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NSF 03-041

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TITLE OF PROPOSED PROJECT **Collaborative Research: Discontinuous Gas Exchange in Insects**

REQUESTED AMOUNT	PROPOSED DURATION (1-60 MONTHS)	REQUESTED STARTING DATE	SHOW RELATED PRELIMINARY PROPOSAL NO. IF APPLICABLE
$ 320,488	36 months	01/01/04	

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PROJECT SUMMARY

Many terrestrial arthropods perform cyclic, discontinuous release of carbon dioxide through precise regulation of the valves (spiracles) that control access to their air-filled respiratory structures (tracheae). For over 50 years our understanding of gas exchange in these organisms has been dominated by the view that water balance was the primary selective force behind this discontinous gas exchange (DGE). This viewpoint arose from early research on pupae from a limited number of insect taxa, primarily several giant silkmoth species. Numerous recent studies, however, have questioned the applicability of the water conservation model to terrestrial arthropods in general, and several alternative hypotheses have been proposed. The chthonic hypothesis proposes that DGE arose as a means to increase the gradients for carbon dioxide release and oxygen uptake in burrows and other hypercapnic/hypoxic habitats. Another hypothesis holds that DGE acts to reduce internal oxygen levels and thereby minimize cellular damage from free radicals. A non-adaptive hypothesis views DGE as emerging from the simple regulatory mechanisms of insect ventilation. None of these hypotheses have been subjected to systematic investigation, and no experiments have been performed to distinguish between models in a rigorous manner.

We propose to test these hypotheses by thoroughly analyzing ventilatory patterns in closely related insects from arid and mesic habitats, from burrowing, arboreal and surface habitats, and from different altitudes. This work will be performed in a rigorous phylogenetic context, using families of beetles from the New and Old World (Tenebrionidae, Carabidae, Scarabaeidae) and fruit flies (*Drosophila*), for which phylogenetic information is available. In addition to testing specific predictions of each model, we will perform critical tests that will enable us to distinguish between models. For example, if the water conservation hypothesis is true, then DGE should be more evident in xeric-adapted organisms and DGE cycles should change in other predictable ways. If the chthonic hypothesis is true, then we would predict that DGE would be more prevalent in fossorial taxa, as compared with arboreal forms. Under the oxidative damage hypothesis, high-altitude species should exhibit DGE less often than low-altitude species. In biochemical tests of these hypotheses, we will measure buffering capacities, carbonic anhydrase levels, and oxidative defense enzymes.

This project will involve the laboratories of three individuals from two continents: Dr. Michael Quinlan (Midwestern University), Dr. Allen Gibbs (University of Arizona), and Dr. Steven Chown (University of Stellenbosch, South Africa). All of these individuals have extensive experience with the techniques and concepts discussed in the proposed research. The broader impacts of this project will include the training of a post-doctoral researcher and graduate student, and all participants will present their work at international scientific meetings. We will broaden participation in scientific research by establishing a close research collaboration between Midwestern University (a non-Ph.D.-granting institution) and the University of Arizona, and by recruiting undergraduates from minority programs at the University of Arizona and Pima Community College in Tucson. The project will performed in close collaboration with Dr. Chown's laboratory in South Africa. This international collaboration will be enhanced by the exchange of students and post-docs between our laboratories.

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BACKGROUND and SIGNIFICANCE

Arthropod Respiratory Systems

The majority of terrestrial arthropods have evolved systems in which gases are distributed to tissues via tubular ingrowths of the body surface. The most effective and well developed of these is the insect tracheal system, which consists of a series of semi-rigid, gas-filled tubes (tracheae) that penetrate the body and tissues, and the spiracles which connect the tracheae to the exterior. The morphology and physiology of insect respiratory systems have been comprehensively reviewed on several occasions (Kaars 1981; Miller 1981; Kestler 1985; Nation 1985).

The spiracles are usually located laterally in pairs (8-10) along the thorax and abdomen. Structurally they are highly variable. Typically the opening leads to a cavity (atrium) from which tracheae arise. The spiracles of most terrestrial insects have closing mechanisms (movable valves) which may be located in the opening itself or at the junction of the atrium and a trachea. The valves may be operated by an opener and a closer muscle, or by a closer muscle that acts against cuticular elasticity. From the atria, the main tracheae branch dichotomously to give rise to smaller diameter tubes, the tracheoles, the smallest of which (< 0.2 μm) penetrate the tissues and become intimately associated with individual cells. Distally, the tracheoles end blindly or may anastomose (Chapman 1998).

Many terrestrial insects, especially those inhabiting dry environments, have their spiracles positioned below the general level of the cuticle or sometimes superficially depressed due to a thickened (raised) surrounding cuticle. Such an arrangement lengthens the diffusion pathway for water and thus increases the resistance to the outward diffusion of water. This feature is often accompanied by the presence of filamentous structures that guard the openings to the various subchambers and/or line the walls of these cavities. For example, spiracles in the cockroach *Periplaneta americana* have honeycomb-like structures lining the atria, either inside or outside the valves, an arrangement that may offer resistance to water passage out of the tracheal system (Miller 1982). Similar structures can be seen in the spiracles of centipedes, particularly those from xeric habitats (Klok et al. 2002).

Another way to reduce the diffusion gradient for water between the tracheal system and the external air is to have the spiracles open into a humid cavity rather than directly to the outside. The subelytral cavity present in flightless desert tenebrionid beetles may serve such a function. The subelytral cavity is an air-filled space between the fused elytra and the abdomen. The abdominal spiracles open into this cavity, and the expired air exits through a single aperture above the anus. It is believed that this arrangement helps lower the rate of respiratory transpiration (Ahearn 1970); however, the extent of this reduction still awaits experimental verification. Recent studies indicate that during rest, air flow is tidal through a single thoracic spiracle, raising concerns regarding the contribution to water economy of the subelytral cavity (Duncan 2002; Duncan and Byrne 2002).

Gas Exchange and Ventilatory Patterns

The movement of atmospheric oxygen via the tracheal tubes to the tissues and the reverse flux of carbon dioxide can occur strictly by diffusion or by a combination of diffusion and convection. In small insects (less than ca. 0.3 mm long), diffusion alone can provide sufficient oxygen to meet tissue needs, including the demands of flight. In larger insects, especially fliers, diffusion must be supplemented with ventilation of the tracheae to provide an adequate supply of oxygen. During rest, pumping movements of the abdominal musculature increase tracheal gas pressures and expel tracheal gas into the atmosphere. Relaxation of these same muscles allows the tracheal system to expand, thus driving the movement of gas into the tracheae. These movements typically continue during flight, and thoracic pumping due to wing movements will provide additional convective flow. Air flow may be in and out of the same spiracles (tidal) or directed, entering the thoracic spiracles and exiting the abdominal spiracles (unidirectional).

In another type of ventilation, the precise control of the spiracles allows a sub-ambient pressure to be maintained in the tracheae with small amounts of air being periodically inspired (Miller 1981). This pattern is referred to as "passive suction ventilation" or "discontinuous gas exchange" (DGE). This process was first reported in the early 1950's (Punt 1950; Punt et al. 1957; Buck & Keister 1955; Schneiderman & Williams 1953, 1955), but a comprehensive physiological explanation was not elucidated until the 1960s by Schneiderman and his colleagues (Schneiderman 1960; Levy & Schneiderman 1966a,b,c; Schneiderman & Schechter 1966; Brockway & Schneiderman 1967).

"Classical" Discontinuous Gas Exchange

The ventilatory pattern of diapausing pupae of the silkmoth *Hyalophora* (Figure 1) is representative of DGE. During the spiracular closed (C) period, O_2 consumption by the tissues lowers the endotracheal PO_2 causing a small drop in the total endotracheal pressure (CO_2 is buffered by extracellular fluids). When endotracheal O_2 levels fall to about 5% (from 21%), the spiracular valves open for very short periods (flutter, or "F" period). Spiracular fluttering allows the bulk flow of atmospheric air into the tracheae, thereby raising the PO_2. The convective flow of air into the tracheae is thought to have the additional effect of retarding the diffusive loss of water vapor from the tracheae. This process continues until CO_2 levels become so high that the spiracular closer muscles relax and the spiracle opens (open, or "O" period). The accumulated CO_2 is discharged during the O period, and the spiracles close again, thereby repeating the cycle. In larger species capable of abdominal ventilation, a period of pumping (ventilation, or "V" period) may occur in place of the O period (Miller 1981).

The primary controller of ventilation and spiracular caliber in insects appears to be CO_2 (Wigglesworth 1972; Miller 1974, 1981; Kaars 1981). The influence of CO_2 is usually mediated through changes in the pH of the hemolymph and body fluids (Snyder et al. 1980). pH changes, in turn, are determined by the buffering capacity of these fluids and the rate of CO_2 excretion. Oxygen tension can also exert control over spiracular movements, although its influence is usually secondary to that of CO_2. The spiracular muscles, whose contractions cause the spiracular apertures to either open or close, are controlled by motor neurons and, in some cases, by local levels of CO_2.

Spiracular movements are also influenced by the humidity and the hydration state of the arthropods. These factors exert their influence directly or in association with CO_2 levels or hypoxia. Miller (1964a,b) was one of the first investigators to show that fully hydrated insects (dragonflies) tend to relax spiracular control mechanisms more so than do individuals which are partially desiccated. In mosquitos, spiracles open for a shorter period of time at 5% RH than they do at 90% RH, regardless of the hydration state of the animal (Krafsur 1971). Bursell (1970) found a strong correlation between the rate of water loss from tsetse flies at different relative humidities and the degree of spiracular opening. Similar relationships between humidity and/or hydration state and spiracular control also have been reported for crickets and locusts (Edney 1977). Abdominal pulsations have also been cited as

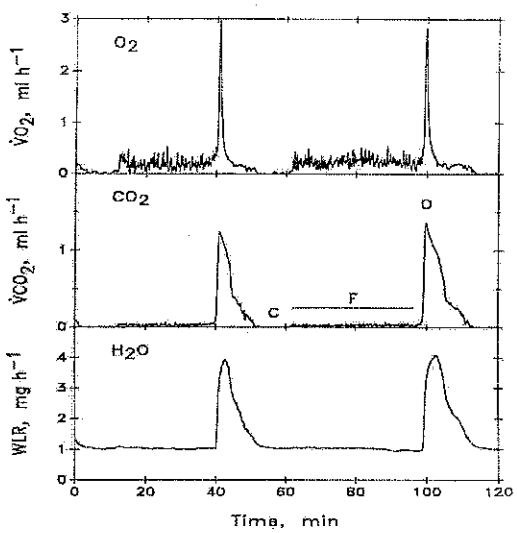

Figure 1. O_2 uptake, CO_2 excretion and water loss (WLR) from a diapausing *Hyalophora* pupa at 20 °C. Note the **closed (C)**, **flutter (F)** and **open (O)** periods in each cycle of discontinuous ventilation. The small excursions in the O_2 and CO_2 traces during the F period represent spiracular fluttering (data from M. Quinlan, unpublished).

important contributors to the regulation of gas exchange cycles (see Sláma 1994, 1999), but recent work suggests that their role is much less significant than originally thought (Tartes et al. 1999, 2000).

DGE cycles are known from a variety of insects and other arthropods. However, the extent to which this phenomenon is consistently present within a higher taxon is not clear (Lighton 1994, 1996). In large measure this is due to the narrow range of taxa that have been examined. DGE cycles were originally described from lepidopteran pupae (Punt 1950; Miller 1974; see also Hetz et al. 1999) and were subsequently found in cockroaches (Wilkins 1960; Kestler 1985), some beetles (Punt 1950, 1956; Lighton, 1991; Davis et al. 1999), a variety of ants (Lighton 1988, 1992; Appel et al. 1991; Lighton et al. 1993), grasshoppers (Hamilton 1964; Hadley & Quinlan 1993; Rourke 2000), wasps (Gibbs and Klein, in prep), and honeybees (Lighton & Lovegrove 1990). Outside the insects, some ticks (Lighton et al. 1993; Lighton & Fielden 1995; Fielden et al. 1999), soliphuges (Lighton & Fielden 1996), pseudoscorpions (Sláma 1995; Lighton & Joos 2002) and centipedes (Klok et al. 2002) exhibit DGE. However, velvet mites (Lighton & Duncan 1995) and harvestmen (Lighton 2002) do not, nor do many tenebrionid beetles from North America (Lighton 1996; Quinlan & Lighton, unpublished). Few other higher-order arthropod groups have been examined, and careful characterization of gas-exchange cycles is rare outside of a few species of ants and beetles (Lighton 1996). Hence, the broad applicability of hypotheses regarding the origin and adaptive significance of DGE remains uncertain (Lighton 1996, 1998).

Adaptive Significance of DGE - Water Conservation?

Because of the prolonged periods during which the spiracles remained closed, DGE often has been cited as being an adaptive mechanism for conserving water. The airways inside the tracheal system are saturated with water vapor, so there will typically be a gradient for diffusion of water vapor out of the insect into the surrounding atmosphere. Closing the spiracles blocks this route for water loss. If gas exchange during the flutter period is primarily convective (as has been demonstrated in moth pupae, roaches and some ants; Levy & Schneidermann 1966c; Kestler 1985; Lighton et al. 1993), then water loss will also be reduced. However, it has not been experimentally verified that intermittent release of CO_2 results in an overall savings of water relative to that which would be lost during a more regular pattern of spiracular opening and closing. To the contrary, Kanwisher (1966) found that the spiracular valves of *Hyalophora* pupae remained open longer than was necessary for effective CO_2 release, resulting in a greater loss of water than CO_2 during the later portions of the burst period. Similar results have been obtained in ants (Gibbs and Johnson, submitted).

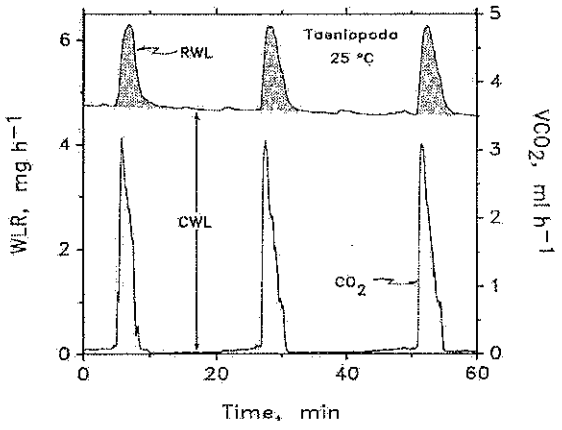

Figure 2. Superimposed traces of water loss (upper trace) and CO_2 release (lower trace) from the lubber grasshopper *Taeniopoda eques* at 25 °C. Stippled areas represent respiratory water loss (*RWL*); the balance is presumed to be cuticular water loss (*CWL*).

Recent advances in flow-through respirometry have made it possible to quantify spiracular water loss by simultaneously measuring carbon dioxide release and water loss from a single individual (Figure 2). Water-loss rates do increase when the spiracles open, but in many cases the increase is comparatively small. Furthermore, in several studies (e.g. Williams and Bradley 1998; Shelton & Appel 2001b) water loss was not lower during cyclic respiration than during acyclic patterns. These results suggest that spiracular water loss is a minor component of total losses, with cuticular losses accounting for the major fraction. Thus, reductions in cuticular permeability are more likely to be

important in adaptation to xeric habitats. Only when cuticular transpiration has been drastically reduced do respiratory losses begin to constitute a significant fraction of total water loss (Zachariassen et al. 1987; Zachariassen 1991; Hadley 1994; Chown 2002).

As more species have been examined, it has become apparent that the specific details of ventilatory patterns differ widely. For example, the F period is often lacking (Quinlan & Lighton 1999; Rourke 2000; Duncan & Byrne 2000; Shelton & Appel 2001a,b; Chown 2001; Gibbs et al. 2003). Recall that the F period is a critical feature of the water-conservation model, since it is during this period that convective air flow allows oxygen uptake with minimal water loss. In some cases, fluttering occurs during the open period, when convective air flow would not be expected at all (Duncan and Byrne 2000), while in others, the F period is virtually continuous (Tartes et al. 2000). Another confounding factor is intra-specific variation. The regularity, or even presence, of DGE can vary between conspecifics of some taxa (Hadley & Quinlan 1993; Rourke 2000; Chown 2001). Furthermore, individual insects may exhibit respiratory behaviors that range from regular DGE cycles to chaotic gas exchange patterns in the same experiment (Chown 2001; Klein and Gibbs, in prep). In some cases this variability may be related to measurement temperature (see Table 1), but in many cases the cause of the variation is unclear (Chown 2001). Clearly there is a requirement to assess variability in gas exchange parameters within and between individuals using standard estimates of repeatability (Lessells & Boag 1987). This has rarely been done for insect gas exchange and metabolism.

In addition, DGE tends to break down at higher temperatures, when the vapor-pressure gradient for water loss is greatest (Quinlan & Hadley 1993; Quinlan & Lighton 1999; Chappell & Rogowitz 2000; Rourke 2000). Thus, insects appear to lose spiracular control when it would be most advantageous for water conservation. Finally, in the few cases where desiccated arthropods have been examined, all have abandoned DGE when total body water was reduced (Hadley & Quinlan 1993; Hadley 1994). These data do not support the hypothesis that DGE is an adaptation for water conservation. Together with studies indicating that spiracular water loss is minor relative to cuticular transpiration, many researchers have begun to question the traditional, textbook explanation for DGE (Chown 2002).

Alternative Models for Discontinuous Gas-Exchange Cycles

The renewed interest and controversy surrounding DGE has stimulated several alternative hypotheses. None has achieved anything near universal support, even from their own authors. Indeed, none of them have really been subjected to serious testing. The primary goal of the proposed research is to distinguish among these models and to identify how DGE originated and is maintained in extant insects.

Hypercapnia (Chthonic)

Lighton and Berrigan (1995) proposed that DGE originated in subterranean arthropods that were exposed to hypercapnia. The fundamental idea behind this "chthonic" model is that carbon dioxide produced by metabolism can not be released against a high environmental PCO_2. By maintaining closed spiracles, insects could increase internal CO_2 levels enough to provide a strong gradient for diffusion out of the tracheal system. Of course, even with open spiracles carbon dioxide levels will eventually build up enough for release to occur; the potential benefit of DGE is that CO_2 is released rapidly, thereby minimizing water loss via the spiracles. This model postulates that maximizing the CO_2:H_2O loss ratio is the primary function of DGE. Alternatively, this model allows that DGE may have originated in hypoxic environments (which are also often hypercapnic), for similar reasons. By allowing internal PO_2 to drop, closed spiracles allow a larger oxygen gradient to develop, so that oxygen can be taken up with minimal water loss.

Note that, no matter whether hypercapnia or hypoxia was the original selective factor, reduction of respiratory water loss while ensuring adequate gas exchange is the important consideration. DGE may be retained in insects that do not currently experience these conditions, because of its benefits for water

conservation. It should otherwise not be retained, however, due to the physiological costs associated with fluctuating internal conditions (especially pH). Of the alternatives to the water-loss hypothesis, the chthonic hypothesis has received the most attention. Chown and Holter (2000) argued that the chthonic hypothesis was unlikely to explain ventilatory patterns in the dung beetle they studied, which often occupies low O_2 and high CO_2 environments in dung pads (Holter 1994; Holter & Spangenberg 1997). Likewise, Shelton and Appel (2001b) considered this hypothesis unlikely to explain cyclic ventilation in the termite they examined.

Oxidative Damage

Another hypothesis has been published only as an abstract (Bradley 2000) and therefore has received no experimental attention to date. It argues that normoxic internal oxygen levels are deleterious, primarily due to cellular damage caused by oxygen free radicals. Under this hypothesis, DGE acts to restrict oxygen levels and is relinquished only during high metabolic demand.

Interacting Oxygen and CO_2 Setpoints

Finally, DGE may not have a specific adaptive function, or at least may not have originated for one. At the cellular level, spiracular opening is regulated by the internal partial pressures of O_2 and CO_2. The spiracles close when O_2 levels rise, and open when CO_2 levels increase (or, in the case of pseudoscorpions, internal hypoxia triggers spiracle opening; Lighton & Joos 2002). A simple, two-setpoint regulatory system such as this can easily exhibit a biphasic pattern, rather than a continuous open or closed condition. Thus, DGE cycles may arise simply as an emergent property of the regulatory mechanisms (Bustami & Hustert 2000; Chown & Holter 2000). This does not preclude their evolutionary modification for purposes such as water conservation, so this non-adaptive model is not mutually exclusive from the adaptive models proposed. In the context of this proposal, this is the null hypothesis.

Rationale for the Proposed Research

The mechanisms and evolution of gas exchange are critical to understanding the success of any group of organisms. Insects are the most successful animal taxon, but major questions in insect respiration remain. Until the last decade, it was "clear" that insects used DGE as a mechanism to conserve water. Now the field is wide open, with multiple competing hypotheses (Chown 2002). We are not aware of a single study that rigorously distinguishes between any two of these hypotheses. Our goal is to use the complementary strengths of three labs (two North American, one South African) to test these hypotheses and to determine the function of DGE. The labs involved are those of Michael Quinlan (Midwestern University), Allen Gibbs (University of Arizona), and Steven Chown (University of Stellenbosch). This collaboration will provide a unique opportunity to examine DGE in a wide range of mesic and xeric arthropods.

Preliminary Data

Each of our lab groups has independently performed comparative studies of DGE, primarily in the context of water balance. In several of these cases, DGE was most pronounced in xeric species. Taxa examined include ants (Quinlan & Lighton 1999), beetles (Davis et al. 1999, 2000; Bosch et al. 2000; Chown & Holter 2000, Chown & Davis, in press), centipedes (Klok et al. 2002), grasshoppers (Quinlan & Hadley 1993) and fruitflies (Gibbs et al. 2003). Within a single species, Bryan Rourke, a student in the Gibbs lab, demonstrated that DGE is much more prevalent in grasshopper populations from lower elevations and more xeric habitats (Rourke 2000). These studies tend to support the water conservation model, but they do not address any of the competing hypotheses.

North American tenebrionids: differences from South African relatives

Preliminary work on North American tenebrionid beetles by Quinlan and colleagues does not support the water conservation model. Tenebrionids from southern Africa provide one of the best

examples of DGE in xeric insects (Lighton 1991). DGE is universal in species from the Namib Desert and less common in more mesic species (Lighton 1991; Chown, unpublished). In contrast, a survey of North American tenebrionids indicates that DGE is rare, even in beetles from an extremely arid region of the Mojave Desert (Table 1). When DGE does occur, it is at temperatures well below even the nighttime temperatures experienced in nature.

Table 1. Ventilatory patterns in tenebrionid beetles collected in July from sand dune areas around the Desert Studies Center (DSC) at Zzyxx, CA (Quinlan and Lighton, unpublished). The DSC is a field station near Barstow CA. Mean temperature in July at Zzyxx is 34°C and the mean yearly rainfall is 3.5", so this site is clearly very xeric.

Species	Temp	N	DGE
Eleodes armata	15 & 25 C	8 per temp	no, though bursts present
Eleodes carbonaria	25 C	5	no, though bursts present
Cryptoglossa verrucosa	15 & 25 C	8 per temp	some DGE activity at 15, none at 25
Cryptoglossa muricatus	15 & 25 C	8 per temp	some DGE activity at both temps
Edrotes ventricosus	15 & 25 C	8 per temp	strong DGE in most at 15, none at 25
Trogloderus castatus	25 C	2	none
Triorophus laevis	25 C	3	none
Eusattus muricatus	25	2	none

Ventilation in carabid beetles

Arizona has a diverse and abundant carabid fauna, including numerous tiger beetles, bombadier beetles, etc. In comparison to other beetle families, the phylogeny of the carabids is well-described, including a taxon-wide analysis of the evolution of arboreality by Karen Ober, a recent graduate from the University of Arizona (Ober 2001). Gibbs has recently been working with Wendy Moore, a graduate student in Entomology, who is developing a molecular and morphological phylogeny of bombadier beetles in the Carabidae. Her research includes fossorial, surface-living, and arboreal species. Our preliminary data indicate that DGE occurs in some, but not all carabids.

Non-classical ventilatory patterns

As noted above, the textbook description of DGE involves the closed, flutter and open (or sometimes V) period. The flutter period is a critical element of the water conservation model, since this is the stage when convective air flow allows oxygen uptake with minimal water loss. However, many species that exhibit discontinuous gas exchange lack the F period, all or part of the time, including ants (Quinlan & Lighton 1999), grasshoppers (Rourke 2000), termites (Shelton & Appel 2001a, b), weevils (Chown 2001), centipedes (Klok et al. 2002), and wasps (Klein and Gibbs, in prep). Single individuals may even switch between cycles with and without the flutter period.

Fruitflies of the genus *Drosophila* frequently exhibit this "non-classical" (no F period) variant of DGE. Fruitflies are small enough that diffusion can support all of their necessary gas exchange, at least while at rest. Gibbs et al. (2003) have observed cyclic CO_2 release in six species of *Drosophila,* with two desert species exhibiting cycles much more often than four mesic congeners. In addition, the amplitude of the CO_2 peaks is greater in xeric *Drosophila*, and DGE occurs when flies are active (walking) and not only while they are quiescent. Yet all of the species examined by Gibbs cycle without F periods, so it appears unlikely that DGE benefits the water balance of these organisms. Another example of "no F period" DGE can be seen in the harvester ant genus *Pogonomyrmex* (Quinlan & Lighton 1999; Gibbs and Johnson, submitted). Cyclic CO_2 release is common in these ants, yet F periods are rare, even in quiescent ants.

EXPERIMENTAL PLAN

Our labs have been examining the role of discontinuous gas exchange independently of each other. We have used similar, but not identical, techniques and have sometimes even used the same species (e.g. *Pogonomyrmex* spp. by Quinlan and Gibbs). As noted above, some of the controversy over the function of DGE can be attributed to experimental differences. By working together and developing standardized measurement procedures, we will remove the problems associated with techniques and be able to concentrate on the biology of the process.

A. General Experimental Procedures

We will use flow-through respirometry to study ventilatory patterns in insects (see Hadley 1994, for an overview of techniques). Each lab will use similar equipment, and we will coordinate to standardize measurement conditions across labs. The measured physiological parameters will include:

- Mass and standard metabolic rate
- Length of the C, F and O periods (when present)
- Whole animal water loss
- Presence or absence of DGE
- Volume of CO_2 released during each period
- Respiratory water loss (when possible)

Test animals will be used as soon after capture as possible. We will also conduct repeatability experiments to ensure that ventilation patterns are not affected by acclimation to laboratory conditions. These experiments will require re-testing the animals 48 to 72 h after their initial test.

B. Choice of Species

B.1. Broad comparisons of ventilatory patterns

Very few arthropod taxa have been examined with regard to ventilatory patterns (mostly lepidopteran pupae, grasshoppers, ants, and some South African tenebrionid beetles). Because the water-conservation model of DGE has been so widely accepted, almost all of these studies have examined only insects from arid habitats. In addition, there is probably a severe reporting ("file-drawer") bias; physiologists tend to stop working on species that do not exhibit DGE (Lighton 1998). An important aspect of this project will be to examine as diverse a series of arthropods as we can, from a diverse array of habitats. We need to identify which species, castes, developmental stages, etc. exhibit DGE and which do not, and we need to report this information.

The Chown lab is investigating the occurrence of DGE across all major insect orders, under funding from the National Research Foundation of South Africa. They will obtain representative material from several different species within each of the orders, because the absence of DGE from one species does not signify its general absence from the taxon. We recognize that, although the evolutionary relationships of insect orders are relatively clear (Beutel & Haas 2000), these broad comparisons will not provide a proper phylogenetically-based understanding of the evolution of DGE. In many orders, however, the Chown lab will be able to obtain information on multiple species from different habitats (e.g. flies, ants, beetles and grasshoppers from arid and mesic habitats). If each habitat comparison within an order provides a similar result, then conclusions regarding the adaptive significance of DGE across insects in general are strengthened considerably. The Quinlan and Gibbs labs will contribute data from additional species to complement the Chown lab's efforts, but will concentrate on a few target taxa for which phylogenetic information is available.

B.2. Phylogenetic analyses of ventilatory patterns

Although it is important to examine the broad-scale diversity of insect respiration, focussed studies of specific taxa are also critical. In particular, the adaptive significance of DGE can best be understood within a phylogenetic context. Each of the investogators has easy access to at least one well-

studied group of insects with a reasonably well-known phylogeny. These include tenebrionid, carabid and scarab beetles (Quinlan, Chown) and *Drosophila* (Gibbs). Each taxon includes representatives from different habitats, and therefore each allows specific tests of hypotheses, as described below.

B.2a. North American Beetles - In contrast to southern Africa, the beetle fauna of the American Southwest has not been widely studied with regard to respiration and water balance (Quinlan and Lighton, unpublished data). The families Tenebrionidae, Carabidae and Scarabaeidae are all well-represented and can be found in a wide range of habits, from open desert to well-watered riparian areas (Table 2). Both the tenebrionids and carabids are readily obtainable during all but the coldest months. We plan to study ventilatory patterns soon after collection, but both families can easily be maintained in captivity if necessary. Scarabaeids are much more seasonal and tend to have patchy distributions. When present, however, they tend to be available in large numbers. Both Quinlan and Gibbs are acquainted with experienced coleopterists in Arizona, and we expect to be able to locate adequate numbers of all beetle families. The Chown lab will continue its work on South African scarabs (Davis et al. 1999, 2000; Bosch et al. 2000), thereby providing a second hemispheric comparison, in addition to the tenebrionids.

Table 2. A sampling of potential Coleoptera test species, based on the insect holdings of the School of Life Sciences at Arizona State University. Only common Arizona species that are well represented in the ASU insect collection were included. The terms "xeric" and "mesic" are approximate and were assigned based on the collection sites. *Eleodes* species from mesic and xeric habitats occur over wide elevational ranges. Species marked with an asterisk (*) are dung beetles.

Family	Xeric	Mesic
Tenebrionidae	*Eleodes armata, E. caudiferus*	*E. carbonaria, E. extricata, E. obscura, E. hispilabris, E. gracilis, E. longicollis*
	Centrioptera muricata, Ce. variolosa *Cryptoglossa verrucosa, Cr. muricata* *Eusattus reticulatus, Eu. muricatus, Eu. convexus* *Edrotes ventricosus, Ed. orbus, Ed. rotundus*	
Carabidae	*Pasimachus elongatus, P. californicus* *P. neomexicanus* *Scarites californicus*	*Calosoma scrutator, C. prominens, C. parvicolli, C. peregrinator, C. affine* *Brachinus costipennis, B. hirsutus, B. mexicanus*
	Scaphinotus petersi	*Bembidion bifossulatus*
Scarabaeidae	*Canthor imitator*, C. indigaceus**	*C. pilularius** *Dichotomius colonicus*, D. carolinus*
	*Copris lecontei** *Phanaeus quadridens*, P. vindex**	*Co. fricator*, Co. arizonensis** *P. amithaon** *Dichelonyx backi, D. truncata, D. sulcata, D. elongata* *Polyphylla hammondi, P. cavifrons, P. diffracta, P. decemlineata*

The Carabidae are the subject of intensive phylogenetic study by David Maddison and his lab at the University of Arizona (Maddison et al. 1999; Ober 2001) and will be a rich source of habitat comparisons. Of particular relevance to this proposal is Maddison's current work on a detailed phylogeny of the genus *Bembidion*, and that of his student, Wendy Moore, on the Paussinae (bombadier beetles and their relatives). Members of these taxa are locally abundant, and occur in a wide variety of habitats and altitudes. In addition, *Scaphinotus petersi* occurs as multiple subspecies, each restricted to a different mountain range of the "sky islands" of southern Arizona (Ball 1966). Once we have identified appropriate comparison species, this species will allow us to examine fine-scale evolution of ventilatory patterns.

The phylogenetic relationships of North American tenebrionids and scarabs are less well understood than those of carabids, but some information is available. Charles Triplehorn, a systematist at Ohio State, has agreed to help us with the tenebrionids (see Triplehorn 1990, 1998). The phylogenetic relationships of the Scarabaeidae have recently been reviewed (Davis et al. 2002), and are also being investigated under an NSF-PEET (Partnerships to Enhance Expertise in Taxonomy) award at the University of Nebraska (see www.museum.unl.edu/research/entomology/scarabcentral.htm for more information). We have been in contact with Brett Ratcliffe, the leader of this project, to obtain information on which species are available and appropriate to test our hypotheses. This project is mostly devoted to the Neotropical scarabaeids, so we may be able to obtain additional study species from tropical habitats.

B.2b. Fruitflies - Numerous phylogenetic studies of the genus *Drosophila* have been published. Of particular relevance are those by Pitnick et al. (1999) and Durando et al. (2000), which focus mainly on the *repleta* group. This large (>100 species) group contains three of the four species endemic to the Sonoran Desert, and the majority of the others are also cactophilic and occur in arid environments. The *repleta* group also includes numerous mesic species, including *D. mercatorum*, which occurs in moist forests in the southeastern United States, and the cosmopolitan *D. hydei*. These species are in culture in the Gibbs lab, and at least 30 other *repleta*-group species are available in southern Arizona or from the Drosophila Species Stock Center at the University of Arizona. Other cactophilic species from the *nannoptera* group are also available, including the fourth Sonoran endemic, *D. pachea*. We recognize that adaptation to laboratory culture may have affected the respiratory patterns of some species, although it is also true that desert species remain highly desiccation-resistant even after nearly 20 years in culture (Gibbs & Matzkin 2001). In addition, the Stock Center has recently replaced many older stocks with fresh ones, including several cactophilic species collected by T.A. Markow's laboratory at the University of Arizona. We will minimize potential problems with laboratory culture by using the most recently collected stocks available or by collecting our own.

B.3. Data analysis

Comparative studies require assessment of the effects of phylogenetic non-independence (see Felsenstein 1985; Harvey & Pagel 1991; Garland & Adolph 1994; Freckleton 2000). Failure to incorporate phylogenetic information overestimates the available degrees of freedom and can lead to false results (see Nee et al. 1991, for an example of the benefits of the method). While the difference between the outcomes of analyses based on phylogenetically independent contrasts and those using non-corrected data are often small (Ricklefs & Starck 1996; Price 1997), the consensus view is that both approaches should be adopted wherever this is possible in evolutionary physiology (Feder et al. 2000). Our phylogenetic analyses will be undertaken using PDAP software (Garland et al. 1999), and non-corrected data will be analyzed using standard parametric and non-parametric methods. When detailed phylogenetic information is not available, other approaches will be used (e.g. nested ANOVAs that partition variation among different taxonomic levels; Addo-Bediako et al. 2001, 2002).

Within-individual variation in DGE is an important variable that has been almost completely overlooked thus far (see Chappell & Rogowitz 2000, for an exception). As Chown (2001) has pointed

out, the adaptive significance of DGE comes into doubt if within-individual variation is large with respect to between-individual variation. Put differently, DGE is unlikely to be of adaptive value to an insect that switches in unpredictable ways between DGE and chaotic modes of gas exchange. We will therefore use nested ANOVAs and measures of repeatability (Lessells & Boag 1987) to assess variation within individuals of a given species.

B.4. Assessing habitat differences

To make appropriate comparisons and test our hypotheses properly, we need to accurately assess the test species' habitat conditions and to design respirometry conditions that resemble these. Unfortunately, published life-history and ecological data are not ideal for this purpose, for two reasons. First, such data will be incomplete for many of our test species. Second, ecological data, when present, can be very misleading, as many arthropods occupy microhabitats whose conditions differ markedly from those of the macrohabitat. For example, *D. melanogaster* is easily collected in Arizona because of its association with humans, not because it is a desert fruitfly. We will therefore measure microclimate variables (temperature and humidity) at our collection sites with portable data loggers, to gain an understanding of the actual conditions experienced by our insects in nature. We will also obtain published rainfall and temperature data whenever possible. For fossorial species, we will measure soil water content and collect air samples from soil or burrows in gas-tight syringes. We will then measure oxygen and CO_2 levels using our respirometry equipment, which is portable enough to be used in the field if power is available.

C. Testing Individual Hypotheses

The experiments described below are designed to test four hypotheses: 1) that DGE is a water-conservation adaptation, 2) that DGE is an adaptation to life in hypercapnic environments, 3) that DGE serves to reduce oxidative damage to tissues, and 4) that DGE is a non-adaptive byproduct of spiracular regulatory mechanisms. These experiments will be performed using appropriate members of the taxa discussed in section B. In this section, we describe tests of specific predictions derived from individual hypotheses; section D describes experiments designed to distinguish one model from another.

C.1. Testing the water-loss hypothesis

DGE is most effective as an adaptation to minimize respiratory water loss if certain conditions prevail during the F period. Recall that endotracheal pressures during the F period are thought to be lower than ambient pressure due to the consumption of O_2 and storage of CO_2 by various extracellular buffers. This pressure gradient is thought to drive convective (bulk) movement of air into the tracheal system, thereby raising endotracheal PO_2 while retarding the outward movement of water vapor. This model has significant experimental support in large saturniid pupae where endotracheal pressures have actually been measured (Hetz et al. 1994; Slamá 1994). However, it has never been positively confirmed in other arthropods, primarily due to the difficulty in determining endotracheal pressures in smaller organisms (Hadley 1994), and Slama (1999) has since argued that the appearance of the F period is an experimental artefact (but see Hetz et al. 1999; Tartes et al. 2000). Another way to test the hypothesis that DGE is beneficial to water balance is to test the following comparative predictions:

Prediction 1: DGE should be more prevalent in xeric-adapted insects than in mesic-adapted insects.

Prediction 2: The F period should be longer in xeric-adapted insects than in mesic insects. Conversely, the O or V periods should be shortest in xeric-adapted insects.

Rationale: O_2 uptake during the F period entails a smaller loss of water than O_2 uptake during the O period due to convective airflow inwards. Xeric insects should therefore maximize their use of the F period for oxygen uptake.

Prediction 3: Carbon dioxide excretion during the F period should be lowest in xeric-adapted insects.

Rationale: The F period of many arthropods exhibiting DGE is marked by the low-level excretion of CO_2. Detectable levels of CO_2 loss during the F period suggest that convective movement of air into the tracheae is not occurring at significant rates. This puts the classic water retention model into question. Further, when CO_2 excretion is present during the F period, it is certain that water loss is also occurring, even if the water vapor cannot be detected (technical limitations make water vapor detection much more difficult than CO_2 measurement). In other words, it is very likely that CO_2 and water loss during the F period are directly correlated. We will test these hypotheses primarily in *Eleodes, Cryptoglossa* (Tenebrionidae) and carabids from xeric and mesic habitats (Table 2).

C.2. Testing the chthonic hypothesis

The chthonic hypothesis predicts that species from enclosed habitats will exhibit more pronounced DGE. It was originally proposed in the context of burrowing species (Lighton, 1996, 1998), but would also apply to wood borers and coprophilic species.

Prediction 4: DGE should be least prevalent in insects that are arboreal or surface-dwelling.

Beetles are an excellent group for this analysis, because they occupy habitats ranging from fossorial (most tenebrionids, carabids in the genus *Brachinus*) to surface dwelling and/or arboreal (many carabids and scarabs). Dung beetles and carrion beetles (scarabs) may experience very high levels of carbon dioxide in combination with hypoxia and therefore provide an excellent comparison to arboreal relatives. Scarabaeids are very abundant in Arizona, so we do not anticipate any difficulty in obtaining numerous species from both hypercapnic and normocapnic microhabitats.

The chthonic model can also be tested by exposing insects to increasing concentrations of atmospheric CO_2. As noted in the preliminary data, certain species are inconsistent with regard to the expression of DGE (Figure 3). All of the tenebrionid species studied so far are fossorial to some degree, and the chthonic hypothesis would predict that DGE should be most evident in conditions where ambient CO_2 is highest.

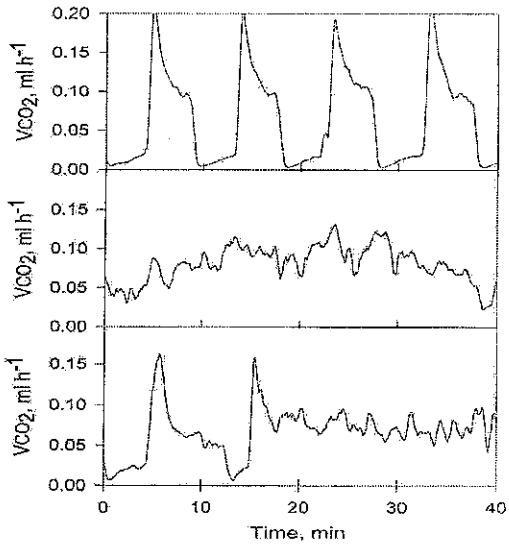

Figure 3. Variation in carbon dioxide excretion from three different *Cryptoglossa muricatus* at 25 °C. All three animals were motionless during the periods shown above.

Prediction 5: Individuals that exhibit DGE weakly or not at all might intensify or begin the behavior when exposed to hypercapnic gas mixtures.

Technical challenges are associated with these experiments. One of us (Quinlan) has attempted these experiments using ant queens. The high levels of atmospheric CO_2 envisioned for the experiments (up to 10%) will completely saturate the CO_2 detector (LiCor LI-6262). Further, high CO_2 levels also affect the ability to detect water vapor, so some independent means of detecting DGE will have to be employed. Fortunately, there are a number of solid state devices currently available that detect water vapor levels by electronic means (e.g., Sable Systems RH-100). These devices are not sensitive to CO_2, so they can be used to detect bursts in water loss associated with the O or V periods of DGE. The down side is that these instruments can be a

challenge to work with, but Quinlan has extensive experience with them (Hadley, Stuart and Quinlan 1982; Hadley & Quinlan 1989; see Figure 1).

Biochemical tests of the chthonic hypothesis: The chthonic hypothesis relies on a high partial pressure of CO_2 within the tracheal system to provide the gradient for diffusion into the surroundings. Because of its high solubility, a large fraction of internal CO_2 will be dissolved in the cells and hemolymph, primarily in the form of bicarbonate. Accumulation of CO_2 while the spiracles are closed will cause internal pH to decrease, and the longer they remain closed, the lower pH will be. Thus, all else being equal, species exhibiting DGC will be exposed to larger fluctuations in tissue pH than non-DGC species, with consequent effects on enzyme activities and cellular transport processes. In order to maintain a high gradient for CO_2 excretion when the spiracles open, bicarbonate must be rapidly converted to CO_2 by the action of carbonic anhydrase. These considerations lead to the following predictions:

Prediction 6: To minimize CO_2-induced pH changes, species exhibiting DGE will have higher tissue buffering capacities.

Prediction 7: To maintain a higher gradient for outward diffusion of CO_2, particularly late in the O period, DGE-performing species will have higher levels of carbonic anhydrase.

We will test these hypotheses by measuring buffering capacity and carbonic anhydrase activities in closely related beetles and fruitflies differing in their ventilatory patterns (Harrison 1988; Harrison & Kennedy 1994). These hypotheses are based on differences in ventilatory patterns rather than habitat, so we will decide which particular species to study based on our respirometry results.

C.3. Testing the oxidative stress hypothesis

The fundamental prediction of this model is that oxidative damage should accumulate less rapidly when an individual is using DGE. Unfortunately, the incremental amount of damage is likely to be so low that it can not be detected. One could prevent insects from performing DGE by use of extreme hypercapnia (which will cause the spiracles to remain open). The problem is that this treatment may elevate oxidative damage by other routes (e.g. elevated metabolic rates or stress responses that generate free radicals, such as immune system responses). In addition, free radical scavenging or repair mechanisms may be stimulated. Instead, we will test this model using a comparative approach.

Prediction 8: Non-DGE species will have higher superoxide dismutase and catalase levels than species which perform DGE.

Rationale: We have already determined that DGE patterns differ among tenebrionid beetles and *Drosophila* species. If DGE serves to reduce oxidative damage, then species that do not exhibit DGE will be exposed to higher internal oxygen levels, and therefore to higher levels of free radicals. Thus, they will also need higher levels of free radical-scavenging systems. In the context of respiratory patterns, the critical source of free radicals is superoxide. We will therefore measure the activity of superoxide dismutase (SOD) and catalase, the enzymes primarily responsible for detoxification of superoxide radicals.

We have some preliminary data implicating SOD in water balance. In desiccation-selected populations of *D. melanogaster*, one allele of SOD increased in frequency from less than 10% to become the major allele (Deckert-Cruz 1996). These populations also exhibit cyclic CO_2 release, whereas control populations do not (Williams et al. 1997, 1998). We measured SOD activities (Paoletti & Mocali 1990) in selected and control populations, as well as in two pairs of desert (DGE) and mesic (non-DGE) *Drosophila* species. In all three comparisons, SOD activities were significantly lower in the flies which exhibited DGE (Gibbs and Fukuzato, unpublished data). As with predictions 6 and 7, our respirometry data will guide our ultimate choice of species in the proposed research.

C.4. Testing the interacting setpoints hypothesis

This hypothesis differs from the others in being a mechanistic rather than an evolutionary (adaptive) hypothesis. It is consistent with theory as an explanation for how DGE can occur (Bustami & Hustert 2000; Chown & Holter 2000), but is also mutually compatible with any of the hypotheses above. This is really the null hypothesis, and we do not think a direct test of is possible; instead we must rely on negative evidence. If none of the other three models gains support, we must conclude that insects breathe the way they do simply because that is how the system is regulated. Alternatively, our experiments may suggest new testable hypotheses.

D. Key Tests of Hypotheses: A Strong Inference Approach

Most studies to date have been designed to address only one of the competing DGE hypotheses. Indeed, alternative hypotheses appeared only when a series of papers challenged the water-conservation model. In the experiments outlined above, we provide testable hypotheses for each DGE model, but we must also be able to distinguish between competing models. This can only be achieved if two models make different predictions regarding outcomes (Huey & Berrigan 1996; Huey et al. 1999; Chown 2002; Chown and Davis 2003).

D.1. Water conservation vs. oxidative damage

In many areas of the western United States, high-altitude environments are drier than low-altitude ones. Thus, the water-conservation hypothesis predicts that DGE cycles should be more prevalent in insects from higher elevations. Oxygen levels decline as altitude increases, suggesting that oxidative damage should be reduced. Therefore, the oxygen toxicity model predicts that DGE cycles should be *less* prevalent at higher altitudes. Elevational patterns in DGE will therefore allow us to distinguish between these models.

Prediction 9.1: Under the water conservation model, DGE should be more prevalent, with longer F cycles, in high-altitude insects.

Prediction 9.2: Under the oxygen toxicity model, high-altitude insects will have less need for DGE.

We know of only one study relevant to these predictions. Within the grasshopper species, *Melanoplus sanguinipes*, individuals collected above 2000 meters in California rarely exhibit DGE, whereas most individuals from low-altitude populations do (Rourke 2000). These results do not favor either model, however, because the low-altitude sites in this particular study were also more xeric. We will test our hypotheses using tenebrionids in the genus *Eleodes* and riparian carabids of the genus *Bembidion*, which occur over a wide elevational range in Arizona. If we identify appropriate comparison species, we will also study subspecies of the carabid *Scaphinotus petersi*, each of which is endemic to a local mountain range (Ball 1966).

D.2. Water conservation vs. chthonic

These models are closely related, because both invoke a central role for water conservation. Thus, they are difficult to distinguish from each other, and no studies to date appear to do so. The chthonic hypothesis was developed by Lighton and colleagues, who primarily study ants and other soil-dwelling insects from arid environments. A wider environmental perspective allows us to make these predictions:

Prediction 10.1: Under the water conservation model, insects from exposed habitats (including arboreal insects in riparian regions) will be under greater water stress than fossorial species and will exhibit more pronounced DGE cycles.

Prediction 10.2: Under the chthonic hypothesis, insects from fossorial or other enclosed habitats (e.g. coprophiles and scavengers) will be more exposed to hypercapnia and hypoxia than surface and arboreal species and therefore will exhibit more pronounced DGE cycles.

Scarabs are excellent beetles with which to test these contrasting predictions, as they include locally common dung beetles (*Aphodius*), carrion beetles (*Nicrophorus*), and a wide variety of leaf-eaters (*Polyphylla*) (Arnett 1985). As noted above (section B), an NSF-PEET project devoted to scarabaeids at the University of Nebraska will be a valuable source of phylogenetic information.

A related prediction can be made by considering fossorial insects from regions differing in aridity. Moister soils will have greater microbial activity and lower rates of diffusion between burrows and the surface, thereby resulting in higher CO_2 levels and lower O_2 levels.

Prediction 10.3: Under the water conservation model, fossorial insects from riparian areas will have less pronounced DGE; under the chthonic model, DGE will be more pronounced.

We will use the Carabidae for this comparison, as we can obtain fossorial species from riparian and arid locations in Arizona (W. Moore, personal communication). Our analyses of soil CO_2 and O_2 levels will indicate the relative exposure to hypercapnia or hypoxia for each species. Phylogenetic information will be obtained from Wendy Moore and other members of the Maddison lab at the University of Arizona.

E. Broader Impacts of the Research

In recent years there has been increased recognition that much of what we "know" in ecology and evolution is biased towards the Northern Hemisphere (Lovegrove 2000; Morton & Stuchbury 2001; Addo-Bediako et al. 2002; Chown et al. 2002), but different ecological and evolutionary patterns may develop in the Southern Hemisphere. Indeed, our preliminary data suggest that tenebrionid beetles from North American and southern African deserts differ markedly in their respiratory patterns. This project will enhance research ties between the US and South Africa and will allow for a direct comparison between southern and northern arthropods, particularly tenebrionid and scarabaeid beetles.

Significant research training will be provided by the support of the post-doctoral researcher and graduate student. In addition, the Gibbs lab typically has 2-4 undergraduates participating in research projects, and actively recruits students from campus programs such as the Undergraduate Biology Research Program and the NSF Futurebound Program. The latter is a new joint program with Pima Community College. It provides female, under-represented minority transfer students with mentoring and places them in research labs at the University of Arizona. We have had one student so far and expect more to join the lab in the future. In summary, this project will provide extensive research training.

This work will also provide excellent synergies with other research programs. We have begun working or corresponding with tenebrionid, carabid and scarabaeid systematists, and we anticipate regular exchanges of information and source material. This project will also complement the Gibbs lab's work on the evolution of water balance in laboratory populations of *D. melanogaster*, by providing for direct comparisons between laboratory and natural populations of *Drosophila*.

Division of Labor

This is a collaborative project, involving three labs in two hemispheres. Our goal is to use consistent procedures across all labs, while allowing each group to maximize its research strengths. The post-doc (see Budget Justification) will be based in the Quinlan lab, but will regularly visit and work in the Gibbs lab. He or she will have easy access to facilities at Arizona State, and will become a scientific member of the Center for Insect Science at the University of Arizona. One scientist each year (either the post-doc or the grad student) will travel to South Africa for several months each year to work in the Chown lab, as will Quinlan in the first year, in order to establish the collaboration directly with the Chown lab.

Quinlan lab: Dr. Quinlan has studied insect ventilation for over 15 years and has helped to develop many state-of-the-art techniques (e.g. humidity chips). He and the post-doc will focus on ventilatory patterns in beetles.

Gibbs lab: Dr. Gibbs is a biochemist who has worked on cuticular lipids and other aspects of water balance in insects for over a decade. He will be responsible for studying ventilation in *Drosophila*, as well as for all biochemical assays.

Chown lab: Dr. Chown is an insect physiologist whose major focus is insect metabolism. He will be responsible for work on African beetles, and for broad-scale comparisons of ventilation in all major insect orders, with support from the National Research Foundation of South Africa.

RESULTS FROM PRIOR SUPPORT (Allen G. Gibbs)

IBN-0110626; $378,425 (October, 2001-September, 2004)

Physiological Evolution and Microarray Analyses in Stress-Selected Drosophila

The primary goal of this project is to identify genes involved in insect water balance and relate them to specific physiological processes. We are doing this by subjecting replicated populations of the fruitfly, *Drosophila melanogaster*, to selection for desiccation resistance for a period of 30 generations. At 10-generation intervals, we are using DNA microarrays to identify: (1) genes that have evolved higher or lower expression, relative to control populations; and (2) genes whose regulation has evolved in response to selection. We are also measuring water-loss rates, metabolic rates, activity levels, and energy storage patterns, in order to determine how specific physiological processes have evolved.

There has been a clear selection response; assays performed after 20 generations indicate a doubling in desiccation resistance, increased body size and carbohydrate storage, reduced activity, and reduced metabolic and water-loss rates. In our analyses of phenotypic responses to desiccation and starvation stress, we have identified ~200 candidate genes. Several have been implicated in responses to other stresses, several code for enzymes involved in energy metabolism, and some genes affect behavior (e.g. *foraging*). Post-doctoral researcher Cheryl Vanier has developed statistical techniques to monitor quality control and to analyze our data as they come in. She has tested her techniques in an independent collaboration with David Galbraith's lab in Plant Sciences, in which she is helping to analyze several years' worth of their microarray data.

While selection proceeds, several undergraduates have been doing comparative studies of water balance and energetics in *Drosophila* species from different habitats. Kelley Kain surveyed lipid, carbohydrate and protein storage in 16 species to put the final touches on a project begun by Marilyn Marron in her senior honor's research. An honors student, Michael Perry, is measuring the effects of desiccation and starvation stress on respiratory quotients. Christina Contreras, a participant in the NSF Futurebound Program, measured thermal tolerance in a variety of desert and mesic *Drosophila* species.Results from this award have been presented at the Society for Integrative and Comparative Biology, the Center for Insect Science Poster Hexapodium, the Fourth International Symposium on Molecular Insect Science, the American Physiological Society, the Society for the Study of Evolution, and a Gordon Conference. Two manuscripts have been published:

Marron, M.T., T.A. Markow, K.J. Kain and A.G. Gibbs (2003). Effects of starvation and desiccation on energy metabolism in *Drosophila*. *Journal of Insect Physiology* 49: 261-270.

Gibbs, A.G., F. Fukuzato and L.M. Matzkin (2003). Evolution of water conservation mechanisms in desert *Drosophila*. *Journal of Experimental Biology* 206: 1183-1192.

Two manuscripts directly related to this project are in preparation, and Dr. Vanier is a co-author on four manuscripts in preparation (so far) from her collaboration with Galbraith's group.

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