Torpor Does Not Influence Spatial Memory in Hibernating Golden-Mantled Ground Squirrels (*Spermophilus [Callospermophilus] lateralis*)

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ABSTRACT

Mammalian hibernation in ground squirrels is characterized by periods of torpor wherein body temperature approaches ambient temperature and metabolism is reduced to as low as 1/100th of active rates. It is unclear how hibernation affects long-term spatial memory, as tremendous remodeling of neurons is associated with torpor use. Given the suspected links between remodeling and memory formation and retention, we examined long-term spatial memory retention throughout a hibernation season. Animals were trained on a Barnes maze before entering torpor. Animals were tested for memory retention once a month throughout a hibernation season. Results indicate marked variation between individuals. Some squirrels retained memory across multiple torpor bouts, while other squirrels did not. No relationship was found between the number of torpor bouts, duration of bouts, or time spent torpid on long-term memory retention. However, that some squirrels successfully retain memory suggests that the profound remodeling of dendritic spines during torpor does not always lead to memory loss.

Keywords: hibernation, spatial memory, torpor, Barnes maze.

Introduction

During the winter, many mammals utilize hibernation presumably to conserve energy. Ground squirrels are considered masters of this strategy, as core body temperatures below 0°C and oxygen consumption rates as low as 1/100th of normothermic values have been reported (Barnes 1989; Frank 1992; Geiser and Ruf 1995; Wang and Lee 2000; Geiser 2004). The profound metabolic reduction of torpor is interrupted every 1–3 wk by a brief period (~12–20 h) of euthermia. Thus, the hibernation season is best characterized by oscillations of body temperature and metabolism.

In a typical hibernation season, a ground squirrel may experience 15 or more periods of torpor (e.g., Healy et al. 2012). Although ground squirrel brain temperatures may be ~1°C warmer than other body regions, cerebral blood flow is reduced approximately ninefold during torpor, and metabolic activity is largely depressed (e.g., Barnes 1989; Frerichs et al. 1994). Memory and learning are associated with the formation and maintenance of dendritic spines (Lamprecht 2014), although some authors question the basis of the relationship (reviewed in Segal 2005). There is tremendous remodeling of dendritic spines during hibernation in ground squirrels. In the thalamus and cortex, marked reductions in spine densities, postsynaptic spines, and spine branches are observable within a day of entering torpor (Ruediger et al. 2007; von der Ohe et al. 2007). Indeed, as much as 50%–65% of synapses may be lost (von der Ohe et al. 2007). More gradual losses are observed in the hippocampus (primary site of spatial learning and memory). In CA3 pyramidal neurons associated with later stages of spatial learning, profound changes in cell body volume, apical dendritic length, dendritic intersections, and tau hyperphosphorylation occur during torpor (Popov et al. 1992; Bullmann et al. 2016). For instance, the number of dendritic branches in the distal region of the pyramidal neurons may be reduced by as much as 50%. This distal region is where the CA3 pyramidal neurons synapse with neurons in the entorhinal cortex, which is an important site of learning and memory (Jarrard 1993). Remarkably, arousal from torpor restores dendritic spine parameters within a few hours (Popov et al. 1992; von der Ohe et al. 2006). Given the tremendous amount of dendritic spine...
remodeling, an important question is whether torpor use may be associated with deficits in spatial learning and memory.

Several studies indicate that lowered body temperature leads to short-term memory impairments in mice and rats, animals that do not hibernate (Riccio et al. 1968; Soumireu-Mourat and Cardo 1972). Similarly, hypothermia leads to a loss of dendritic spines (Roelandse and Matus 2004). However, results are mixed regarding animals that naturally hibernate. Some studies indicate that animals have impaired spatial, recognition, odor discrimination, and operant memory after a hibernation season relative to those kept at room temperature (Mateo and Johnston 2000; Millesi et al. 2001; Bullmann et al. 2019). Other studies found no such deficits after a hibernation season for social recognition memory (Millesi et al. 2001) or operant conditioning tasks (McNamara and Riedesel 1973; Clemens et al. 2009). One study established memory retention in salamanders after a 90-d brumation state but also found memory disruption if exposed to cold temperatures immediately after initial testing (Kundey et al. 2018). Another study even suggests an enhancement in contextual learning after hibernation (Welzsin et al. 2006). Although some studies examined the effects of hibernation on memory, the majority of these studies measured memory before and after a hibernation season. Here, we examine the effects of torpor use on long-term spatial memory retention to test the prediction that the tremendous neural remodeling inherent to torpor should disrupt memory retention.

Methods

Subjects

All of the experiments were performed between August 2012 and February 2013. Golden-mantled ground squirrels (Spermophilus [Callospermophilus] lateralis) were livetrapped using Sherman-type box traps from the Duck Creek area of southern Utah on August 1, 2012. Squirrels were treated for external parasites and transported to the University of Nevada Las Vegas (UNLV). Squirrels were singly housed in standard rat polypropylene cages (27 cm × 48 cm × 20 cm) lined with corncob bedding and fed rat chow, supplemented with sunflower seeds, and watered ad lib. throughout the experiment. A total of 11 adult squirrels were deemed appropriate for use in Barnes maze testing (six females, five males).

Surgery

Following the completion of Barnes maze training and the first monthly assessment (see “Barnes Maze Testing” below; mid-September 2012), squirrels were surgically implanted with paraffin-encased iButton temperature loggers (logged every 30 min; Maxim) placed into the abdomen. Briefly, aseptic procedures were followed throughout. Anesthesia was induced using 5% isoflurane in an induction chamber. Anesthesia was maintained with 1%-2% isoflurane delivered through a nose cone. The incision site was shaved and cleaned using Betadine surgical scrub and alcohol. A small incision was made through the skin and abdominal musculature. The paraffin-encased data logger was placed into the animal. The abdominal musculature was closed using interrupted 4-0 braided polyglycolic acid (PGA) sutures followed with subcuticular 4-0 PGA closure. Although wound closure was accomplished with the subcuticular sutures, an interrupted pattern of 5-0 nylon monofilament skin sutures was placed. Surgical glue was applied to reduce risk of postoperative chewing-induced dehiscence. After the surgical glue dried, the wound was covered with triple antibiotic ointment. Squirrels were given a single 0.01-mg/kg injection of buprenorphine at the time of surgery for analgesia. Follow-up monitoring revealed quick healing with no complications. Our experiences are that squirrels heal quickly within a few days of surgery.

Squirrels were allowed to recover at room temperature. At the end of October, squirrels were placed into an environmental chamber. The temperature of the chamber was reduced to 4°C over several days to allow effective hibernation. Squirrels were maintained in constant darkness. Following completion of the trials and near the end of the hibernation season, the data logger was retrieved when tissues were collected for an unrelated study. Body temperature ($T_b$) data were coded as either torpid ($T_b < 20$°C) or aroused ($T_b > 20$°C) for the analyses. All procedures were approved by the UNLV Institutional Animal Care and Use Committee.

Barnes Maze Testing

All behavioral procedures utilized a modified Barnes-type maze (Barnes 1979). This maze was chosen because ground squirrels naturally escape into holes when faced with a predation risk. The maze was housed in an evenly lit and dedicated room near where animals were housed. The maze was constructed from a circular white high-density polyethylene plastic table that was ~1.5 m in diameter (for layout, see schematic in fig. A1). Ten evenly spaced 11-cm holes were drilled along the edge and fitted with polyvinyl chloride (PVC) pipe fittings. All but two nonescape holes were consistently fitted with 10-cm threaded plugs out of sight of the level of the maze. Two holes that were distantly placed were used as possible escape holes. Each squirrel was randomly assigned to one of the two potential escape holes as a goal of the maze. The unused hole was plugged as per the other holes. This assignment was used to ensure that there was no effect of maze position or internal cues in choosing the refuge. The available escape hole led to a PVC chamber that allowed the squirrel to escape the maze. The goal of the maze was for squirrels to learn to seek the escape chamber. The maze was skirted with a ~40-cm-high wall to discourage animals from jumping off the table. Three outer maze geometric cues, distinct in shape and color, were placed around the side of the maze just above the skirt. Trials were recorded and captured using a video tracking system (Smart, San Diego Instruments, San Diego, CA). For each trial, the animal’s latency (time required) to find the escape chamber was determined. Data are reported as means ± SEs.

Following a 1-wk period to allow adjustment to the facility after capture, an initial training period was conducted for 15 consecutive days in August. The squirrels were randomly placed in one of three quadrants (not the quadrant with the
escape hole) near the center of the maze to initiate the trial. Each animal was allowed a maximum of 60 s to complete the maze. The use of wild animals required unusual modifications to typical testing procedures. Two researchers stood at assigned positions along the outside of the maze to assist with keeping the animals inside the maze. Although squirrels ceased leaping from the table during the training period, the researchers were used in all of the subsequent trials to ensure consistent spatial cues. Preliminary efforts revealed that some squirrels would run to a hole and linger. A rubber black bat (similar in shape to a raptor) was hung in one corner of the room from the ceiling and proved effective in motivating most animals to seek out the escape chamber. This stimulus was present throughout the duration of the study. If the animal remained immobile for more than 5 s, the researcher gently waved an arm in the air to simulate an overhead predatory shadow. If after 60 s the animal did not locate the escape chamber, the researcher gently guided the squirrel to the target hole. This guidance of the squirrel to the hole or cues like waving an arm were not required by the conclusion of the training period. On reaching the escape chamber, the animal was allowed to remain for 60 s, then it was returned to its home cage for an additional 60-s rest period, at which point the maze and escape hole were cleaned. After the 60-s period the animal was returned to the maze for another trial. A total of four such trials for each animal were conducted per day for the duration of the training period and when squirrels were tested for memory retention. The maze and escape hole were cleaned with Formula 409 cleaner (Clorox, Oakland, CA) when switching between animals and between each trial.

Memory retention assays were conducted once monthly for the next 6 mo. The onset of torpor use is variable, but all of the squirrels were tested before the onset of torpor use and after experiencing bouts of torpor. Furthermore, torpor bout duration varied between animals. Animals were required to be aroused between bouts of torpor to allow testing. Skin temperature was determined using an infrared thermometer (Fluke model 59, Everett, WA) to check torpor status. Some squirrels were naturally aroused, as evidenced by their warm skin temperature (and later confirmed using the data from the implanted temperature logger). All of the other squirrels were induced to arouse a minimum of 5 h before the start of testing by gentle shaking and manipulation. Squirrels reach normothermic temperatures within ~3 h under these conditions and were fully aroused for a minimum of 2 h before testing (Utz and van Breukelen 2013). Our experiences are that squirrels are fully aware of their surroundings before they even reach normothermia, meaning that all of the squirrels were active and alert for the assays. Subjects were taken individually in their home cage to the dedicated testing room. After testing, squirrels were returned to the environmental chamber. We measured the amount of time required by squirrels to enter the escape hole (latency), the number of times a squirrel first attempted to enter a wrong hole (reference memory errors; nine possible errors per day), and the number of times a squirrel returned to a particular wrong hole (working memory errors) during both initial training and subsequent retention assays.

Stress Minimization

Concerns that wild-caught animals may require precautions to minimize stress prompted us to take the following actions. Following capture, animals were allowed to habituate to the facility for 1 wk before training. A large-diameter PVC tube that was cut in half was placed in each animal’s cage as a hiding area. When transported to the testing room, animals were allowed to habituate for 5 min before the trials. During the trials, animals were allowed 1 min to hide in the escape hole before being placed back in its home cage for 1 min. Minimal contact with the animals occurred to reduce stress. Animals had biweekly cage changes except during the hibernation season, when cage changes would disturb torpor use. We note that animals are spontaneously anorexic during the entire hibernation season despite the availability of food and water. To avoid unnecessary handling, animals were not weighed. Visual appraisal of body condition was used to ensure an animal’s weight was appropriate to the hibernation cycle.

Statistical Analyses

Latency is reported as an average across the four trials of the day for the time required to reach the target hole. Change in latency was calculated by subtracting the average latency value for the monthly retention assays from the average latency value for the final training day. Reference memory errors (first time animal visited each wrong hole for that day) were calculated for the final day of training and each subsequent month. Working memory errors (each time animal returned to incorrect hole) were parsed as intratrial (returned to wrong hole within each trial) and intertrial (return to wrong hole during subsequent trials). A paired t-test was performed between the final monthly testing latency and the final training day latency. Reference errors and working memory errors were compared for the initial day of training and the final day of training using a paired t-test. Correlation analyses were used to compare latency and time spent torpid or number of torpor bouts across testing. A one-way ANOVA was performed to determine whether there were differences in the change of latency as a result of never having been torpid versus type of arousal from torpor (induced alarm vs. spontaneous natural arousal). An unpaired t-test was performed to determine whether sex affected change in latency or reference errors. Significance was set at 0.05.

Results

Squirrels adapt well to the laboratory. All squirrels ate and drank from a water bottle. All animals eventually entered torpor, although the timing was variable. One study animal died late in the season during torpor. This rare occurrence in the laboratory is not unexpected. In the wild, mortality may be quite high (see van Breukelen et al. 2008 for review). We note that even after being induced to arouse from torpor for the monthly behavioral trials, squirrels predictably resumed torpor after being placed back into the environmental chamber.

We attempted to train 17 squirrels on the Barnes maze. In evaluating our results, we focused on the parameters of latency,
reference errors, and working memory errors. Latency was reduced across training days (e.g., there was a marked reduction in latency on the final day of training; final day mean latency = 19.60 ± 1.98 s for all 17 squirrels) compared with the first day of training (mean latency = 47.92 ± 2.58 s; paired t-test, *P < 0.05*). Reference errors were reduced from the initial day of training to the last day of training (mean errors per day for day 1 = 2.3, for day 15 = 1.1; *P < 0.05*). Working memory errors were negligible across training and subsequent retesting trials and were omitted from further analyses (data not shown). We found that some squirrels rarely entered the escape hole even after experimenter prompting. The goal of the current study was to determine whether memory retention was affected by torpor. The goal of the study was not to determine whether squirrels had the ability to learn or whether they would participate in the Barnes maze. As such, only squirrels that entered the holes and with an average final latency of less than 30 s were used for subsequent trials (fig. 1; *n = 11*).

Individual variation in learning was evident, with some squirrels quickly learning to identify the escape hole. These squirrels maintained consistently short latencies and low reference errors throughout the training. Other squirrels seemed to struggle to find the hole throughout training and had consistently longer latencies and higher reference errors. A few squirrels had individual days of short latencies followed by days with long latencies. Some squirrels retained these short latencies throughout the hibernation season (fig. 2). These squirrels did not appear to suffer any deficit in memory retention despite 10 or more torpor bouts. Other squirrels appeared to lose memory (fig. 3). Despite the three illustrated

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**Figure 1.** Effect of training on latency in a modified Barnes maze. By 15 d of training, most squirrels had learned to find the hole. Only squirrels with a final latency <30 s and demonstrated improvement from day 1 (paired *t*-test, *P > 0.05*) were used in subsequent analyses (mean latency = 14.0 ± 2.5 s; *n = 11*).

**Figure 2.** Latency across the hibernation season in three representative squirrels that appeared to retain long-term spatial memory. Mean latency for each monthly trial is represented by the circles. The approximate body temperature (*Tb*; see text) is depicted below the latency data to indicate when torpor occurred.
squirrels in figure 3 having a final training day average latency of 18.5 ± 4.1 s, these squirrels performed relatively poorly throughout the hibernation season regardless of torpor status. Our assumption was that torpor might result in a progressive deterioration of memory. In other words, memory would be most affected by the last monthly retention assay. Importantly, when all 11 squirrels are included in a robust statistical analysis (paired t-test between the latency of the final training day compared with the final monthly retention assay), we found no significant differences, suggesting that the group as a whole retained memory despite the use of torpor (P > 0.05). Data for each of the four trials performed on each individual squirrel for both the final training day and the final retention assay do not suggest that squirrels learn during the retention assay (e.g., latencies did not shorten from trial 1 to trial 4; fig. 4).

There were no significant correlations between time spent torpid and change in latency (P > 0.05; fig. 5) or time spent torpid and reference errors (P > 0.05; data not shown). The squirrels averaged 411.5 ± 77.7 h in the torpid state during the part of the hibernation season included in this study. Similarly, there were no significant correlations between the number of torpor bouts and change in latency (P > 0.05; fig. 6) or the number of reference errors (P > 0.05; data not shown). The squirrels averaged 10.7 ± 1.4 torpor bouts during the part of the hibernation season included in this study. Additional correlation analyses were performed after exclusion of data from animals that had not yet hibernated, and no significant differences were found (data not shown). ANOVA revealed no effect of state (never having experienced torpor vs. induced to arouse vs. naturally aroused) on latency (P > 0.05; fig. 7). Finally, results of an unpaired t-test indicated no effect of sex on memory retention (P > 0.05; fig. 8).

**Discussion**

The current study examined the effect of torpor on long-term spatial memory retention in hibernating ground squirrels. Ground
Squirrels were trained in a Barnes maze and tested at different time points throughout the hibernation season. Squirrels learned to find the escape hole based on the spatial cues. However, there was variability between these wild animals in how well or how quickly they learned. We speculate that this variability may have stemmed from motivation, stress, personality, or something similar. Since our goal was not to determine whether ground squirrels can learn but rather to determine whether torpor use affected long-term memory retention, we restricted our experiments to squirrels that showed marked learning by the end of the training period. Some squirrels retained their long-term spatial memory throughout the hibernation season (figs. 2, 5, 6). Despite numerous torpor bouts, these squirrels consistently demonstrated low latencies. These data suggest that there are no long-term spatial memory deficits simply as a result of torpor use in hibernation. Other squirrels (fig. 3) appeared to lose memory. Hibernation does not affect other exploratory behaviors in ground squirrels, suggesting that our results are not due to simple reluctance of the squirrels to explore (Semenova et al. 2005). We note that in the squirrels shown in figure 3, loss of memory was apparent even before the initial onset of torpor. Of course, repeated trials offer an opportunity for squirrels to learn within an experimental day, but we find no consistent pattern to suggest as much (fig. 4). In our study, squirrels averaged more than 10 torpor bouts, encompassing more than 400 h of torpor, with no indication of progressive loss of memory.

The literature is mixed in terms of whether hibernators retain memory after torpor use. Much of the discrepancies may be the result of the type of animal model. Many studies allowed one group of animals to enter hibernation and kept another group from hibernating (Mihailovic et al. 1968; Mateo and Johnston 2000; Millesi et al. 2001; Boerema 2012; Kundey et al. 2018). These studies conflict as to whether spatial memory was affected. One strong aspect of the current study is that animals were tested several times before entering torpor and at different time points during torpor. This approach allowed us to determine both immediate effects and long-term effects of torpor use on spatial memory retention. Other studies used various animal models of hibernation, such as bats (Ruczyński, and Siemers 2011), hamsters (Boerema 2012; Bullmann et al. 2016, 2019), salamanders (Kundey et al. 2018), marmots (Clemens et al. 2009), or ground squirrels (Mihailovic et al. 1968; McNamara and Riedesel 1973; Mateo and Johnston, 2000; Millesi et al. 2001). Hibernation use may not be synonymous across taxa as diverse as salamanders and ground squirrels. Furthermore, photoperiod may be used successfully to modulate hibernation use in hamsters, with no apparent detrimental effects (e.g., Boerema 2012). However, the prevention of torpor use in ground squirrels to accommodate learning and memory studies is more problematic. Marked physiological differences are apparent in ground squirrels that are competent to enter torpor versus those that cannot enter torpor (Baker and van Breukelen 2009). Squirrels that cannot enter torpor typically become anorexic like squirrels that do use torpor. In contrast to torpor-using squirrels, those that do not use torpor typically lose weight quickly and die during the hibernation season. Maintenance of ground squirrels at warmer temperatures (e.g., 20°C) will result in shorter and more unpredictable torpor bouts (Russell et al. 2010; F. van Breukelen, personal observations). The differences found in the retention of spatial memory may then result from the experimental approach and model of hibernation rather than a direct effect of hibernation per se.

Reports on hibernators also vary depending on methodology—that is, the task used to measure memory. Ground squirrels exhibit better memory retention for less aversive

![Figure 5. Effect of time spent torpid on latency. Different colored symbols represent different squirrels. There is no significant correlation (P > 0.05).](image_url)

![Figure 6. Effect of the number of torpor bouts on latency. Different colored symbols represent different squirrels. There is no significant correlation (P > 0.05).](image_url)
stimuli (Zhao et al. 2004). Many studies in ground squirrels may have been stressful and therefore may have impacted memory. In one study on contextual memory, ground squirrels were subjected to dunking in water (McNamara and Riedesel 1973). One questions whether this paradigm may have been unduly stressful to the animal. Data are also mixed on whether social recognition is impaired after a hibernation season (e.g., Mateo and Johnston 2000; Millesi et al. 2001). Odor discrimination is used to test social recognition memory. One can envision that intrigued squirrels may seek another squirrel based on odor and not memory. Studies that used food reward as a cue found no effect of hibernation on contextual memory in marmots (Clemens et al. 2009) and no effect of hibernation on spatial memory in bats (Ruczynski and Siemers 2011). Such food reward use may be problematic given the precise way that food intake is regulated in preparation for and during hibernation (Mrosovsky 1977). For instance, the hibernation season is characterized by spontaneous anorexia, and food intake during the prehibernation fattening stage is well regulated (see van Breukelen and Martin 2002 for review). It is unclear how effective a food reward may be when animals are spontaneously anorexic. We are unaware of any studies before the current study that examined long-term memory retention during a hibernation season that did not use a food reward. In other words, previous studies that did not use a food reward appear to be restricted to before and after a hibernation season. All of these confounding factors may lead to discrepancies in the literature. Importantly, that we limited our analyses to animals that had demonstrated learning during the training period suggests that our included squirrels were not so adversely affected by the stress of captivity so as not to learn. We recognize that some individual squirrels may have had personalities incompatible with the study design.

Our data do not address whether torpor affects the acquisition of new short-term memories, although we did not find consistent improvement in latency across trials in a single day (fig. 4). Remarkably, enhanced hippocampal-dependent learning for contextual knowledge or fear responses has been demonstrated during the interbout arousal (McNamara and Riedesel 1973; Weltzin et al. 2006). Perhaps this enhancement of learning reflects the tremendous neuronal remodeling that occurs during the interbout arousal. However, if the remodeling of neurons led to greater learning ability, one might have expected a progressive reduction in latency after repeated torpor bouts (i.e., enhanced training). Our squirrels averaged more than 10 torpor bouts before their final retention assay. We find no progressive loss or improvement in memory (fig. 6). Studies in mice and rats indicate that lowered body temperature leads to short-term memory impairments and a loss of dendritic spines (Riccio et al. 1968; Soumireu-Mourat and Cardo 1972; Roelandse and Matus 2004; Popov et al. 2007). Conversely, mice that enter daily torpor do not exhibit impairments in long-term spatial memory in a Morris water maze (Nowakowski et al. 2006).
Do differences in short-term versus long-term memory retention reflect differences in torpor versus hypothermia or an effect of recovery from torpor? Perhaps consolidation processes are adversely affected by torpor use or benefited from the inter-bout arousal. Neurons lose synaptic strengthening or long-term potentiation in the hippocampus at temperatures below 15°C (Hamilton et al. 2017; Horowitz and Horwitz 2019).

We recognize limitations to the current study. Variability in mastery of the task was evident in these wild-caught animals. The high level of variation may simply be the result of motivation. We attempted to control for motivation by using stimuli similar to natural predators. Evidence of lack of memory retention is evident in some squirrels that had not yet experienced a bout of torpor (e.g., fig. 3). Squirrels cannot be manipulated to enter torpor, and the period between the time of training and the onset of torpor was variable. In our experimental design, we focused on the natural hibernation cycle. An alternate experimental design that regularly trained squirrels until the onset of torpor might shed additional light on the process. Importantly, some individual animals maintained stable performances throughout the experiment. These data demonstrate that torpor use does not necessarily affect retention of long-term spatial memory in hibernating ground squirrels. When one considers the degree and extent to which dendritic spines are remodeled during torpor use, such a result warrants additional investigation.

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APPENDIX

Figure A1. Barnes maze schematic. The layout of the Barnes maze is denoted. The maze was ~150 cm in diameter. Ten evenly spaced 11-cm holes were placed around the periphery as indicated. Either hole A or hole B was randomly assigned to squirrels for use as an escape hole. The unused escape hole and the other holes were fitted with plugs to prevent entry. A nest box was attached to the viable escape hole. To discourage jumping, the maze was skirted with a 40-cm-high wall. Researcher and plastic bat presence was used to promote entry of squirrels into the escape hole. See text for details.
Literature Cited


