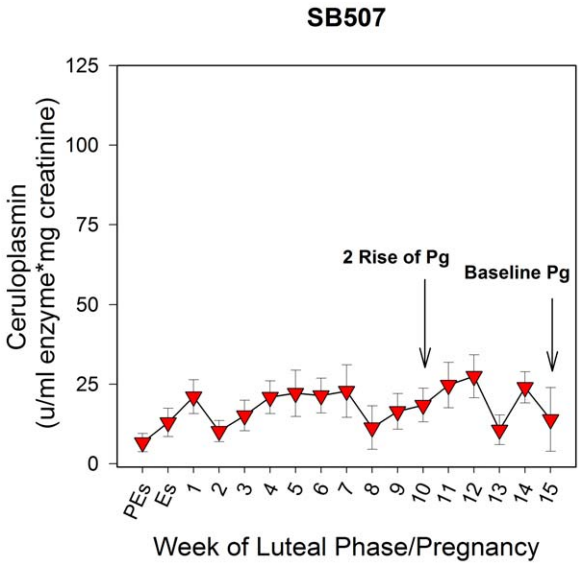

Figure 6. The levels of active ceruloplasmin in urine for a bred/no birth cycle for female SB507: suspected pseudopregnancy/no conception or lost pregnancy that did not elicit an immune response based on ceruloplasmin. In 2009, SB507 (MZS) was artificially inseminated but no change in the levels of active urinary ceruloplasmin was observed throughout the luteal phase and levels were similar to those observed at proestrus and estrus. Pg = Progestagen; PEs = Proestrus; Es = Estrus; Numerical numbers = weeks of the luteal phase. Data are weekly means ± SEM; n = 1 reproductive cycle.
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pregnancies. A schematic model of elevated ceruloplasmin and its relationship with progestagens during the pregnant luteal phase is depicted in Figure 7. Based on the results from this initial study, deviations in the pattern of urinary ceruloplasmin during the secondary luteal phase rise suggest a reduced chance of a term pregnancy. Analysis of additional term and confirmed lost pregnancies will help to elucidate the importance of the temporal pattern of change in relation to term pregnancies compared to lost pregnancies in the giant panda.

Interestingly, in SB507's (MZS) 2009 cycle, no significant increase was observed in the levels of active ceruloplasmin in urine during the luteal phase, indicating that this cycle may have been a pseudopregnancy when analyzing the cycle based on ceruloplasmin (Figure 6). However, it is also possible that this cycle was a lost pregnancy that did not elicit an appropriate inflammatory or immune response. In other species, there is evidence that immunological recognition is important for the maintenance of pregnancy and that an inappropriate maternal response to the fetus can result in fetal death [23,24,25,26,27,28,29]. A failed increase in, or an abnormal pattern of, active ceruloplasmin in urine following breeding or AI may be characteristic of an inappropriate response to pregnancy by the maternal immune system. Moreover, an unusual ceruloplasmin profile could also be a sign of abnormal embryonic development. No studies examining the specific temporal pattern of change in ceruloplasmin during pregnancy in relation to fetal loss have been carried out in other species. However, there is some evidence that either lower or higher levels of ceruloplasmin compared to levels observed during normal pregnancy could be an indication of problems associated with gestation in humans. Abnormal levels of ceruloplasmin have been found in blood and/or in amniotic fluid during pregnancies which resulted in such conditions as spontaneous abortions, preeclampsia, and Klinefelter's syndrome [30,31,32,33,34].

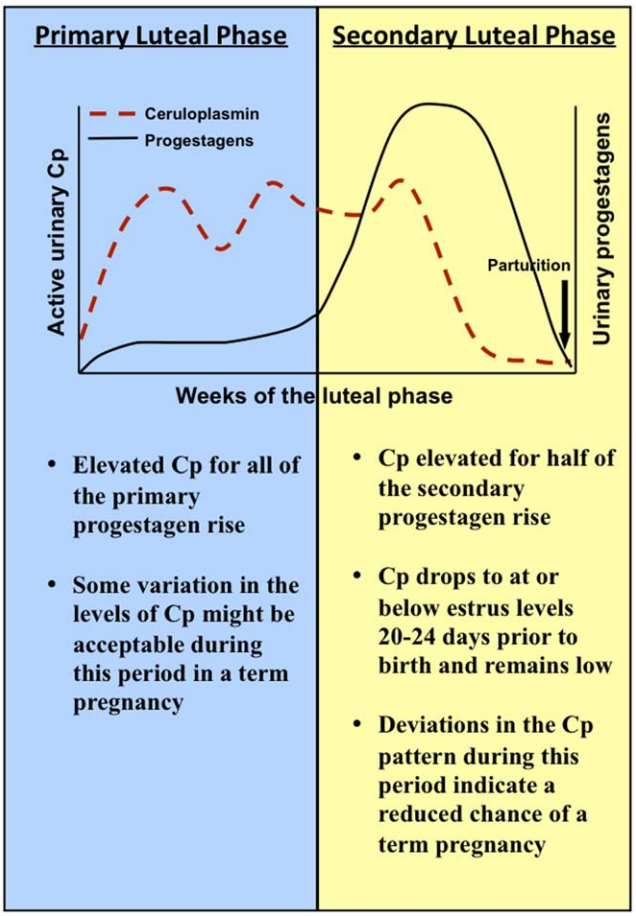

Figure 7. Schematic summary of elevated urinary ceruloplasmin and its relationship with progestagens during the pregnant luteal phase in the giant panda.
doi:10.1371/journal.pone.0021159.g007

These data provide a foundation for understanding conception, fetal loss, and recognition and maintenance of pregnancy in giant pandas. More research will be needed to further verify the rate of lost pregnancies as well as to characterize the abnormal patterns in ceruloplasmin that we observed in known and suspected lost pregnancy cycles. Factors influencing pregnancy loss in captive animals will also need to be examined. If the rate of pregnancy loss is as high as this initial study suggests, ex-situ conservation efforts of this iconic endangered species will improve tremendously by subsequent investigations into the origin and mechanisms of pregnancy recognition and maintenance. Other ursids undergo both pseudopregnancy and delayed implantation of the embryo [35,36,37]. Thus, these studies will provide a background for future research in other endangered carnivores and ursids, such as the polar bear, in which common physiological factors are unreliable at determining pregnancy and the understanding of many components of basic reproductive biology is lacking.

Materials and Methods

Animals and sample collection

Urine from reproductive cycles of the four adult female giant pandas held at North American institutions was used for the study. Females SB507, SB437, SB452 and SB371 were housed at the Memphis Zoological Society (MZS), the Smithsonian National Zoological Park (SNZP), Zoo Atlanta (ZA) and the San Diego Zoo

(SDZ), respectively. All animals were fed a diet consisting of at least 85–95% bamboo, with fruit and high fiber biscuits given as treats, and water was provided *ad libitum*. Urine samples were collected fresh, in a similar manner from each institution, via aspiration from the animal's enclosure and then stored frozen at −20°C. Sub-samples of urine (500 ul aliquots) from SNZP, ZA and SDZ were shipped frozen overnight to the MZS for ceruloplasmin analysis.

Ceruloplasmin assay

Urine samples from sixteen estrous cycles (four cycles from each female) were used in the present study. Three to seven samples per week were analyzed for the weeks of proestrus (when available), estrus, and weeks 1 through the week of baseline progestagen levels or parturition of the luteal phase/pregnancy. Known pseudopregnant cycles when no breeding occurred were used as controls from each female.

Prior to being assayed for ceruloplasmin, urine samples were centrifuged at 3500 RPM for 10 minutes and concentrated to 300 µl. Samples were then assayed for ceruloplasmin through oxidasic activity measurement according to the optimizations of Sunderman and Nomoto, 1970 [38] with modifications. Briefly, the concentration of ceruloplasmin is determined by the rate of formation of a colored product from ceruloplasmin and the substrate, N,N-dimethyl-p-phenylenediamine (Sigma-Aldrich, St. Louis, MO). The rate of colorometric change is proportional to the amount of ceruloplasmin in each sample. The change in absorbance of the photometric product was measured every 30 seconds for a 5 minute interval using a Biomate 3 UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA). The change in absorbance was also measured in a blank control for each run and this value was subtracted from the change in absorbance in each urine sample. Units of ceruloplasmin were calculated based on the difference in the change of absorbance in each sample from the control multiplied by reaction volume per the unit definition of change in absorbance at 550 nm (0.01), volume of enzyme used and the conversion factor for the published unit definition of a 7 ml reaction volume [39]. The inter-assay coefficient of variance was 14.9%. Creatinine was also measured in each urine sample to account for the concentration of water [3,40], and final ceruloplasmin concentration was expressed per mg of creatinine. Ceruloplasmin assay validations are shown in the Materials and Methods S1, Figures S1 and S2 and Table S1.

Progestagen enzyme immunoassays

An enzyme immunoassay (EIA) using the same progestagen antibody (CL425; C. Munro, UCDavis, CA) and conjugate (horseradish peroxidase progesterone conjugate; C. Munro, UCDavis, CA) was utilized to determine urinary progestagen concentrations for animals from SNZP, ZA and MZS. Urinary progestagen concentrations for SB371 (SDZ) were measured at SDZ using a single antibody EIA for the progesterone metabolite, pregnanediol–3-glucuronide (PdG; P-26; C. Munro, UCDavis, CA). Assay details are provided in the Materials and Methods S1. Both the group-specific CL425 progestagen antibody and the PdG specific antibody are widely used for captive management of the giant panda and a similar relationship was observed between active ceruloplasmin and the secondary rise of progestagens in urine for term pregnant cycles when concentrations of progestagens were analyzed using either antibody.

Statistical analysis

Data sets for ceruloplasmin were tested for homogeneity of variance by Hartley's test [41,42] and values were log transformed before statistical analysis, if heterogeneous variance was indicated. Changes in weekly ceruloplasmin levels for each cycle were analyzed using a one-way analysis of variance (ANOVA) using JMP 7 Statistical Discovery (SAS, Cary, NC). Dunnett's test was used to determine significant differences between weekly means of the luteal phase and that of estrus/proestrus (baseline control) level means. Means were considered different at $P \leq 0.05$. Graphed ceruloplasmin values are presented as weekly means ± SEM of non-transformed values and represent 3–7 urine samples/week.

For each luteal phase, the primary and secondary rise in urinary progestagens were determined by an iterative method as described previously for fecal progestagens [5]. Briefly, the primary rise was regarded as the time from the beginning of the luteal phase until the elevation of progestagens to 2 standard deviations above the mean for two or more days, while the secondary rise in urinary progestagens was the duration from the end of the primary rise until the end of the luteal phase. Termination of the luteal phase was defined as either parturition (term pregnancies) or the return to baseline progestagens (all other cycles).

Supporting Information

Figure S1 The effect of freeze-thaw cycles on the levels of active ceruloplasmin in urine obtained from giant pandas. Repeated freeze-thaw cycles could affect the levels of active ceruloplasmin in urine. Ceruloplasmin activity was decreased by an average of 33, 42, 50, and 53% in the 2nd, 3rd, 4th, and 5th thaws, respectively, compared to the activity observed at the initial measurement (set to 100%). Because banked samples had been frozen and thawed on numerous occasions and the exact number of freeze-thaw cycles was unknown and inconsistent between cycles, samples were not analyzed between different cycles. Data are the percent mean decrease in ceruloplasmin activity from the activity found at the initial thaw for the current study ± SEM; n = 3 samples.
(TIF)

Figure S2 The effects of boiling on the levels of active ceruloplasmin in urine obtained from giant pandas. Change in absorbance in each urine sample was measured prior to boiling and then after boiling for 10 or 30 minutes. Boiling for 10 minutes produced a decrease in activity by an average of 34%, while boiling for 30 minutes produced a decrease in activity by an average of 65%, compared to the ceruloplasmin activity observed with no treatment (set to 100%). Data are the percent mean decrease in ceruloplasmin activity from the activity found at the initial measurement ± SEM; n = 4 samples.
(TIF)

Table S1 Comparison of active ceruloplasmin values in sample pools of giant panda urine obtained from the change in absorbance/ml enzyme method and standard curve method. To check the validity that the rate of colorimetric change is proportional to the amount of ceruloplasmin in giant panda urine, values obtained from the change in absorbance/ml enzyme calculation method were compared to values obtained from a standard curve method. For the change in absorbance/ml enzyme calculation method, units of ceruloplasmin in two pools (a high and low pool) were calculated based on the difference in the change of absorbance in each sample from the blank control multiplied by reaction volume per unit definition for the change in absorbance at 550 nm (0.01), volume of enzyme used and the published conversion factor for the unit definition of a 7 ml reaction volume [39]. For the standard curve method, after subtracting the change of absorbance in each sample from the

blank control, units of ceruloplasmin in each pool were calculated both manually using the linear regression (LR) equation obtained from the standard curve; $y = 0.0067x - 0.0011$ and by using Sigma Plot software (Systat Software Inc., San Jose, CA) to calculate the values using a 4-parameter logistic (4PL) curve fit. A. The raw values of active ceruloplasmin for pool dilutions; values are expressed as u/ml enzyme* (change in absorbance/ml enzyme method) or u/ml* (standard curve method). B. Raw values of active ceruloplasmin by dilution factor; values are expressed as u/ml enzyme* (change in absorbance/ml enzyme method) or u/ml*(standard curve method). *Values are not expressed on a per mg of creatinine basis.
(DOC)

Materials and Methods S1
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: ELW. Performed the experiments: ELW. Analyzed the data: ELW. Contributed reagents/materials/analysis tools: DCK BSD AJK. Wrote the manuscript: ELW. Commented on the paper: DCK BSD AJK.

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