RESEARCH ARTICLE

Blood clotting behavior is innately modulated in *Ursus americanus* during early and late denning relative to summer months

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ABSTRACT

Remarkably, American black bears (*Ursus americanus*) are capable of varying their heart rates to coincide with their breathing, creating pauses of 30 s or more, yet they do not appear to suffer from embolic events. We evaluated some features of the clotting cascade of black bears, providing novel insights into the underlying mechanisms they evoke for embolic protection during hibernation. We measured activated clotting time, prothrombin time and activated partial thromboplastin time during early denning (December), late denning (March) and summer (August). Activated clotting time during early hibernation was ~3 times longer than that observed among non-hibernating animals. Clotting time was reduced later in hibernation, when bears were within ~1 month of emerging from dens. Prothrombin time was similar for each seasonal time point, whereas activated partial thromboplastin time was highest during early denning and decreased during late denning and summer. We also examined D-dimer concentration to assess whether the bears were likely to have experienced embolic events. None of the non-parturient bears exceeded a D-dimer concentration of 250 ng ml^{-1} (considered the clinical threshold for embolism in mammals). Our findings suggest there is unique expression of the clotting cascade in American black bears during hibernation, in which extrinsic pathways are maintained but intrinsic pathways are suppressed. This was evaluated by a significant difference between the activated clotting time and activated partial thromboplastin time during the denning and non-denning periods. These changes are likely adaptive, to avoid clotting events during states of immobilization and/or periods of asystole. However, an intact extrinsic pathway allows for healing of external injuries and/or foreign body responses.

KEY WORDS: Clotting cascade, Hibernation, Hibernation physiology

INTRODUCTION

One of the most notable adaptations of the American black bear is its capacity for fasting and inactivity during the winter months. American black bears in the northern parts of their range can remain in their dens for 4–7 months while eliciting minimal movements (Lohuis et al., 2007). Such adaptations would be ideal to replicate for patients in the intensive care unit, as they may lose up to 40–60% of their strength in weeks to months and are often deficient in their healing abilities (Harlow et al., 2001).

Hibernating black bears exhibit dramatic respiratory sinus arrhythmias. Their respiratory rate declines to 2–3 breaths min^{-1}

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and, during inspiration, their heart rate jumps to 60–70 beats min^{-1}. Following exhalation, their hearts may pause for 30 s or more; these asystolic events have been documented by implanted loop recorders (Laske et al., 2010). In humans, asystolic events and irregular heart beats (most notably, atrial fibrillation) can yield aggregation of blood in the extremities and/or within the heart itself, producing consequential blood clots. These, in turn, can result in major medical emergencies such as stroke and/or in some cases myocardial infarction. Thus, significant questions remain as to how hibernating bears are innately capable of undergoing long periods of inactivity and irregular cardiac rhythms without eliciting emboli. One explanation, revealed in previous research on brown bears (*Ursus arctos*), is that bears have more and smaller platelets compared with humans (Fröbert et al., 2010). Interestingly, other studies have demonstrated seasonal changes that may suggest modulation, including previous work done by Chow et al. (2013) and Sheikh et al. (2003) describing differences in immune-related proteins including alpha 2 macroglobulins.

Here, we conducted further investigations of the clotting properties of bears to test the hypothesis that some profound seasonal change accounts for their ability to withstand prolonged asystolic events during hibernation (Iles, 2015). We examined their activated clotting time (ACT), activated prothromboplastin time (aPTT) and prothrombin time (PT) and, additionally, conducted D-dimer tests. ACT and aPTT were evaluated to observe events in the intrinsic clotting cascade, whereas PT was assessed for events in the extrinsic and common pathways. The presence of D-dimer was recorded in order to assess whether bears are likely to suffer from embolic events during the winter months, as evaluated in the fibrinolytic cascade (Fig. 1). Importantly, it has been reported that D-dimer levels may increase as a result of other conditions such as pregnancy, inflammation and liver disease. One needs to consider that the fibrinolytic pathway is very complicated; altered D-dimer levels do not rule out the potential for embolization, but can be used as a relative indication that an embolic event may have occurred.

MATERIALS AND METHODS
Bear handling
This research was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Minnesota (Protocol title: Physiology of hibernation of American black bears). Samples were obtained from free-ranging American black bears, *Ursus americanus* (Pallas 1780), in northern Minnesota, USA. Blood aliquots were compared between early denning (mid-December; 1.5–2 months after entering dens) and late denning (mid-March; ~1 month before emergence). We attempted to handle individual bears during both seasons to obtain matched pairs of samples; however, it should be noted that most of the samples were not sample size or animal matched. In addition, we obtained samples from a small cohort of captive Minnesota black bears in August (non-hibernating).
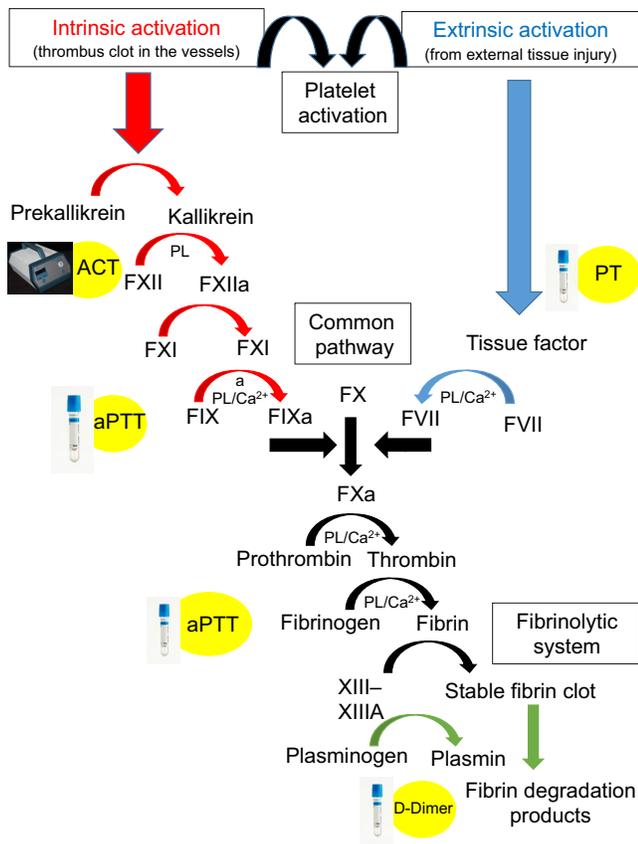
Fig. 1. Blood tests and the clotting cascade in American black bears.
Outline of the mammalian clotting cascade, including the blood tests conducted and where they correlate to the clotting cascade and the fibrinolytic pathway. Activated clotting time (ACT; activation of coagulation via the intrinsic Factor XII pathway) was taken at the den to identify the initial clotting behavior in the intrinsic pathway. Activated partial thromboplastin time (aPTT; a measure of contact factor XII, XI, IX and VIII deficiencies) was recorded to identify the behavior of the intrinsic pathway. Prothrombin time (PT; one-stage test based upon on the time required for a fibrin clot to form after the addition of tissue factor) was used to identify the clotting behavior in the extrinsic pathway. FV, F, FII (prothrombin) and fibrinogen: 'common pathway' clotting factors. D-Dimer, fibrin degradation product from the fibrinolytic system. PL, phospholipids from activated platelets.

Radio-collared bears were studied at their den sites. The hibernating bears were anesthetized with an intramuscular injection of a 50:50 mixture of tiletamine:zolazepam ($3.9–5.3$ mg kg^{-1}) delivered by a jab pole or dart. In previous studies, we observed that under this anesthesia, the bears' cardiac function increased, lacking sinus pauses, but resumed normal hibernation cardiac modulations within several hours post-den visit (Laske et al., 2011). The active, captive bears were immobilized with ketamine hydrochloride ($11–13$ mg kg^{-1}) and xylazine ($0.6–0.7$ mg kg^{-1}). The chemical immobilization was done in accordance with the generally accepted methods for safety of the animal during the different seasons (Kreeger, 2012). Specifically, each bear was continuously monitored for anesthetic status while morphological and physiological measurements were taken. Blood samples were drawn from an animal's femoral vein using a sterile needle and collected into a vacutainer. Upon completion of the examination, each bear was returned to its original location (den site or holding facility).

Determination of clotting properties
Each ACT was determined on site, immediately after blood was drawn (Fig. 2). During the winter sampling, a coagulation analyzer

(Hemochron 401, Soma Technology, Bloomfield, CT, USA) was housed in a styrofoam case that was maintained at approximately 37°C using a warming unit within the coagulation analyzer, which was assessed during these samplings to ensure it was maintaining the proper temperature. Blood (2 ml) was collected and transferred to ACT tubes containing diatomaceous earth and glass beads. Each tube was agitated 10 times and placed in the analyzer. A coagulation time was then determined and data were recorded. A second aliquot of blood was collected in sodium citrate tubes in the field. These were spun for plasma isolation using a centrifuge (Model E8, LW Scientific, Lawrenceville, GA, USA) at 3300 rpm for 20 min within 10 h of collection. Blood samples displaying any level of hemolysis from visual inspection were excluded from the study. The plasma from each sample was transferred to cryo-containers and stored at −70°C. Subsequently, PT, aPTT and D-dimer tests were performed at the University of Minnesota Veterinary Clinic (St Paul, MN, USA). Standard methods for determining PT and aPTT were used as per their instrumentation (Instrumentation Laboratory, Werfen, Bedford, MA, USA).

The concentrations of D-dimers were also determined by standard methods, i.e. by analyzing the level of fibrin degradation products from the fibrinolytic pathway (the normal concentration value for mammals is defined as approximately <250 ng ml^{-1}) (Righini et al., 2014). Increased D-dimer levels are considered an indicator of thrombus; however, false positives for an embolic event can occur from conditions such as pregnancy (Righini et al., 2014). Here, we defined a potential embolic event as a D-dimer concentration of >250 ng ml^{-1}. We excluded pregnant females from overall averages as D-dimer concentrations can increase during pregnancy, although their subgroup results were used as a comparison. All data are presented as mean values with standard deviations. Raw data are presented in Table S1. Statistical comparisons were performed using unpaired t-test ($P<0.05$ was considered statistically significant).

RESULTS
ACT
Blood samples ($N=31$) from 12 female black bears were evaluated for their ACT during early denning, late denning and/or in the summer. An unpaired t-test demonstrated a statistically significant difference amongst these populations of female bears, when comparing the summer cohort with the early-denning and late-denning bears: early denning $217±30$ s ($N=10$), late-denning $164±33$ s ($N=11$) and summer $81±12$ s ($N=3$) (Fig. 3). Our sample of male bears was smaller, but followed the same declining trend in ACT from early denning ($215±22$ s, $N=3$) to late denning ($189±16$ s, $N=3$) to summer (56 s, $N=1$). Note that average human clotting time ranges from 90 to 120 s and therapeutic anticoagulation clotting time ranges from 200 s to over 300 s, depending on clinical needs. These data suggest that black bears modulate their intrinsic clotting cascade during hibernation, potentially as a protective mechanism.

aPPT and PT
Mean aPTT was highest during early denning ($29.7±7.9$ s, $N=8$) and decreased during late denning ($24.5±2.1$ s, $N=11$), and in the summer months averaged $17.6±0.8$ s ($N=2$) (Fig. 4). Summer samples compared with early denning and with late denning were found to be significantly different ($P<0.05$).

PT of black bears during early denning and late denning was comparable to that for the summer months. Specifically, PT remained similar within all animals studied: $7.6±1.5$ s for early denning ($N=9$), $8.1±2.2$ s in late denning ($N=11$) and $8.6±0.3$ s in the summer months $N=2$) (Fig. 5); no statistical differences were
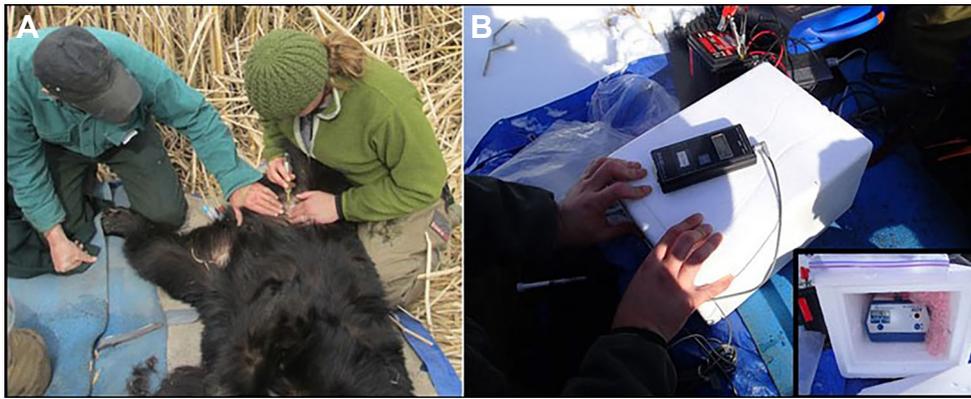
Fig. 2. Point of care ACT test in the field. (A) In the field, researchers took blood samples from the femoral vein of the black bear. (B) A 2 ml sample of the femoral blood was immediately transferred to the ACT tube and agitated, then the tube was placed in a thermally controlled Hemochron unit and relative clotting time was documented at the den.

found. This suggests that bears have similar extrinsic clotting behaviors between seasons.

Determination of D-dimer levels

For the non-pregnant bears, the average D-dimer concentration was 152 ng ml^{-1} (N=8) in early denning and 124 ng ml^{-1} (N=8) during late denning (Fig. 6). We had a very limited sample size for pregnant females (N=2); the average was 349 ng ml^{-1}, although one had a very high D-dimer of 548 ng ml^{-1}, which can be expected with pregnancy.

DISCUSSION

In the clinical setting, properties of a patient's clotting cascade are observed by utilizing laboratory measures of parameters such as ACT, PT and aPTT. These diagnostic tools help clinicians to recognize the relative activity within the complex multi-stepped clotting cascade (Fig. 1). Here, we have applied these same measures to the American black bear in order to better understand the properties of their clotting mechanisms during early-denning, late-denning and active summer months. The evaluation of these parameters provided us with the novel insight that these bears modulate their clotting mechanisms/cascades during hibernation, in comparison to non-hibernating mammals including humans.

In the present study, we observed that clotting times were nearly 3-fold higher for hibernating black bears than for summer active ones. It should be noted that the ACT test has been used historically as a point of care diagnostic to evaluate the levels of unfractionated heparin in a patient's blood, generally during surgery or for management of anti-coagulation therapy. This test has been

extremely valuable for looking at the clotting properties of the intrinsic pathway, specifically the initial aspect of the clotting cascade that involves factor XII10 (Fig. 1). The clinical use of anticoagulation therapy ranges from a clotting time of approximately 200 s for long-term therapy to over 300 s for acute surgical procedures with a high risk of clot formation. In comparison, the normal range of human ACT is 90–110 s (not treated with an anti-coagulant). It is also important to note that all ACT evaluations were performed with blood samples warmed to 37°C, yet the average rectal temperature of black bears during hibernation in this study was found to be 35.6°C during early denning and 34.8°C during late denning.

It is well documented that mild hypothermia can prolong coagulation times (Sun et al., 2015). More specifically, in the clinical setting, it has been reported that a decrease in core temperature by 3–4°C may cause a decrease in the enzymatic reactions associated with coagulation and thus increase clotting time (Liu and Yenari, 2007).

In contrast, other clinical studies have demonstrated the need for a decrease up to 5–10°C in order for the clotting time to be statistically significant (Valeri et al., 1995). Thus, it is important to note that Minnesota black bears are typically mildly hypothermic during hibernation. Nevertheless, the ACT analyzer we employed performed an automated temperature correction, so it is likely that this part of the clotting cascade should not have been affected by their state of mild hypothermia. In addition to the effect on enzymatic activity, hypothermia can also induce an inhibition of platelet function (Ahmed, 2011; Polderman, 2012).

Paradoxically, in humans, there is a suspected increase in fibrinolytic activity with mild hypothermia, whereas in our study, bears elicited normal D-dimer levels. In other words, it is probable that temperature regulation contributes to the bears' adaptation of anti-coagulation, although it is most likely not the only mechanism for clot inhibition. D-Dimer concentrations can be used as a clinical
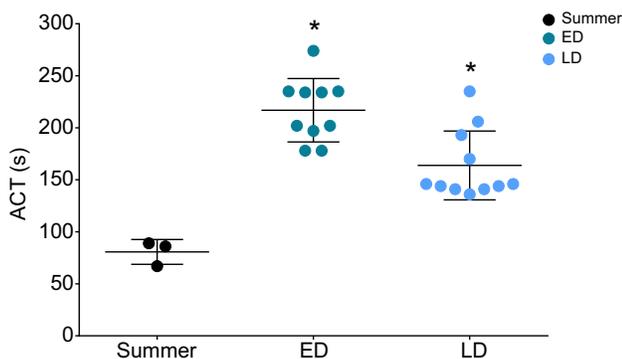

Fig. 3. ACT of the black bear. ACT of female black bears during early denning (ED, N=10), late denning (LD, N=11) and in the summer (N=3) were assessed. Black bears had significantly higher ACT during hibernation compared with the active summer months (unpaired t-test; *P<0.01).
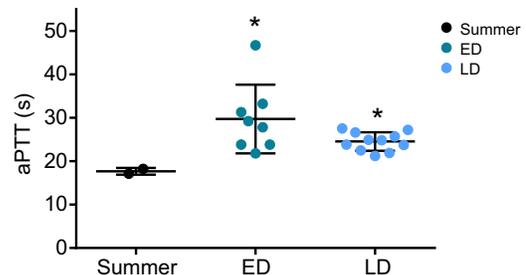

Fig. 4. aPTT of the black bear. aPTT was significantly different between summer months (N=2) and early (N=8) and late denning (N=11), representing a significant downregulation of the intrinsic pathway (*P<0.01).
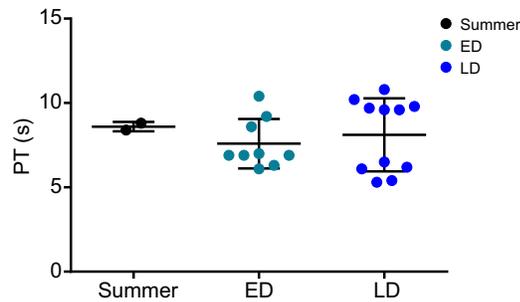
Fig. 5. PT of the black bear. No significant differences in PT were identified when comparing bears during the active summer months (N=2) with early denning (N=9) and/or late denning (N=11) periods. This suggests that bears have similar extrinsic clotting behavior between seasons.

means for evaluating excessive clotting or embolic events. The concentration of D-dimers in the bears' plasma was evaluated in order to make a reasonable conclusion as to whether bears are affected by embolic events during hibernation. The current clinical standard to assess the lack of an embolism (in non-pregnant mammals) is a D-dimer concentration of <250 ng ml^{-1} in plasma. Conditions such as pregnancy, as discussed above, can increase the levels of circulating fibrinolytic degradation products. Thus, we consider it possible that the increase in D-dimer concentration from the pregnant bear may have been caused by her pregnancy. Individually, none of the non-pregnant/nursing bears exceeded a D-dimer concentration of 250 ng ml^{-1}.

Additional information about the intrinsic pathway was collected from the aPTT. This pathway is triggered by blood itself and is produced within the cardiovascular system; abnormal increases in the aPTT can be a reflection of malabsorption and increased plasma potassium levels. Here, we noted further modulation of the intrinsic pathway by increased aPTT during early denning. In order to evaluate the extrinsic pathway, the PT was evaluated. This cascade produces clots from tissue damage externally. The preservation of the extrinsic pathway is consistent with the observed ability of black bears to maintain their responses to foreign bodies and healing of external injuries (Lohuis et al., 2005, 2007; Iaizzo, 2009; Iaizzo et al., 2012; Laske et al., 2005, 2010, 2011).

We consider here that black bears adapt their clotting pathways during hibernation in order to avoid intravascular blood clots from forming. They are also capable of reversing the process as they are preparing themselves to emerge from hibernation. None of the non-pregnant bears that we studied elicited increased fibrinolytic degradation products. Once again, this is a clinical approach to
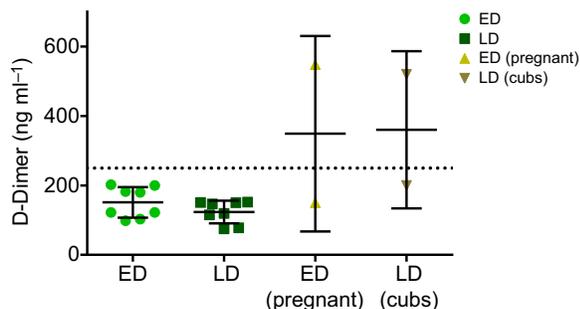
determine whether a patient has undergone an embolic event by evaluating this product in the plasma, which is indicative of embolism or excessive clot formation. Thus, our data support the hypothesis that bears elicit protective mechanisms to avoid clotting events during hibernation. The preferential maintenance of the black bears' extrinsic pathway may be extremely beneficial because, if needed, black bears can be roused to defend themselves and/or their young and still have protection against excessive blood loss if injured. On occasion, we have observed injuries such as a gunshot wound.

It was also observed that females that were pregnant/nursing elicited lower ACT than female bears without cubs. It could be speculated that the regulation of the intrinsic pathways is slightly attenuated during pregnancy to minimize the potential for blood or fluid loss during the birthing process, which occurs while hibernating. Nevertheless, in the future it would be of interest to increase our sample size as well as investigate clotting pathways during several time periods, in order to determine whether this pattern is cyclic. We recognize the limitation of the current studies in the relatively low number of animals studied and the fact that our summer cohort consisted of captive animals. Ongoing work by our group is intended to increase sample size and attempt to use wild animals for all seasonal measurements. In addition, we plan to further assess the clotting factors by various computational methods in upcoming studies. Again, we consider that better understanding of modulations within the mechanisms of the clotting cascade in black bears may introduce opportunities for employing novel clinical mechanisms for anti-coagulation therapy and targeted treatments. It should be noted that in a very recent publication the clotting behavior in hibernating brown bears (*Ursus arctos*) has been described to elicit similar responses (Welinder et al., 2016).

Conclusions

To our knowledge, this is one of the first studies to explore the seasonal clotting properties of free-ranging American black bears. These animals elicit unique modulations in their intrinsic clotting cascades during early denning, late denning and non-hibernating states (summer months). These adaptations have remarkable survival advantages in terms of the avoidance of embolic events during states of immobilization, starvation and renal shut-down. Yet, the bears maintain their extrinsic clotting pathways, which allows responses to foreign bodies and external wound healing. A detailed understanding of these modulations may have translational applications to human medicine for the improved management of the clotting cascades in various clinical situations.

Author contributions
T.G.L., D.L.G., P.A.I., T.L.I. conducted the fieldwork. T.G.L., D.L.G. and P.A.I. offered expert advice in the areas of bear ecology, engineering and physiology. T.L.I. and P.A.I. designed the experiments. T.L.I. prepared the manuscript. All authors read and contributed to the final manuscript.

Fig. 6. D-Dimer levels in the hibernating black bear. D-Dimer plasma concentrations for non-parturient black bears during early denning (N=8) and late denning (N=8), versus early denning pregnant bears (N=2) and late denning bears with cubs (N=3). The normal range is indicated between 0 and the dotted line.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.141549.supplemental

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