First *in vitro* cycle of the chicken mite, *Dermanyssus gallinae* (DeGeer 1778), utilizing an artificial feeding device

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SUMMARY

The red poultry mite, *Dermanyssus gallinae*, is one of the most economically deleterious ecto-parasites of layer hens worldwide. *D. gallinae* is difficult to eliminate from infested poultry farms, and even to study, because it resides on the host only during the bloodmeal at night, and hides in the crevices of poultry houses during the day. Here, the life-cycle of *D. gallinae* was reproduced entirely *in vitro*. Mites were incubated in a glass pipette at 30 °C, 60–95 % relative humidity and total darkness. A feeding apparatus, composed of a membrane, reservoir and blood was fitted on the pipette during bloodmeals. We tested feeding rates on blood mixed with 1 of 3 anti-coagulants (EDTA, heparin and trisodium citrate) at different concentrations, and biological and artificial membranes. The best engorgement and survival rates for all 3 haematophagous life-stages of the parasite were observed in 1-day-old chick membranes and heparinized (0·02 mmo/ml) blood. We then describe the steps in developing a complete self-sustaining *in vitro* life-cycle. A colony of mites was maintained *in vitro* for 7 generations. Losses in the first generation were heavy, but survival had multiplied 5-fold by the fifth generation. We hypothesize that heavy mortality rates during the first life-cycle correspond to selective pressure: only the mites which fed and survived *in vitro* were able to reproduce.

Key words: *Dermanyssus gallinae*, *in vitro* life-cycle, blood, anticoagulant.

INTRODUCTION

The poultry red mite, *Dermanyssus gallinae*, is the most economically deleterious ectoparasite of layer hens (Chauve, 1998). The red mite is an obligatory haematophagous ectoparasite of both domestic and wild birds (Arends, 1997), but occasionally feeds on mammals, including man (Auger *et al*. 1979). Dermatitis has been reported by poultry house workers in infested poultry houses (Evans, 1992; Chauve, 1998). Furthermore, *D. gallinae* has been suspected as a vector of several major pathogenic diseases, i.e. eastern equine encephalomyelitis, avian smallpox, avian cholera and salmonellosis (Zeman *et al*. 1982; Durden, Linthicum & Turell, 1992; Wallade *et al*. 1993).

D. gallinae lives in the cracks and crevices of poultry houses. The parasite hides during the day, and takes its bloodmeals at night. Under optimal conditions, *D. gallinae* can complete its life-cycle in as little as 10 days (Reynaud, Chauve & Beugnet, 1997). After mating, females take a bloodmeal on their host, then lay 3–4 eggs. A larva hatches out of each egg, and moults into a protonymph after only 2 days. The protonymph takes a bloodmeal before moulting into the second nymphal stage, or deutonymph, which in turn takes a bloodmeal before moulting into an adult.

Unfortunately, it is extremely difficult to free poultry houses of mites once an infestation has occurred. Not only are the mites nocturnal, highly prolific, and only remain on the host for short periods of time, but they are also developing acaricide resistance (Beugnet *et al*. 1997; Beugnet, Chauve & Zenner, 1999). These properties render the study of mites extremely necessary but difficult.

Mite populations have already been raised in the laboratory (Reynaud *et al*. 1997). But until present, the bloodmeals of laboratory-raised mites took place on live chickens. This system limited the scope of research to individual life-stages: it was impossible to collect all the mites after a bloodmeal, and therefore to control the population composition and genetics over several generations. Kirkwood (1971) and Zeman (1988) attempted to replace bloodmeals on live animals with *in vitro* bloodmeals on artificial membranes. Although these authors obtained *in vitro* bloodmeals, they did not report subsequent mating, moulting, or egg laying. Accordingly, there are no reports of complete *in vitro* life-cycles of haematophagous acarians, ticks or mites.

This report describes the development of an *in vitro* feeding technique for *D. gallinae* which ultimately resulted in complete *in vitro* life-cycles. We

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discuss factors that affect the feeding response, survival rate and moulting/egg laying rate of gorged poultry mites. Finally, we report a mite population which was raised entirely *in vitro* for over 7 generations.

MATERIALS AND METHODS

In vitro *storage conditions*

Mites were kept in an incubator at 30 °C, 60–95 % relative humidity and total darkness.

Parasites

D. gallinae mites were collected from the litter of infested poultry houses. Mites were separated from the litter with a suction pump and a collector. They were then identified in the laboratory, placed in labelled glass vials and stored at least 1 week prior to trials to eliminate the effects of any previous bloodmeals i.e. digestion, moulting, and/or egg laying.

Feeding device

The *in vitro* feeding device consisted of a Pasteur pipette and a blood reservoir separated by an artificial membrane. The main chamber of the pipette, which contained the mites, was fitted with a folded piece of filter paper (Papier à filtrer 'blanc Prolabo', Prolabo Creteil, France). Mites were sucked into the chamber by fitting the larger diameter of the pipette with a filter and tubing attached to a suction pump. Once the mites were in the chamber, the larger opening was covered with a filter tip (Filter Tips 1000 (G), Greiner Labortechnik Kremsmünster, Austria), and the smaller opening was sealed with parafilm (Parafilm 'M' laboratory film, Prolabo). During bloodmeals, a 2×2 cm square of membrane was stretched over the large opening and held tightly in place with a 1 ml pipette tip, which also served as a blood reservoir.

Artificial membranes

Biological membranes were prepared from the following animals: adult chickens (*Gallus gallus*), 1-day-old chicks, turkey hens (*Meleagris gallopavo*) (Grimaud frères, Roussay, France), and nude mice (*Mus musculus* Nu/Nu) (Iffa-Credo l'Arbresle, France). All animals were received live, euthanized and, if necessary, hand plucked. Skin was then carefully dissected from the corpses, scraped free of fatty subcutaneous tissue, rinsed in 0·09 % saline solution, dried with paper towels and either stored at +4 °C or frozen at −20 °C. Biological membranes were placed on the feeding device so that the external side faced the mite chamber. Parafilm membranes were stretched to 10 times their original size. Red mite feeding preferences for 2 types of synthetic membranes (Film plastique Parafilm 'M', Prolabo Creteil, France) and a commercial skin substitute (pansement hydrocolloïde 0·3 mm Urgomed N.D., Urgo Reims, France) and 4 biological membranes were tested. The influence of membrane freezing or refrigeration on mite feeding was also tested. Each trial was conducted on 3 pipettes of 30 *D. gallinae* each, and repeated 3 times. All animals were housed and treated in accordance with animal experimentation French law (October 19, 1987).

Blood

Blood was drawn from the occipital sinus of live chickens, mixed with an anticoagulant, and stored either at +4 °C or frozen at −20 °C. Adult female mite preferences for 3 anti-coagulants, heparin (héparine ammonium salt from porcine intestinal mucosa, Sigma, l'Isle d'Abeau, France), trisodium citrate (citrate tri-sodique analar, Polylabo, Strasbourg, France), or EDTA (ethylenediaminetetraacetic acid disodium salt, Sigma, St Louis, USA), at concentrations ranging from 0·005 to 1 mmol/ml were tested. We also tested survival rates 5 days after a bloodmeal with each anti-coagulant. Fresh heparinized (0·02 mmol/ml) chicken blood was utilized for the complete feeding cycle trials. Each trial was conducted on 3 pipettes of 30 *D. gallinae* each, and repeated 3 times.

Feeding trials

Initially, each bloodmeal step was produced separately *in vitro*. Trial 1 was started with unengorged adults and ended after protonymph feeding. Trial 2 was started at the unengorged protonymphs stage, and ended with unengorged adults. Trial 3 began with unengorged deutonymphs, and ended after protonymph feeding. In trial 4, we then linked the steps, in order to obtain a complete cycle, from hatching through egg laying. For the complete cycle to take place, nymph engorgement rates were enhanced by repeating bloodmeals 3 times at 24 h intervals. Similarly, egg laying was increased by repeating bloodmeals 4 times at 24 h intervals. Adults were observed closely, and eggs harvested quickly after laying. Eggs were placed in fresh pipettes in batches of approximately 30, where a new cycle could begin. Finally, an *in vitro* cycle was begun anew, and pursued over 7 successive generations. Trial 1 was conducted on 4 pipettes of 20 *D. gallinae* each, whereas trials 2, 3, and 4 were begun with 3 pipettes of 20 *D. gallinae* each. The final self-sustaining *in vitro* cycle was begun with 5 pipettes of 20 *D. gallinae* protonymphs each.

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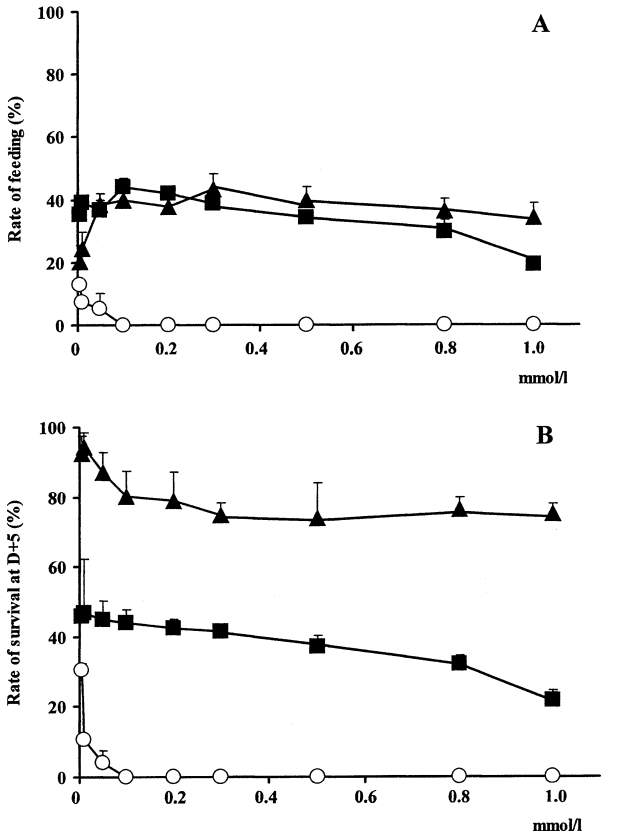

Fig. 1. Effect of anti-coagulant type and concentration on the feeding and moulting rate of adult female *Dermanyssus gallinae*. Values are mean ±S.E.M. (n = 270). (A) Feeding rates of adult female *D. gallinae* fed with blood where coagulation has been prevented by heparin (▲), trisodium citrate (■) or EDTA (○) at different concentrations. (B) Rate of surviving adult female *D. gallinae* fed with blood where coagulation has been prevented by heparin (▲), trisodium citrate (■) or EDTA (○) at different concentrations.

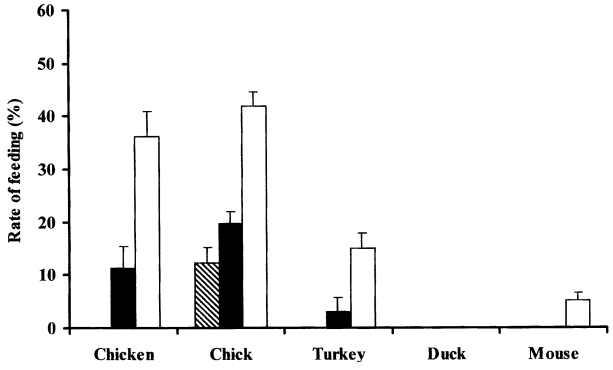

Fig. 2. Effect of skin membrane type on the feeding success of *Dermanyssus gallinae* protonymphs, deutonymphs and adults. Values are mean ±S.E.M. (n = 270). (□) Rate of adult female fed; (■) rate of deutonymph fed; (▨) rate of protonymph fed.

Statistical analysis

The percentage of mites that successfully completed a stage change was compared with a χ^2 test at a 5% confidence level. The overall return on a generation, defined as the ratio of eggs produced in one generation in relation to the previous generation was also compared with a χ^2 test at a 5% confidence level.

RESULTS

Anti-coagulant effects

The *in vivo* engorgement rates of adult female mites fed on chicken blood supplemented with an anti-coagulant at different concentrations are given in Fig. 1. The engorgement rates with 0·01 mmol/ml EDTA was 12·8%, but decreased rapidly as anti-coagulant concentrations increased, and stopped altogether at EDTA concentrations greater than 0·1 mmol/ml. The best engorgement rates were obtained with heparin at 0·1–0·3 mmol/ml and trisodium citrate at 0·05–0·3 mmol/ml. Survival rates 5 days after a bloodmeal decreased as anti-coagulant concentrations increased, irrespective of anti-coagulant used. However, day 5 survival rates greater than 50% were only obtained with heparin; 75% of engorged females survived at 1 mmol/ml heparin, while the best survival rates were obtained at 0·02 mmol/ml. *D. gallinae* survival rates fed on trisodium citrate-treated blood ranged from 21·7% to 45·9%, while EDTA was 100% toxic at doses greater than 0·1 mmol/ml.

Membrane effects

Mites did not feed on artificial non-biological membranes. Engorgement rates of protonymph, deutonymph and adult *D. gallinae* fed *in vitro* on different artificial biological membranes are given in Fig. 2. Protonymphs only fed on 1-day-old chick skin membranes (12·6%). The highest deutonymph and adult engorgement rates were also observed on 1-day-old chick skin membranes (20·6% and 44·2% respectively), although adult engorgement rates on chicken skins were also quite high (36·2%). Only 15·1% adult mites and 3·1% deutonymph fed on a turkey skin membrane. Finally, no bloodmeals occurred on a duck skin membrane, although a few adult mites did feed on a nude mouse skin membrane.

The temperature at which membranes are stored influences feeding success: per day of storage, engorgement rates on refrigerated skins decreased more rapidly than with frozen skins (Fig. 3). Feeding rates for adult female *D. gallinae* on refrigerated or frozen skins dropped from 47·5% on 1-day-old skins to 22·8% and 38·4% respectively after 4 days of storage. The same tendency was observed among the other haematophagous life-stages of *D. gallinae*. Protonymph stopped feeding altogether on skins that had been refrigerated for 3 or more days, and deutonymph stopped feeding on 6-day-old refrigerated skins.

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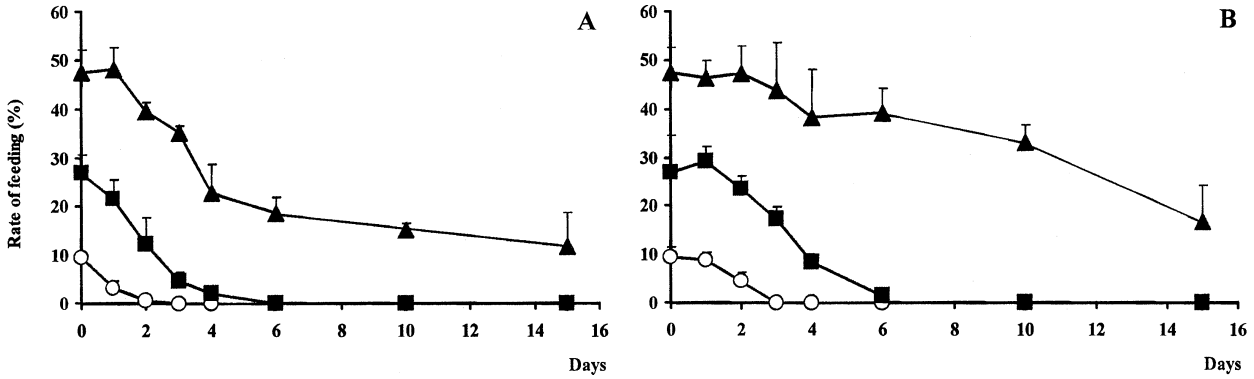
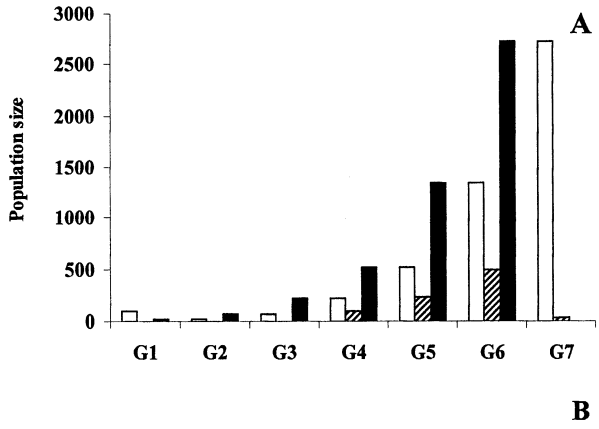


Fig. 3. Effect of membrane conservation technique on the feeding rate of *Dermanyssus gallinae* protonymphs, deutonymphs and adults. Values are mean ±S.E.M. ($n = 270$). (A) Rate of feeding for all haematophagous stage of *D. gallinae* fed with a membrane of chick flesh stored at +4 °C. (B) Rate of feeding for all haematophagous stages of *D. gallinae* fed with a membrane of chick flesh stored at −20 °C. (▲) Adult female; (■) deutonymph; (○) protonymph.

Table 1. Population dynamics of four trial *Dermanyssus gallinae* colonies reared *in vitro*

	Adults				Protonymphs		Deutonymphs		Adults				Protonymphs	
	Non-fed	Fed	Eggs	Larvae	Non-fed	Fed	Non-fed	Fed	Non-fed	Fed	Eggs	Larvae	Non-fed	Fed
Trial 1	80	66	259	232	186	42								
Trial 2					60	37	29	25	13					
Trial 3							60	47	22	10	23	21	16	13
Trial 4			60	57	42	31	27	25	17	16	96	91	85	72



B

	Generation 1	Generation 2	Generation 3	Generation 4
Number of eggs in previous generation (A)	100	27	70	228
Number of eggs in current generation (B)	27	70	228	518
Output	0.27*	2.6	3.26	2.27
Metamorphosis of larva to protonymph (in %)	76.1*	95.2	93.4	90.6
Protonymph feeding rate (in %)	53.7*	90.0	92.9	92.4
Deutonymph feeding rate (in %)	87.5	100.0	93.6	95.5

Fig. 4. *In vitro* cycle of *Dermanyssus gallinae*. (A) Evolution of the *in vitro* population size during 7 generations. (□) Number of eggs of the previous generation; (▨) number of adults of the current generation; (■) number of eggs laid during this generation. (B) Returns on the passage of certain stages to another obtained during the first 4 generations. Values marked with an asterisk are significantly different from unmarked values of the same line ($\chi^2 < 0{\cdot}05$).

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Development of the in vitro *cycle*

Initially, partial development *in vitro* cycles were obtained starting from either protonymph, deutonymph or adults (Table 1). In trial 1, adult mites were placed into the Pasteur pipette and given only 1 bloodmeal. Consequently, egg laying occurred only once per engorged adult (66 individuals), and egg numbers were low (259 eggs). Protonymph feeding and survival rates were low, and the trial was stopped. In trial 2, only 13 adults were obtained from an original 60 unengorged protonymphs. Similarly, in trial 3, 13 gorged protonymphs were obtained from an original 60 deutonymphs. Each trial was stopped when of *D. gallinae* numbers were considered insufficient. However, all 3 trials demonstrated that a complete *in vitro* cycle could possibly be obtained, if *D. gallinae* numbers could be kept sufficiently high to stop the population from going extinct. In trial 4, the feeding, and therefore survival and metamorphosis/egg laying rates were enhanced, and 16 gorged adults were obtained from an original 60 eggs. These adults laid 96 eggs, of which 72 survived to the gorged protonymph stage. Egg numbers were amplified by approximately 50 % from one generation to the next.

In vitro *colony*

The final self-sustaining *in vitro* cycle (Fig. 4A), was pursued for over 7 generations. The third generation egg production (228) more than doubled in relation to the initial egg population (100) and multiplied 5-fold by the fifth generation. Fig. 4B shows that the overall return on a generation increased significantly after the first generation.

DISCUSSION

A controlled, *in vitro* development cycle of *D. gallinae* is an important step towards understanding, and perhaps controlling this parasite. In this study, we defined the feeding apparatus and substrates which were utilized by all haematophagous life-stages of *D. gallinae*. We then produced the parasite's entire life-cycle *in vitro*.

In vitro feeding success can be defined as the number of mites who take a bloodmeal, the number of mites surviving after a bloodmeal, according to results, that is, a bloodmeal is successful if it results in the passage of a haematophagous life-stage to the next (Chabaud, 1950). In this study, the first 2 definitions were utilized to define optimal feeding apparatus and substrate conditions. The latter definition was applied to the final outcome of work: a complete, self-sustaining, *in vitro* life-cycle.

Several authors previously worked on *in vitro* bloodmeals for either *D. gallinae* (Kirkwood 1971; Zeman 1988) or *Ornithonyssus sylviarum* (Crystal, 1986). These authors noted that temperature, light and relative humidity influence feeding rates of both parasites. Here, environmental conditions were fixed at 30 °C, 60–95 % RH and complete darkness, and we focused on the feeding apparatus and substrate which would produce successful bloodmeals.

We first tested feeding preferences for blood mixed with 1 of 3 different anti-coagulants, heparin, trisodium citrate, and EDTA at different concentrations. EDTA was the least preferred anticoagulant, and appeared to be toxic at even low concentrations. *D. gallinae* feeding rates of 40 % on blood at 0·07 mmol/ml EDTA have been reported (Zeman, 1988) under optimal environmental conditions (40 °C, and RH at a constant rate of 95 %). Voigt *et al.* (1993), obtained results similar to ours with *Amblyomna variegatum*. The causes of high mortality rates in this experiment are unknown. However, we suspect that EDTA may interfere with Ca^{2+}-dependent mechanisms in the mite.

Forty percent of mites fed on blood at 0·02 mmol/ml trisodium citrate were alive 5 days after the bloodmeal. Survival rates decreased slightly as anticoagulant concentrations increased. In agreement with Voigt *et al.* (1993) and Kirkwood (1971), the best feeding and survival rates were obtained with heparin, a protein which is naturally present in blood. We therefore utilized heparin at 0·02 mmol/ml blood throughout the remainder of our work.

Voigt *et al.* (1993) and Ward, Lainson & Shaw (1977) noted that the bite itself conditions the successful bloodmeal. Blood-sucking parasites are not attracted to all membranes equally. In fact, all forms of *D. gallinae* 'rejected' both synthetic membranes, but several life-forms fed on 1-day-old chick, chicken and even nude mouse skin membranes. Zeman (1988) suggested that surface lipids may repel or attract biting mites (and therefore promote the subsequent bloodmeal). This may explain why both synthetic and duck skin membranes were rejected by *D. gallinae*. In the first case, no surface lipids are present, and in the second, the surface lipids of aquatic animal skins are chemically far removed from those of terrestrial animal skins (Aslan, Ozcan & Kurtul, 2000; Gutierrez *et al.* 1998). During the preparation process, only subcutaneous fatty tissue was removed. The skins were rinsed in saline solution and patted dry: the surface lipids were left intact. Thus, synthetic membranes were definitely abandoned in favour of biological membranes.

Among all the biological membranes tested, only 1-day-old chick skin membranes were fed upon by all 3 haematophagous stages of *D. gallinae*. In preparing the membranes, we did not specifically select the skins from the finest or thickest part of the

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body surface. Protonymphs have small chelicera (Evans, 1992; Baker & Wharton, 1952), and may not be able to pierce thicker membranes.

Finally, *D. gallinae* will feed on skins that have been frozen for 4 days at −20 °C, as will *O. sylviarum* (Crystal, 1986). Furthermore, *D. gallinae* preferred frozen and newly thawed skins to several day old refrigerated skins. The membranes used in a feeding device can therefore be prepared in advance, frozen and only thawed as needed.

It was necessary to determine what the optimal feeding apparatus and substrate conditions were in order to proceed with the second phase of our work: a complete, self-sustaining, *in vitro* life-cycle. Successful bloodmeals, as defined by Chabaud (1950), were obtained for each haematophagous life-stage with our feeding apparatus. However, after only 1 bloodmeal, the number of individuals completing the step change was judged insufficient to produce a complete life-cycle, and the population was expected to decline to extinction rapidly.

Adult mites may lay up to 5 times in a row, provided they take a bloodmeal between each clutch (Hutcheson & Oliver, 1988). We hypothesized that the number of individuals produced at each step, regardless of life-stage, would be multiplied by increasing feeding opportunities. Therefore, at each step, the parasites were given the opportunity to feed on several successive days. In this manner, the adult feeding rate jumped from 40 to nearly 100%, although, protonymph and deutonymph feeding rates did not increase as dramatically.

This *in vitro* system appears to mimic natural selection. Mites that were unable to feed in the experimental system died, while the adapted animals continued to feed, moult and reproduce. This may explain why the highest mortality rates occurred among first generation protonymph. Egg production rates confirm this hypothesis: the egg population at the end of the first generation was only 27% of the initial load, but rose to 260% and 326% after the second and third generations. At the time of writing, we had raised 7 generations entirely *in vitro*. Nonetheless, new questions will arise as the *in vitro* cycle continues to evolve. Will the fecundity and fertility rates of an inbred mite population drop after several generations more? Will a population raised entirely *in vitro*, and therefore adapted to these conditions, be representative of poultry house mites?

The benefits of an *in vitro* mite rearing system are multiple. We can now study the dynamics of an *D. gallinae* population experimentally, and perhaps even extend this system to other competing mite species. Furthermore, mite populations of interest, for example acaricid-resistant strains, can be maintained and manipulated in the laboratory. Finally, the study of the biology and genetics of the parasite may be facilitated and lead to greater understanding of these pests.

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