti-bacterial activities to enable the development of new treatments¹⁷.

Analysis of immune and inflammatory responses against *Hypoderma* and the sheep blowfly began in the 1970s^{20, 21}. However, a concentrated effort to develop vaccines was coincident with the development of recombinant technology and the ability to carefully dissect host pathological and immune responses to maggots and their components. This technology led to work on both natural antigens – exposed to the host during infection and hidden antigens – only exposed to the host via vaccination but accessible to host immune attack. As with most complex metazoan parasites the extracts of larvae initially tested for vaccine effects were not particularly effective²² though the work on *Hypoderma* was always more rewarding than similar studies on the sheep blowfly²³.

To explain this difference and the quite different results that are explored later in this review a short examination of life cycles and host reactions is necessary. Sheep blowflies lay their eggs communally on stained or wet wool allowing the larvae to establish on the skin^{24,25}, they then degrade the epidermis²⁴ which causes an exudative wound that spreads as the larvae grow until it can cover a significant fraction of the animal's skin. This wound results in water

110 loss as the larvae inhibit coagulation and release a range of active enzymes and other toxins
111 ^{26,17}. Depending on the size and health of the host around 1000 larvae (approx. five female
112 egg masses) can have significant negative effects on sheep health ²⁷. Female flies are
113 attracted to ovipositing females and recently laid eggs, as a result, one egg mass can soon be
114 joined by number of others causing rapid morbidity and mortality in a significant percentage
115 of a flock ². To date there have been no studies that show any consistent resistance to repeated
116 infections ²⁸ though there are certainly animals that are resistant to infection and it is possible
117 to breed resistant animals by direct selection ²⁹.

118 The warble flies show quite a different infection strategy and resultant host response. These
119 flies lay their eggs singly or in groups of up to twenty on the skin surface usually on host hairs
120 ³⁰. The larvae hatch and penetrate the skin, then migrate into the deep tissues of the body
121 where they 'rest' and grow for a period of several months before migrating to their site of
122 predilection usually the back of the bovid host ³¹. Here the host forms a warble or lump with
123 a central hole to the surface through which they respire ³¹. On reaching larval maturity after
124 about a month, they leave the host through the hole and drop to the ground to pupate. The
125 host usually shows significant resistance to the larvae after the first season of exposure and
126 this is further enhanced after subsequent exposures. Thus fewer warbles are seen on older
127 animals ^{32,33}.

128 A number of other infections have been analysed for host immune activities and these will be
129 discussed in the article but the clear majority of work has involved the species described
130 above. It is also interesting that despite the quite different outcomes in terms of the
131 application of these immune studies to each of these infections, the current controls for both
132 forms of myiasis are still reliant on the technologies developed in the 1970s.

133

134 SHEEP BLOWFLY INFECTIONS

135 **Overview**

136 A study of the immune response to blowfly infections in sheep spans over 35 years of
137 research in which a range of observations have been reported in the literature ^{12,34,35}. Despite
138 these observations it is clear that we still know comparatively little about the detailed immune
139 responses induced by larvae upon primary and subsequent infections of the host. Furthermore
140 we know even less about the changes that take place in the larvae themselves in response to
141 their environmental stimuli. Clearly it is only through a greater understanding of this intricate
142 relationship that we can attempt to better manage this important ectoparasite especially if the
143 aim is to develop an effective prophylactic vaccine or other immune based intervention
144 strategies.

145 This section of the review will focus on the literature associated with describing the immune
146 response in sheep to the sheep blowfly. Specific areas addressed will include the humoral and
147 inflammatory response of the host to the larvae and the implications for the development of
148 immunity, immunosuppression, host response to vaccination and impediments to vaccine
149 development. Commentary will also be made on the development of new technologies and
150 approaches to identifying possible vaccine targets.

151 The humoral immune responses to infection was first reported back in the early 1980's ³⁶ and
152 several groups have further investigated the nature of both the systemic and local humoral
153 response during infection ^{28,37-41}. These humoral responses have been shown to be directed
154 against E/S products of the larvae ^{42,43} and the salivary antigens ³⁷ following natural infection.
155 While the response has been characterized by the production of IgG ^{36,41} with subsequent
156 analysis identifying a number of isotypes including IgG1, IgG2, IgA and IgM ⁴⁴ and IgE ⁴⁵
157 with the primary isotype being identified as IgG1 ⁴².

Along with the humoral responses a series of studies also investigated the cellular immune responses occurring during infection with the larvae^{38,46}. The responses following both primary and secondary infections are characterized by massive cellular infiltrations within 48 h of wound initiation, with the majority of cells having the CD45 phenotype and neutrophils comprising the major cell type at the skin surface. Other phenotypes reported in the skin of flystruck sheep included CD1+ Langerhans' cells, CD4+ T helper, $\gamma\delta$ -TCR+ cells and T19+ (CD4-, CD8-) T cells⁴⁶⁻⁴⁸. Changes noted in local cytokine responses in the skin and draining lymph nodes^{47,49} included increases as detected via RT-PCR, in expression of IL-1 α , IL-1 β , IL-8 and in IL-6 via northern analysis⁵⁰. For a more detailed review of the cellular events occurring following blowfly larval infections refer to the review by Elkington and Mahony³⁴.

Based on the descriptions of the cellular responses it is clear that a complicated series of inflammatory events occur during flystrike infections. While the findings might not be too surprising given the nature of the acute insult to the skin by the larvae, an important question is whether this knowledge provides some insight firstly into explaining the lack of a protective acquired immune response to the larvae and/or whether it might provide important clues into the development of a vaccine against the larvae.

Inflammation versus immunity

Previously the composition of the cells that infiltrate the site of infection was briefly described as comprising neutrophils, eosinophils, macrophages and lymphocytes of which CD4 and $\gamma\delta$ -TCR+ make up a large proportion of the infiltrating lymphocytes. Associated with this knowledge is the finding that sheep are able to mount a variety of hypersensitivity responses post challenge to larval antigens with some of these responses notably immediate-

type and Arthus responses bearing some correlation with partial acquired resistance to the larvae³⁸. An important point to note is that while it has been possible to demonstrate partial protection to the larvae in an experimental setting following repeated larval challenge, it is still not clear what key components of the immune system are contributing to this transient immune response. Furthermore, the apparent lack of protective immunity to the larvae in the field provides strong evidence that whatever immune responses are induced by the larvae they are either irrelevant or not of sufficient magnitude to control the larvae during natural challenge in an outbred flock. The reference to outbred flocks is interesting based on the significant amount of research conducted on the Trangie line of sheep that had been selected for resistance/susceptibility to fleece-rot and flystrike since 1974⁵¹. One key finding from these sheep is that under natural flystrike conditions in one year old sheep it was reported that 19% of sheep classed as susceptible suffered body strike compared to only 1% of the resistant flock⁵¹. Further analysis of the hypersensitivity response to larval excretory/secretory proteins reported a more sustained hypersensitivity response in rams selected for innate resistance (R) compared to the susceptible (S) rams⁵³. It was also suggested that this difference in hypersensitivity response implied a genetic difference between flocks which could possibly be used for selection of resistant animals. These R versus S sheep were also monitored for a range of molecules present in exudates collected at varying times from flystrike wounds. Significant differences were detected in the exudate compositions for Complement C3, a fragment of IgG, α 1-antitrypsin and for a range of unidentified peptides and it was suggested at the time that the more rapid exudation of acute-phase and serum proteins at infection sites on R sheep may allow the inhibition of the establishment of fleece rot bacteria or *L. cuprina* larvae under natural challenge⁴¹. The Trangie flock may therefore harbor important information on the innate response to challenge infection with the larvae in the field. How to translate these responses or assess their importance to outbred animals is a

difficult question to answer, however given that a variety of responses had been documented some of which correlated with protection and coupled with the finding that sheep can show resistance to natural challenge drove research into the direction of exploring vaccination as an alternative to chemical control of this parasite.

Vaccine responses

Over the years a number of vaccination strategies have been investigated using a wide range of larval antigens, reviewed by Elkington and Mahony³⁴. The basic premise for vaccination is to induce an immune response in the host that would inhibit larval growth and ultimately survival. Ideally such an approach would result in a reduced dependence on insecticides providing protection over critical periods during the season when flies are most prevalent. A number of reports identified the production of serum antibodies to various larval antigens post vaccination and subsequent anti-larval growth effects *in vitro*⁵⁴⁻⁶¹ however these results generally did not translate into significant protection following implants with first stage larvae *in vivo*. The reasons for this apparent inability to translate *in vitro* effects to the *in vivo* situation were suggested to include insufficient antibody titres present in the skin, the degradation of IgG at the wound site and the time required to reach peak antibody titres in the sera^{40,62,63}. In contrast to the reports where vaccination failed to illicit a protective response *in vivo*, was the study by Bowles *et al*⁶⁴ in which vaccination with four partially-purified antigens induced an 86% (Trial 1) and a 67% (Trial 2) reduction in the incidence of strike compared to unvaccinated controls. In addition, larvae recovered from vaccinated animals (Trial 1) were up to 85% smaller than their control counterparts. It was also noted that antibody titres from the protected sheep failed to correlate with protection which is not inconsistent with previous efforts to correlate protection with antibody titres. However, what

was very interesting was the presence of cellular foci described at the site of challenge in protected animals, which consisted of CD1+ Langerhans' cells, CD4+ T helper and $\gamma\delta$ -TCR+ cells, and these foci may have provided an early immune response upon larval challenge in primed animals that significantly affected the ability of the larvae to successfully establish⁶⁴. While delayed-type hypersensitivity responses (DTH) were also measured post larval implantation this parameter did not correlate with protection.

Inhibitors of immune control - immunosuppression

Producing an effective vaccine against the sheep blowfly presents numerous significant challenges as attested by the results obtained thus far for vaccination attempts. There are many factors to consider including the choice of antigen(s), the type of immune response to be elicited (humoral vs cellular or both), the route of administration, (subcutaneous, intramuscular or possibly intradermal), the choice of adjuvant or immunomodulators and the number and timing of immunisations. These factors are not trivial issues to be researched and whilst some progress has been made a significant body of research remains incomplete if a blowfly vaccine is to be effective. Another important point to consider when seeking to produce an effective vaccine is the ability of the larvae to influence the immune response of the host. Such interactions are common place in the parasite world⁶⁵. With respect to the sheep blowfly the ability of the larvae to degrade host immunoglobulin⁶² is one clear example whereby the larvae are capable of avoiding the host humoral immune response. In addition, excretory-secretory (E/S) products produced by the larvae have been shown to be able to directly influence the immune response at both the cellular and humoral levels⁶⁶. The results from this study demonstrated that the proliferation of ovine lymphocytes could be suppressed *in vitro* following exposure to the larval products but interestingly this suppression

could be reversed. Elkington et al.⁶⁷ reported that a 56 kDa protein from E/S material was shown to be capable of significantly inhibiting lymphocyte proliferation and it was proposed that this particular protein may have an immunomodulatory role during blowfly infections. These types of responses are not surprising given the lack of protective immunity demonstrated against this parasite in the field and what is known from other parasites as they have evolved to avoid host immune responses⁶⁸. What will be challenging from a vaccine perspective is to identify the key immunomodulatory molecules produced by the larvae as presumably these too would be useful vaccine candidates.

New approaches to control.

As mentioned previously, an understanding of the interaction between the sheep host and the larvae is critical to the development of an effective vaccine strategy. Previous research on this interaction has been performed largely in the absence of genomic data and produced a few, partial sequences of potential vaccine candidate proteins⁶⁹⁻⁷¹. An exception to this is the extensive work that was conducted on the peritrophins from *Lucilia* in which a number were cloned and sequenced^{57,58,72}. The sheep genome (<http://www.sheephapmap.org>) and the nearly completed *L. cuprina* genome (5K genome project) (http://www.arthropodgenomes.org/wiki/Main_Page), combined with the power of mass spectrometry and transcriptomic data analyses, should result in the completion and characterization of these partial proteins and in the identification of additional novel potential candidate target genes for both vaccine and drug development purposes. Transcriptomic and proteomic analyses of blowfly larvae and sheep during infection, should deliver novel insights into the sheep's immune response and result in the assembly of blowfly secretomes; a wide range of secreted proteins of different larval stages that are potential candidates for vaccine

design. Furthermore, essential genes are ideal candidates for drug targeting in the blowfly and can now be inferred from the available information on the related non-parasitic fly species, *Drosophila melanogaster* and validated using molecular knockout techniques, such as CRISPR⁷³. The missing component in the delivery of these molecular techniques and vaccine development is adequate and sustained funding which has been largely unavailable from industry or other sources for over ten years, despite the mulesing controversy and the obvious importance of developing alternative therapies.

Other Blowflies

Vaccine research in other blowfly species has been very limited and has concentrated mainly on the screwworms which penetrate deep into their hosts after infecting through wounds or body orifices. As a result they can cause rapid mortality especially in young and stressed animals. They are also non-selective in their host range, successfully infecting most available mammals and some birds⁷⁴. *Cochliomyia hominivorax*, the new world screwworm has been controlled in a large part of its previously endemic range via the release of genetically-altered ‘sterile’ male flies⁵. However, its persistence in South America and occasional incursion elsewhere⁷⁵ suggests that additional controls are still needed. *Chrysomya bezziana*, the old world screwworm fly, is endemic in Asia and Africa⁷⁶. It has long been a major quarantine concern in Northern Australia with significant populations of the fly in the Torres Strait⁷⁷.

Chrysomya bezziana

In common with *Lucilia*, the screw-worm, *Chrysomya bezziana* secretes highly active products onto the host. These consist of a mixture of enzymes and toxins that inhibit coagulation, degrade complement components, lyse cells and degrade skin matrix proteins⁷⁸. Analysis in the 1980s confirmed that skin reactions to the screwworms were similar to *Lucilia*

with neutrophil accumulation followed by fibrosis, eosinophilia and mast cell proliferation after the larvae had dropped off the host ⁷⁹.

Analysis of the protease enzymes present in the larval secretions again shows similar composition to *Lucilia* ⁷⁸. This paper also reports unpublished vaccination trials with these proteases though without significant effect, a finding which again mimics the *Lucilia* data.

In keeping with the *Lucilia* lead, the peritrophin 48 gene was isolated from *Chrysomya*, expressed in bacteria and compared to both the *Lucilia* and *Drosophila* homologues ⁸⁰. This antigen and two other peritrophin molecules (Cb15 and Cb42) were then used in vaccine trials in sheep ⁴¹. No significant differences were found *in vitro* with Cb15 and Cb42 and only a small negative effect on growth occurred with Cb48. *In vivo* the vaccination apparently caused a small increase in larval weight over controls ⁴¹. The results suggest that these proteins are less effective as antigen targets in *Chrysomya* than they are in *Lucilia* though additional studies on adjuvants and protocols may improve such responses. However, the lack of significant effect was not conducive to further funding and the program was not extended beyond the initial trials.

WARBLE INFECTIONS

Overview

As long as records have been kept researchers and cattlemen have noted that cattle develop resistance to cattle grub infections. This translates into fewer grubs per animal in older animals and has been reported in the literature numerous times ⁸²⁻⁸⁴.

Confusion reigns with regard to whether the impact of immune responses mostly affects early first instars as they migrate through the internal tissues of the host ⁸³ or whether the impact of the immune response mostly affects late second and early third instars as they reside in the

warble^{84,85}. This dichotomy is of particular relevance to the commercial success of a vaccine which would ideally prevent damage to hide and sub-dermal tissues that results from formation of warbles.

Cellular responses to invading first instars have been described in artificial infections and show very little change in cell types associated with primary infections⁸⁶. The infiltration of B cells and IgG positive cells shortly after challenge infections in perivascular areas was dramatic and rapid⁸⁶. Local and systemic cytokine responses in the first few days of both primary and challenge infections have been reported by⁸⁷ who suggest that the bovine response is framed by both Th1 (increase in IFN- γ) as well as Th2 (increases in IL-4 and IL-10) responses. Similarly, inflammatory cell responses in skin of primary and challenge infections⁸⁸ showed an increase in CD4⁺ during the early phase of primary infections while B cells were predominant in challenge infections and the numbers increase in association with the number of previous infections.

Cytokine and antibody responses have been recently characterized in naturally infested animals during the later phases of the infection by Vasquez et al⁸⁹ and Panadero et al⁹⁰. These authors suggest that in natural infections the cytokine profiles were less clear than in artificial, pulse infections^{87,88} and a similar situation was observed for inflammatory cells. IL-10 was higher in challenge infections which they interpreted as allowing reduced inflammatory responses which increased the survival of migrating first instars. They noted that the inflammation regulatory cytokine IL-10 declined rapidly after larvae had exited the warbles which was consistent between primary and challenge infections, suggesting that this cytokine was important in maintaining the host granuloma from which the second and third instars derive their nutrient.

Vaccine natural antigens

The earliest work on vaccines dates to the 1950's⁹¹ and 1960's^{92,93}. These studies used whole third instar antigens and the vaccine was delivered after animals were naturally infested. Baron and Colwell⁹⁴ reported the use of native hypodermins as vaccine components with the addition of monophosphoryl lipid A as an adjuvant. Pruett⁹⁵ reported an increase in cattle grub mortality in cattle vaccinated with native HyA, but the bulk of the mortality was observed at the second and third instar stage in the host's subdermal tissues. This aspect was detrimental to the vaccine approach as while it had a measure of population control the immediate damage to hides and carcasses was not eliminated. This was followed by studies reporting the production of recombinant antigens (reviewed by^{95,96}).

Panadero et al.⁸⁵ described the immunomodulatory effect of three serine proteinases from first instars of *H. lineatum*, reporting that lymphocyte proliferative responses were down regulated particularly by HyA as had been previously noted by Nicolas-Gaulard et al.⁹⁷. In addition these authors reported a down regulation of cytokine responses that was also strongly mediated by HyA. Hypodermin C had a much less significant effect while HyB was intermediate in effect.

Vaccine hidden antigens

Colwell⁹⁸ reported up to 100% mortality of cattle grubs in calves immunized with soluble extracts of third instar fat body formulated with Quil A as the adjuvant. Significant increases in mortality of migrating first instars was noted as well as increased mortality of second and third instars in vaccinated animals in comparison with untreated controls and adjuvant only treated animals. Subsequent LC – MS/MS analysis of four bands (29, 50, 60 and 80 kDa)

from SDS-PAGE separated proteins that were subjected to MASCOT database searches revealed peptides with similarities to several proteins.

Interesting proteins that had protein scores of greater than 400 included the Hexamerins/arylphorins (also known as larval serum proteins), which are storage proteins of the hemocyanin family that act as amino acid pools for reconstruction associated with insect metamorphosis and in some cases support egg production⁹⁹. These proteins have also been identified by Roelfstra et al⁸ in second and third instar *Gasterophilus intestinalis*. A Glutathione-S-transferase from a multifunctional family of enzymes that protect cells by preventing the damaging effects of oxygen and other free radicals. These are widely used in anti-parasite vaccines; e.g. *Haemaphysalis longicornis* and *Rhipicephalus microplus*¹⁰⁰ *Schistosoma mansoni*¹⁰¹, *Psoroptes ovis*¹⁰², *Necator americanus*¹⁰³. Arginine kinase is a crucial enzyme that catalyzes the transfer of phosphoryl groups from ATP to arginine in arthropods and has been noted as an excellent target for drug development. It has also been implicated as a major human allergen¹⁰⁴ and is found in parasitic nematodes where it has been suggested as a potential vaccine candidate¹⁰⁵. Phenoloxidase is a major component of the insect immune system that participates in encapsulation and wound healing through formation of melanin¹⁰⁶.

Recombinant antigens

The gene sequences for the three major proteins secreted by first instar *Hypoderma* spp, were first reported in the early 1990s¹⁰⁷, but work with recombinant versions of these enzymes predated that description. Recombinant versions of these serine proteinases were formulated and expressed as inclusion bodies in *E. coli* at both the Lethbridge Research Centre and at the

USDA Kerrville laboratory. Hypodermin A was chosen to be the primary component of the vaccine which was formulated with alhydrogel/amphigen as the adjuvant ⁹⁶.

Current state of potential vaccines

All research into the use of recombinant hypodermins for cattle grub control effectively ended in the late 1990`s. This was the result of difficulty obtaining patents for the production of the antigens and the advent of macrocyclic lactone endectocides. A recent attempt to use `hidden` antigens as vaccine components while highly successful relied on the use of a cocktail of uncharacterized native antigens. Other than what has been mentioned in the previous section there has been no further work to determine the most active components in the cocktail or to develop recombinant proteins for evaluation of their effect.

Inhibitors of immune control

Immunosuppression

HyA has been shown to have a potent inhibitory effect ^{97,85} through down regulation of lymphocyte proliferation. Hypodermin C has been reported to degrade complement component C3 ¹⁰⁸. Other cattle grub immunosuppressive effects have been noted for HyC ¹⁰⁹.

Macrocyclic lactone endectocides with their extremely high efficacy against Hypoderma ¹¹⁰ and their ease of use have effectively spelled the end of vaccine research and development on cattle grubs. The macrocyclic lactones are so effective against cattle grubs that eradication campaigns in which `micro-dose` applications at 1/10 the recommended dose have been proposed ^{111,112} With the appearance of drug resistance to these products in cattle gastro-

419 intestinal nematodes ^{113,114} there has been a push to develop new active ingredients such as
420 monepantel ¹¹⁵ and to develop combination treatments which will undoubtedly have a
421 macrocyclic lactone as a component. This will continue to have an effect on vaccine research
422 against bot flies.

423

424 **Other bot flies**

425 *Dermatobia hominis*

426 Work with *Dermatobia hominus* has been stimulated by its increasing incidence in travellers
427 returning from, and its continuing importance in, South America ¹¹⁶⁻¹¹⁸ though identification
428 is not always proven in travellers ¹¹⁹.

429 *Dermatobia hominis* females capture mammophilic flies, in mid flight, depositing several
430 eggs onto their abdomens. These flies visit a host, often for a blood meal, and the increased
431 temperature stimulates eggs to hatch releasing the larvae. Larvae penetrate the host skin and
432 begin to develop without deep tissue migration such as occurs with some other cuterebrid
433 larvae.

434 Studies of this fly have again analysed E/S products for protease activity with a serine
435 protease mix again found to be dominant ¹²⁰. A notable finding is a high molecular weight
436 metalloprotease produced by the first instar. This is suggested to be active during skin
437 invasion though further work is required to confirm the hypothesis. Other warble flies seem to
438 be more reliant on the serine proteases and particularly collagenolytic serine proteases for
439 invasion, so further work would be interesting.

440 A recent study used immunodominant antibodies to identify and isolate an antigen for a
441 vaccine trial in cattle. Thus whole larval somatic extracts were used to immunize cattle and

then identify an antibody in immune sera that recognised an immunodominant 50 kDa antigen¹²¹. This was isolated from the soluble extracts of whole *Dermatobia* larvae, of all three instars and used to vaccinate cattle. This approach yielded a greater than 90% reduction in the number of surviving larvae after challenge.

The success of the approach suggests alternative vaccine antigens to the established use of the Hypodermins in other warble flies⁸⁸ as the molecular weight of this antigen is almost twice that of these serine proteases. Other studies in *Hypoderma* have indicated the presence of tissue-derived antigens at about this molecular weight⁹⁸.

Oestrus ovis

The final group of Oestridae with recent work reported are the nasal bots which includes *Oestrus ovis* and *Rhinoestrus* spp, parasites of sheep/goats and equines respectively. These parasitic flies are inhabitants in the upper sinus of their hosts and tend to be specific to their hosts, which include equids, artiodactyls and even a species in macropods (kangaroos)¹²².

Oestrus ovis females forcefully expel fully developed first instars directly onto the nose of the host. The larvae are encased in a viscous fluid that aids them staying on the host. Shortly after deposition the larvae migrate initially into the outer nasal passages where development begins. Later the larvae will move to the nasal sinuses and other cavities within the head.

These larvae tend to be very common in their hosts infecting from 30 to 50% of flocks in most studies (reviewed in¹²³). The larvae produce very similar enzymes to the more invasive oestridae¹²⁴ and the major antigens recognized by the host are excretory/secretory products including salivary gland antigens¹²⁵. However, immunisation with E/S products did not show any protective effect other than limited growth inhibition¹²⁶. Use of 'hidden antigens' derived from washed third instar digestive tracts had also no effect on larval survival, but reduced the size of the larvae, primarily of second instars¹²⁶.

466 *Gasterophilus*

467 *Gasterophilus* spp. females deposit eggs onto the host hairs where they remain attached until
468 they are induced to hatch by the friction and or moisture of host grooming activity. Larvae
469 penetrate the oral mucosa and undergo migration within the oral cavity for some days
470 (specifics of the migration and site vary by species). Following a brief period of development
471 the larvae drop into the gastro-intestinal tract, find species-specific attachment sites and
472 complete development.

473 Despite the importance of these both flies to the horse industry worldwide there has been little
474 effort to describe immune responses or to develop vaccines. Roelfstra et al.⁸ have described
475 horse immune responses to crude extracts of whole second and third instar *G. intestinalis*
476 along with a description of some of the proteins identified by tandem mass spectroscopy. As
477 outlined in the Hypoderma discussion several of the proteins appear similar to those described
478 from the fatbody of *Hypoderma lineatum* (e.g. larval serum protein, arylphorin).

479

480 CONCLUSION

481 The issue for those concerned with the development of new controls for myiasis diseases is
482 the continuing reliance in insecticides over the last 50 years and the lack of current research
483 towards alternatives. This is especially true of vaccine work where no significant funding has
484 been available since 2000. Although this could be argued as due to a lack of potential
485 candidate vaccines in the case of the sheep blowflies (*Lucilia*) there have been good
486 candidates for *Hypoderma* control for at least this period⁹⁴.

487 Clearly the drugs currently available, which include the macrocyclic lactones and the insect
488 growth regulators (esp. chitin synthesis inhibitors), are very effective and relatively cheap

though resistance is known in field populations of *Lucilia* ^{128,129}. Drugs have allowed virtual eradication of *Hypoderma* populations in most western European countries apparently without the induction of resistant strains ¹²⁹. Such ready availability of cheap and effective control has inhibited research into other measures especially in the last ten years. The only issue with this suppression is that once resistance does develop, as it has in *Lucilia* to almost all commercial drugs used to date ¹²⁸, then there will be little if anything available other than drug combinations and a scramble to rapidly reassess past research for alternative controls.

An example of the effect of the lack of funding into novel controls is the situation with mulesing and the Australian wool industry, mentioned in the introduction to this review. Complaints about the welfare and cruelty issues involved in mulesing sheep are not new and animal welfare groups have been active against the operation for many years ¹³⁰. The involvement of PETA who threatened an international boycott of wool from mulesed sheep in 2004 was merely the final act of a long building issue ¹³⁰. The response of the industry in 2004 was to fund research into a limited range of alternatives to mulesing ¹³¹, this amounted to other methods of performing the same operation and an analysis of breeding options to select plain bodied sheep ¹³², more recently selection for blowfly resistance has joined the list ²⁹, though both these genetic options are obviously long term. As a result the declaration by the Australian Wool Corporation that they would stop mulesing by 2010 has not been achieved and the boycotts are extending ¹³³. Although funding vaccine research may not have resolved the issue it should be noted that even vaccines with 60% protective effect can add significant control options in integrated management programs ¹³⁴, which are the only currently available strategies for blowfly control in unmulesed sheep ¹¹. In addition and as discussed earlier, changes in technology over the last ten years have added significantly to our ability to find and test target molecules in parasites and these technologies are rapidly advancing our understanding of a wide range of other parasite-host interactions. The same

514 cannot be said of myiasis infections and a reassessment of the potential for immune based
515 control of these infections is surely in order.

516

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