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Parasite Immunology Invited Review

The immunobiology of myiasis infections – whatever happened to vaccination?

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SUMMARY

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

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

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 parasites [1]. 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 flies [2]. 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 hides [4]. 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 Europe [17]. 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 treatments [18]. 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 healing [19]. 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 treatments [17].

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 effective [22] though the work on Hypoderma was always more rewarding than similar studies on the sheep blowfly [23].

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 epidermis [24] 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

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

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

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

SHEEP BLOWFLY INFECTIONS

Overview

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

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

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

158 Along with the humoral responses a series of studies also investigated the cellular immune
159 responses occurring during infection with the larvae [38,46]. The responses following both
160 primary and secondary infections are characterized by massive cellular infiltrations within 48
161 h of wound initiation, with the majority of cells having the CD45 phenotype and neutrophils
162 comprising the major cell type at the skin surface. Other phenotypes reported in the skin of
163 flystruck sheep included CD1+ Langerhans' cells, CD4+ T helper, γδ-TCR+ cells and T19+
164 (CD4−, CD8−) T cells [46-48]. Changes noted in local cytokine responses in the skin and
165 draining lymph nodes [47,49] included increases as detected via RT-PCR, in expression of IL-1α,
166 Il-1β, IL-8 and in IL-6 via northern analysis [50]. For a more detailed review of the cellular
167 events occurring following blowfly larval infections refer to the review by Elkington and
168 Mahony [34].

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

175

176 **Inflammation versus immunity**

177 Previously the composition of the cells that infiltrate the site of infection was briefly
178 described as comprising neutrophils, eosinophils, macrophages and lymphocytes of which
179 CD4 and γδ-TCR+ make up a large proportion of the infiltrating lymphocytes. Associated
180 with this knowledge is the finding that sheep are able to mount a variety of hypersensitivity
181 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 larvae [38]. 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 1974 [51]. 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 flock [51]. 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) rams [53]. 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, α1-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 challenge [41]. 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

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

211

212 **Vaccine responses**

213 Over the years a number of vaccination strategies have been investigated using a wide range
214 of larval antigens, reviewed by Elkington and Mahony [34]. The basic premise for vaccination is
215 to induce an immune response in the host that would inhibit larval growth and ultimately
216 survival. Ideally such an approach would result in a reduced dependence on insecticides
217 providing protection over critical periods during the season when flies are most prevalent. A
218 number of reports identified the production of serum antibodies to various larval antigens post
219 vaccination and subsequent anti-larval growth effects *in vitro* [54-61] however these results
220 generally did not translate into significant protection following implants with first stage larvae
221 *in vivo*. The reasons for this apparent inability to translate *in vitro* effects to the *in vivo*
222 situation were suggested to include insufficient antibody titres present in the skin, the
223 degradation of IgG at the wound site and the time required to reach peak antibody titres in the
224 sera [40,62,63]. In contrast to the reports where vaccination failed to illicit a protective response *in*
225 *vivo*, was the study by Bowles *et al* [64] in which vaccination with four partially-purified
226 antigens induced an 86% (Trial 1) and a 67% (Trial 2) reduction in the incidence of strike
227 compared to unvaccinated controls. In addition, larvae recovered from vaccinated animals
228 (Trial 1) were up to 85% smaller than their control counterparts. It was also noted that
229 antibody titres from the protected sheep failed to correlate with protection which is not
230 inconsistent with previous efforts to correlate protection with antibody titres. However, what

231 was very interesting was the presence of cellular foci described at the site of challenge in
232 protected animals, which consisted of CD1+ Langerhans' cells, CD4+ T helper and γδ-TCR+
233 cells, and these foci may have provided an early immune response upon larval challenge in
234 primed animals that significantly affected the ability of the larvae to successfully establish[64].
235 While delayed-type hypersensitivity responses (DTH) were also measured post larval
236 implantation this parameter did not correlate with protection.

237

238 **Inhibitors of immune control - immunosuppression**

239 Producing an effective vaccine against the sheep blowfly presents numerous significant
240 challenges as attested by the results obtained thus far for vaccination attempts. There are
241 many factors to consider including the choice of antigen(s), the type of immune response to
242 be elicited (humoral vs cellular or both), the route of administration, (subcutaneous,
243 intramuscular or possibly intradermal), the choice of adjuvant or immunomodulators and the
244 number and timing of immunisations. These factors are not trivial issues to be researched and
245 whilst some progress has been made a significant body of research remains incomplete if a
246 blowfly vaccine is to be effective. Another important point to consider when seeking to
247 produce an effective vaccine is the ability of the larvae to influence the immune response of
248 the host. Such interactions are common place in the parasite world [65]. With respect to the
249 sheep blowfly the ability of the larvae to degrade host immunoglobulin [62] is one clear
250 example whereby the larvae are capable of avoiding the host humoral immune response. In
251 addition, excretory-secretory (E/S) products produced by the larvae have been shown to be
252 able to directly influence the immune response at both the cellular and humoral levels [66]. The
253 results from this study demonstrated that the proliferation of ovine lymphocytes could be
254 suppressed *in vitro* following exposure to the larval products but interestingly this suppression

255 could be reversed. Elkington et al.[67] reported that a 56 kDa protein from E/S material was
256 shown to be capable of significantly inhibiting lymphocyte proliferation and it was proposed
257 that this particular protein may have an immunomodulatory role during blowfly infections.
258 These types of responses are not surprising given the lack of protective immunity
259 demonstrated against this parasite in the field and what is known from other parasites as they
260 have evolved to avoid host immune responses [68]. What will be challenging from a vaccine
261 perspective is to identify the key immunomodulatory molecules produced by the larvae as
262 presumably these too would be useful vaccine candidates.

263

264 **New approaches to control.**

265 As mentioned previously, an understanding of the interaction between the sheep host and the
266 larvae is critical to the development of an effective vaccine strategy. Previous research on this
267 interaction has been performed largely in the absence of genomic data and produced a few,
268 partial sequences of potential vaccine candidate proteins [69-71]. An exception to this is the
269 extensive work that was conducted on the peritrophins from Lucilia in which a number were
270 cloned and sequenced [57,58,72]. The sheep genome (http://www.sheephapmap.org) and the
271 nearly completed *L. cuprina* genome (5K genome project)
272 (http://www.arthropodgenomes.org/wiki/Main_Page), combined with the power of mass
273 spectrometry and transcriptomic data analyses, should result in the completion and
274 characterization of these partial proteins and in the identification of additional novel potential
275 candidate target genes for both vaccine and drug development purposes. Transcriptomic and
276 proteomic analyses of blowfly larvae and sheep during infection, should deliver novel insights
277 into the sheep's immune response and result in the assembly of blowfly secretomes; a wide
278 range of secreted proteins of different larval stages that are potential candidates for vaccine

279 design. Furthermore, essential genes are ideal candidates for drug targeting in the blowfly and
280 can now be inferred from the available information on the related non-parasitic fly species,
281 Drosophila melanogaster and validated using molecular knockout techniques, such as
282 CRISPR [73].The missing component in the delivery of these molecular techniques and vaccine
283 development is adequate and sustained funding which has been largely unavailable from
284 industry or other sources for over ten years, despite the mulesing controversy and the obvious
285 importance of developing alternative therapies.

286

287 **Other Blowflies**

288 Vaccine research in other blowfly species has been very limited and has concentrated mainly
289 on the screwworms which penetrate deep into their hosts after infecting through wounds or
290 body orifices. As a result they can cause rapid mortality especially in young and stressed
291 animals. They are also non-selective in their host range, successfully infecting most available
292 mammals and some birds [74]. *Cochliomyia hominovorax*, the new world screwworm has been
293 controlled in a large part of its previously endemic range via the release of genetically-altered
294 'sterile' male flies [5]. However, its persistence in South America and occasional incursion
295 elsewhere [75] suggests that additional controls are still needed. *Chrysomya bezziana*, the old
296 world screwworm fly, is endemic in Asia and Africa [76]. It has long been a major quarantine
297 concern in Northern Australia with significant populations of the fly in the Torres Strait [77].

298 *Chrysomya bezziana*

299 In common with Lucilia, the screw-worm, *Chrysomya bezziana* secretes highly active
300 products onto the host. These consist of a mixture of enzymes and toxins that inhibit
301 coagulation, degrade complement components, lyse cells and degrade skin matrix proteins [78].
302 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 trails 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

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

330 Cellular responses to invading first instars have been described in artificial infections and
331 show very little change in cell types associated with primary infections [86]. The infiltration of
332 B cells and IgG positive cells shortly after challenge infections in perivascular areas was
333 dramatic and rapid [86]. Local and systemic cytokine responses in the first few days of both
334 primary and challenge infections have been reported by [87] who suggest that the bovine
335 response is framed by both Th1 (increase in IFN-γ) was well as Th2 (increases in IL-4 and IL-
336 10) responses. Similarly, inflammatory cell responses in skin of primary and challenge
337 infections [88] showed an increase in $CD4^+$ during the early phase of primary infections while B
338 cells were predominant in challenge infections and the numbers increase in association with
339 the number of previous infections.

340 Cytokine and antibody responses have been recently characterized in naturally infested
341 animals during the later phases of the infection by Vasquez et al [89] and Panadero et al [90].
342 These authors suggest that in natural infections the cytokine profiles were less clear than in
343 artificial, pulse infections [87,88] and a similar situation was observed for inflammatory cells.
344 IL-10 was higher in challenge infections which they interpreted as allowing reduced
345 inflammatory responses which increased the survival of migrating first instars. They noted
346 that the inflammation regulatory cytokine IL-10 declined rapidly after larvae had exited the
347 warbles which was consistent between primary and challenge infections, suggesting that this
348 cytokine was important in maintaining the host granuloma from which the second and third
349 instars derive their nutrient.

350

Vaccine natural antigens

The earliest work on vaccines dates to the 1950's [91] and 1960's [92,93]. These studies used whole third instar antigens and the vaccine was delivered after animals were naturally infested. Baron and Colwell [94] reported the use of native hypodermins as vaccine components with the addition of monophosphoryl lipid A as an adjuvant. Pruett [95] 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

Colwell [98] 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 production [99]. These proteins have also been identified by Roelfstra et al [8] 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], *Psoropotes 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 allergen [104] and is found in parasitic nematodes where it has been suggested as a potential vaccine candidate [105]. Phenoloxidase is a major component of the insect immune system that participates in encapsulation and wound healing through formation of melanin [106].

Recombinant antigens

The gene sequences for the three major proteins secreted by first instar *Hypoderma* spp, were first reported in the early 1990s [107], 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

442 then identify an antibody in immune sera that recognised an immunodominant 50 kDa antigen
443 [121]. This was isolated from the soluble extracts of whole Dermatobia larvae, of all three
444 instars and used to vaccinate cattle. This approach yielded a greater than 90% reduction in the
445 number of surviving larvae after challenge.

446 The success of the approach suggests alternative vaccine antigens to the established use of the
447 Hypodermins in other warble flies [88] as the molecular weight of this antigen is almost twice
448 that of these serine proteases. Other studies in Hypoderma have indicated the presence of
449 tissue-derived antigens at about this molecular weight [98].

450 *Oestrus ovis*

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

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

459 These larvae tend to be very common in their hosts infecting from 30 to 50% of flocks in
460 most studies (reviewed in [123]). The larvae produce very similar enzymes to the more invasive
461 oestridae [124] and the major antigens recognized by the host are excretory/secretory products
462 including salivary gland antigens [125]. However, immunisation with E/S products did not show
463 any protective effect other than limited growth inhibition [126]. Use of 'hidden antigens' derived
464 from washed third instar digestive tracts had also no effect on larval survival, but reduced the
465 size of the larvae, primarily of second instars [126].

Gasterophilus

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

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

CONCLUSION

The issue for those concerned with the development of new controls for myiasis diseases is the continuing reliance in insecticides over the last 50 years and the lack of current research towards alternatives. This is especially true of vaccine work where no significant funding has been available since 2000. Although this could be argued as due to a lack of potential candidate vaccines in the case of the sheep blowflies (Lucilia) there have been good candidates for Hypoderma control for at least this period [94].

Clearly the drugs currently available, which include the macrocyclic lactones and the insect 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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