Szkoła Główna Gospodarstwa Wiejskiego
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Funkcjonowanie populacji wiewiórki pospolitej na terenach o różnym stopniu przekształcenia antropogenicznego – w rezerwacie leśnym i parku miejskim

Functioning of red squirrel population in areas under different levels of anthropopression – in a forest reserve and an urban park

Rozprawa doktorska
Doctoral thesis

Praca wykonana pod kierunkiem
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Oświadczam ponadto, że niniejsza wersja rozprawy jest identyczna z załączoną wersją elektroniczną.

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Spis treści

1. Wykaz publikacji stanowiących rozprawę doktorską

1. **<u>Beliniak A.</u>,** Krauze-Gryz K., Jasińska K., Jankowska K., Gryz J. 2021. Contrast in daily activity patterns of red squirrels inhabiting urban park and urban forest. Hystrix, the Italian Journal of Mammalogy, 32(2):159–164. https://doi.org/10.4404/hystrix-00476-2021.
   (IF: 1,5; 140 pkt)

2. **<u>Beliniak A.</u>,** Gryz J., Klich D., Jasińska K., Krauze-Gryz D. 2022. Body Condition and Breeding of Urban Red Squirrels: Comparison of Two Populations Affected by Different Levels of Urbanization. Animals, 12(23): 3246. https://doi.org/10.3390/ani12233246.
   (IF: 3,0; 100 pkt)

3. **<u>Beliniak A.</u>,** Gryz J., Klich D., Łopucki R., Sadok I., Ożga K., Jasińska K.D., Ścibior A., Gołębiowska D., Krauze-Gryz D. 2024. Long-term, medium-term and acute stress response of urban populations of Eurasian red squirrels affected by different levels of human disturbance. PLoS ONE. https://doi.org/10.1371/journal.pone.0302933.
   (IF: 3,7, 100 pkt)

4. Jackowiak M., Krauze-Gryz D., **<u>Beliniak A</u>.,** Jasińska K. D., Gryz J., Żyfka-Zagrodzińska E., Matracka A. The heavy burden of city life: factors affecting mercury bioaccumulation in urban red squirrels.
   Manuskrypt w trakcie recenzji w Environmental Science and Pollution Research
   (IF: 5.8, 100 pkt)

2. Streszczenie

Urbanizacja w dużym stopniu wpływa na środowisko naturalne, a zwierzęta żyjące na terenach miejskich muszą mierzyć się z warunkami, które mogą być inne niż te spotykane w naturalnych warunkach. Jednym z gatunków, który przystosował się do życia w miastach i osiąga w nich wysokie zagęszczenia populacji jest wiewiórka pospolita *Sciurus vulgaris*. Wiedza na temat funkcjonowania zwierząt w środowiskach poddanych w różnym stopniu antropopresji wciąż jest niewielka. W mojej pracy doktorskiej, składającej się z cyklu publikacji, zostały porównane dwie miejskie populacje wiewiórek, żyjące na terenach różniących się od siebie pod względem przekształcenia antropogenicznego. Jedna populacja zamieszkiwała park miejski w centrum miasta (Łazienki Królewskie w Warszawie), chętnie odwiedzany przez mieszkańców i turystów, a wiewiórki tam są dokarmiane cały rok. Druga badana populacja żyła na terenie leśnego rezerwatu przyrody położonego na obrzeżach miasta i zamkniętego dla odwiedzających (Rezerwat Przyrody Las Natoliński w Warszawie). W skład cyklu publikacji wchodzą cztery artykuły, w których porównywane były m.in.: 1) aktywność dobowa i sezonowa, 2) kondycja zdrowotna i aktywność płciowa, 3) reakcje na stres (wyrażone kilkoma wskaźnikami) oraz 4) poziom zanieczyszczenia organizmu metalem ciężkim, na przykładzie rtęci. Wykazano szereg różnic pod względem funkcjonowania obu populacji. Wzorzec aktywności dobowej różnił się w zależności od pory roku oraz terenu badań: w rezerwacie wzorzec ten bardziej przypominał tereny pozamiejskie, gdzie widoczne były dwa szczyty aktywności (jeden po wschodzie słońca, a drugi przed zachodem), w parku natomiast wiewiórki prezentowały głównie jeden szczyt aktywności (który zaczynał się po wschodzie słońca i trwał do popołudnia). Wykazano też różnice w kondycji zdrowotnej oraz aktywności płciowej: wiewiórki zamieszkujące park miały niższą masę ciała i gorszą kondycję, choć jednocześnie zaobserwowano tam więcej aktywnych płciowo samic. Nie wykazano natomiast różnic pod kątem reakcji na stres pomiędzy obiema populacjami, choć wiewiórki żyjące w rezerwacie leśnym wykazywały wyższy poziom stresu podczas bezpośredniego kontaktu z człowiekiem (mierzonego częstotliwością oddechu). Na poziom zanieczyszczenia sierści metalem ciężkim (rtęcią) wpływ miały teren badań, płeć oraz status płciowy – wartości te były wyższe u wiewiórek zamieszkujących park oraz u aktywnych płciowo samic.

Wyniki badań przedstawionych w artykułach sugerują, że wiewiórki zamieszkujące tereny o różnym stopniu przekształcenia antropogenicznego różnią się od siebie pod wieloma aspektami – te zamieszkujące rezerwat leśny mają więcej cech wspólnych z populacjami zasiedlającymi naturalne środowiska, a te z parku miejskiego, funkcjonują odmiennie. Zwierzęta z parku najprawdopodobniej zmodyfikowały swoją aktywność dobową, by dostosować ją do obecności odwiedzających, co zwiększa szanse na zdobycie pokarmu i jednocześnie pozwala na skrócenie czasu potrzebnego na znalezienie odpowiedniej ilości pożywienia. Różnice w masie ciała oraz kondycji mogą być spowodowane dużą konkurencją między osobnikami w populacji o dużym zagęszczeniu (tj. w parku), choć z drugiej strony, stabilny dostęp do pokarmu uzupełniającego przez cały rok może zmniejszać znaczenie zapasów tłuszczu dla przeżycia i sukcesu rozrodczego. Dostępność pokarmu może również skutkować większą liczbą samic aktywnych płciowo i potencjalnie wpływać pozytywnie na rozrodczość. Brak różnic w reakcji na długo i średnioterminowy stres (tj. w poziomie metabolitów hormonów stresu w sierści i odchodach) pomiędzy populacjami wskazuje na podobny wpływ czynników środowiskowych (m.in.. pogody, pory roku), a zmniejszona reakcja na bezpośredni kontakt z człowiekiem wiewiórek z parku może wynikać z przyzwyczajenia do jego obecności. Większe zanieczyszczenie sierści metalem ciężkim w populacji wiewiórek zamieszkujących park miejski może być wynikiem zmian w sposobie żerowania (co może mieć bezpośredni wpływ na poziom metali ciężkich w organizmie). Wyniki badań zawartych w czterech artykułach potwierdzają dużą plastyczność wiewiórek pospolitych zamieszkujących obszar o dużej antropopresji oraz dostosowanie się tych zwierząt do specyficznych miejskich warunków. Ponadto wyniki podkreślają rolę miejskich środowisk leśnych jako ważną ostoję (refugium) dla dzikich zwierząt.

Słowa kluczowe: aktywność dobowa, aktywność sezonowa, kondycja, masa ciała, *Sciurus vulgaris*, stres, urbanizacja, zaburzenia środowiskowe

3. English summary

Urbanization highly affects the environment, and animals living in urban areas encounter conditions that may differ from rural habitats. One of the species that has adjusted to these specific conditions and can reach high population density is the red squirrel *Sciurus vulgaris*. Knowledge of how wildlife cope with this anthropogenic disturbance is still scarce. In this thesis, consisting of a series of papers, two populations of red squirrels inhabiting utterly different habitats are compared. One population lived in an urban park in a city centre (Royal Łazienki Museum in Warsaw), which is popular among inhabitants and tourists, and squirrels have access to supplementary feeding throughout the year. The second population lived in an urban forest reserve (Las Natoliński Nature Reserve), which is closed to the public and located in a suburban area. This thesis consists of four papers, in which, among others, I compared 1) daily and seasonal activity, 2) body condition and sexual activity, 3) reaction to stress, and 4) level of intoxication by heavy metal (mercury). A number of differences between both populations were found. Daily and seasonal activity patterns differed: in the urban forest, activity patterns were similar to rural populations, with two activity peaks (one after sunrise and a second before sunset); in the urban park, squirrels presented mostly one peak of activity (which started after sunrise and lasted until the afternoon). Differences in body condition and sexual activity were also shown: squirrels from the park had lower body mass and worse body condition. At the same time, more sexually active females and more juveniles were observed there. In contrast, there were no differences in terms of stress reaction between the two populations, although squirrels from the urban forest showed higher reactions to stress during direct contact with humans (measured by breath rate). Levels of heavy metal (mercury) contamination in fur were influenced by study area, sex, and sex status – values were higher in squirrels from the urban park and sexually active females. The results suggest that squirrels inhabiting areas of different anthropogenic disturbances may differ in many aspects – those living in an urban forest are similar to populations inhabiting rural habitats. In contrast, those from an urban park show a number of changes. Animals from the park probably have modified their diurnal activity to adjust to the presence of humans visiting the park, which increases the chances of obtaining food and, simultaneously, reduces the time needed to find enough food. Differences in body weight and fitness may be due to high competition between individuals in a high-density population (i.e., urban park). On the other hand, stable access to supplemental food throughout the year may reduce the importance of fat reserves for survival and reproductive success. Food availability may also result in more sexually active females and potentially positively affect reproduction. The lack of differences in reactions to stress indicates that both populations are similarly subject to environmental stressors (e.g., weather, season), and the reduced response to direct contact with humans of park squirrels may be due to habituation to their presence. Higher heavy metal contamination may be due to changes in foraging patterns. The results of the studies included in the four articles confirm the high plasticity of red squirrels living in environments affected by anthropopression and the adjustment of these animals to specific urban conditions. In addition, the results highlight the role of urban forests as important refuges for wild animals, where their populations may remain relatively natural.

Keywords: body mass, daily activity, condition, environmental disturbances, hormone stress metabolites, seasonal activity, *Sciurus vulgaris*, stress, urbanization.

4. Wstęp i cel badań

Urbanizacja to proces, który ma bardzo duży wpływ na dziką przyrodę i oddziałuje na nią na wiele sposobów (Grimm i in. 2008; Johnson i Munshi-South 2017; McDonald i in. 2019). W ciągu ostatnich dekad miasta się gwałtownie powiększają i przewiduje się, że w kolejnych latach wzrost liczby ludzi mieszkających w miastach będzie jeszcze większy (Li i in. 2021). Tereny zurbanizowane są znacząco odmienne od środowisk naturalnych, a ich ekspansja powoduje m.in. utratę lasów (Birnie-Gauvin i in. 2016; Alberti i in. 2017; McDonald i in. 2019). Wiele gatunków zwierząt nie jest w stanie poradzić sobie ze specyficznymi warunkami miejskimi, co prowadzi do zmniejszenia różnorodności biologicznej (McDonald i in. 2019; Łopucki i in. 2019). Zmiany wprowadzone przez człowieka oddziałują bezpośrednio na dzikie zwierzęta, a specyficzne warunki miast mogą negatywnie wpływać na populacje i/lub poszczególne osobniki. Przykładem takich zaburzeń środowiskowych, które wpływają na zwierzęta jest hałas, który może zmieniać poziom czujności, sposób szukania pożywienia (Sweet i in. 2022), może powodować wyższy stres, obniżoną odporność i zmiany w ekspresji genów (Kight i Swaddle 2011; Francis i Barber 2013). Zanieczyszczenie światłem z kolei powoduje zmiany w aktywności, interakcjach między osobnikami, wykorzystaniu przestrzeni (Ciach i Fröhlich 2019; Hoffmann i in. 2019), strategiach żerowania i reprodukcji (Longcore i Rich 2004; Gaston i in. 2014). Intensywny miejski ruch uliczny może prowadzić do kolizji pojazdów ze zwierzętami, a ulice zwiększają fragmentację środowiska, co prowadzi do utrudnia przemieszczania się osobników, ogranicza dostępność środowisk oraz przepływ genów między populacjami (Fahrig 2003; Johnson i Munshi-South 2017; Seiler i Bhardwaj 2020). Zwierzęta żyjące w miastach narażone są również na różnego rodzaju zanieczyszczenia (Murray i in. 2019), a zwiększone stężenie metali ciężkich w miastach może być potencjalnym zagrożeniem dla populacji oraz ekosystemów (Dietz i in. 2006; Poissant i in. 2008; Dietz, Outridge, i Hobson 2009; Rea i in. 2013; Herring, Eagles-Smith, i Varland 2018; Dietz i in. 2022). To może skutkować obniżeniem odporności organizmu i powodować nieprawidłowości rozwojowe u poszczególnych osobników (Serieys i in. 2018). Biorąc pod uwagę rosnącą ilość terenów zurbanizowanych i ich wpływ na dzikie zwierzęta (Baker i Harris 2007) oraz potencjalnie wyższą ekspozycję zwierząt na zanieczyszczenia w miastach (Newman 2006), wpływ metali ciężkich na populacje ssaków powinien być monitorowany. Dodatkowo choroby i pasożyty mogą rozprzestrzeniać się szybciej na terenach zurbanizowanych (Bradley i

Altizer 2007; Oro i in. 2013), a zwierzęta są bardziej podatne na choroby z powodu obniżonej odporności (Birnie-Gauvin i in. 2016). Stała obecność ludzi może także być czynnikiem stresowym dla zwierząt (Rangel-Negrín i in. 2009; Zwijacz-Kozica i in. 2013), choć wyniki są niejednoznaczne (Iglesias-Carrasco i in. 2020).

Pomimo wymienionych powyżej negatywnych konsekwencji, środowiska zurbanizowane mogą mieć również pozytywny wpływ na zwierzęta (Jakubiak i Klich 2021). W miastach obserwowane jest tzw. zjawisko miejskiej wyspy ciepła, czyli inne ruchy wiatrów, wyższe temperatury powietrza oraz gleby w porównaniu do okolicznych terenów pozamiejskich (Rizwan i in. 2008; Han i in. 2014; Droste i in. 2018), co ułatwia przeżycie i zdobycie pokarmu, zwłaszcza zimą (Bateman i Fleming 2012). Co więcej, okres rozrodczy może być wydłużony w związku z korzystniejszymi warunkami środowiska (Dominoni i in. 2013), a część osobników zimuje w mieście bez podejmowania migracji sezonowej (Partecke i Gwinner 2007). Ponadto zabudowania oraz inne konstrukcje znajdujące się w mieście, mogą zapewniać zwierzętom dodatkowe schronienie (Herr i in. 2010; Lowry i in. 2013). Co więcej, liczba naturalnych drapieżników w miastach może być niższa (Shochat i in. 2006), choć z drugiej strony ich rolę przejmują drapieżniki udomowione, np. koty (*Felis catus*) (Jokimäki i in. 2017). Pokarm uzupełniający, oprócz wspomnianych wcześniej negatywnych efektów, może również pozytywnie oddziaływać na zwierzęta (Bateman i Fleming 2012; Lowry i in. 2013), np. powodować wzrost masy ciała, czy zwiększoną aktywność płciową samic i przeżywalność (Oro i in. 2013; Łopucki i in. 2019). Niektóre gatunki w miastach mogą też osiągać wyższe zagęszczenia niż w naturalnych środowiskach (McCleery 2009). Dodatkowo zwierzęta z miast najczęściej są bardziej śmiałe oraz prezentują mniejszą neofobię (Uchida i in. 2019). Z drugiej strony jednak miasta mogą być swojego rodzaju "pułapką ekologiczną" – zwierzęta zachęcone dostępnością pożywienia zajmują środowiska miejskie, które jednak w dłuższej perspektywie czasowej okazują się być niskiej jakości, co wpływa negatywnie na przeżywalność zwierząt, ich reprodukcję i kondycję (Lepczyk i in. 2017; De Satgé i in. 2019). Wciąż nie wiadomo czy środowiska miejskie są takimi pułapkami czy raczej pełnią rolę refugium w długiej perspektywie czasowej (Lepczyk i in. 2017).

Liczba badań podejmujących tematykę wpływu urbanizacji na środowisko naturalne w ostatnich latach rośnie (McDonnell i Hahs 2015), jednak ekologia środowisk miejskich (ang. „urban ecology") jest względnie młodą dziedziną biologii. Wciąż niewiele wiadomo na temat mechanizmów pozwalających na przeżycie zwierzętom w środowisku miejskim oraz jak i dlaczego osobniki zamieszkujące te środowiska różnią się od przedstawicieli tego samego gatunku żyjących w środowiskach naturalnych (Ouyang i in. 2018). Lepsze zrozumienie, jak zwierzęta dostosowują się do środowisk zurbanizowanych pozwala na podejmowanie odpowiednich decyzji odnośnie planowania przestrzeni miejskiej i ochrony przyrody.

Wiewiórka pospolita *Sciurus vulgaris* jest jednym tych z gatunków, który występuje w miastach i osiąga w nich duże zagęszczenia (Rézouki i in. 2014; Fey i in. 2016; Reher i in. 2016; Haigh i in. 2017; Uchida i in. 2019; Kostrzewa i Krauze-Gryz 2020; Krauze-Gryz i in. 2021a*a,b*). Choć pierwotnie była związana z lasami (Bosch i Lurz 2012), tereny zielone w miastach mogą stanowić ważną ostoję dla tego gatunku (Jokimäki i in. 2017; Fingland i in. 2022). W ciągu ostatnich lat zainteresowanie badaczy miejskimi populacjami wiewiórek wyraźnie wzrasta (Fingland i in. 2022). Zauważono wiele różnic między populacjami żyjących na terenach o różnym stopniu urbanizacji: osobniki miejskie zajmują mniejsze areały osobnicze (Hämäläinen i in. 2018; Krauze-Gryz i in. 2021*a*), korzystają z różnych dostępnych struktur, np. budynków (Hämäläinen i in. 2018), więcej czasu spędzają na ziemi oraz częściej wchodzą w interakcje z człowiekiem (Krauze-Gryz i in. 2021*a,b*). W przypadku terenów zurbanizowanych, wiewiórki często dokarmiane są bezpośrednio przez ludzi, jak również korzystają z pokarmu przeznaczonego dla ptaków w karmnikach (Bosch i Lurz 2012; Krauze-Gryz i Gryz 2015; Reher i in. 2016; Thomas i in. 2018; Kostrzewa i Krauze-Gryz 2020; Krauze-Gryz i in. 2021*a*).

Celem badań było porównanie dwóch populacji wiewiórek zamieszkujących to samo miasto (Warszawę), ale dwa bardzo różniące się od siebie środowiska: park miejski oraz rezerwat leśny. Oba tereny są w różnym stopniu przekształcone antropogenicznie: park jest silnie zmodyfikowany przez człowieka, a zwierzęta są tam regularnie dokarmiane, rezerwat natomiast jest zamknięty dla odwiedzających, a ingerencja człowieka w środowisku jest niewielka.

Wcześniejsze badania wiewiórek prowadzone na tych samych terenach wykazały istotne zmiany w funkcjonowaniu populacji zamieszkującej park w porównaniu do rezerwatu (Krauze-Gryz i in. 2021*a,b*). W obecnych badaniach skupiono się na tych aspektach populacji, które pozostają niezbadane lub wyniki innych badań są niejednoznaczne. W pracy zostały porównane różne aspekty funkcjonowania populacji: 1) dzienny i sezonowy rytm aktywności, 2) masa ciała, kondycja fizyczna i aktywność rozrodcza, 3) poziom stresu, 4) poziom zanieczyszczenia organizmu metalem ciężkim (rtęcią).

5. Główne założenia badawcze

1) Dobowy i sezonowy wzorzec aktywności wiewiórek żyjących na obu terenach jest odmienny. Wiewiórki w rezerwacie leśnym, gdzie obecność ludzi jest ograniczona, prezentują typowy wzorzec aktywności dla gatunku w jego naturalnym środowisku, wiewiórki z parku dostosowują swą aktywność do obecności ludzi dostarczających pokarm.

2) Wiewiórki zamieszkujące park, gdzie są dokarmiane, będą miały wyższą masę ciała oraz lepszą kondycję, niezależnie od pory roku. Dzięki większej dostępności pokarmu zimą, wiewiórki w parku pozostaną w lepszej kondycji, dzięki czemu będą mogły wcześniej przystąpić do rozrodu.

3) W rezerwacie, gdzie jest mniejsze zagęszczenie wiewiórek, zwierzęta będą miały mniejszy poziom metabolitów tzw. hormonów stresu. Odpowiedź stresowa na bezpośredni kontakt z człowiekiem (wokalizacja, częstotliwość oddechu oraz poziom zaniepokojenia, ang. „struggle rate”) w parku miejskim będzie słabsza, ponieważ osobniki tam żyjące mają częstszy kontakt z ludźmi.

4) Wiewiórki zamieszkujące park w centrum miasta są bardziej narażone na zanieczyszczenia, dlatego będą się charakteryzowały wyższym stężeniem zawartości rtęci w sierści niż wiewiórki w rezerwacie. W związku z tym, że rtęć podlega bioakumulacji, poziom zanieczyszczenia rtęcią poszczególnych osobników będzie zależał od ich wieku i kondycji.

6. Materiał i metody

Badania prowadzone były na obszarze dwóch terenów zielonych w Warszawie, różniących się stopniem przekształcenia antropogenicznego: w parku miejskim (Muzeum Łazienki Królewskie) oraz w rezerwacie leśnym (Rezerwat Przyrody Las Natoliński).

Początki Muzeum Łazienki Królewskie sięgają XVII wieku. Obecnie teren znajduje się w centrum miasta i jest bardzo popularny wśród mieszkańców i turystów. Według raportu Polskiej Organizacji turystycznej Muzeum Łazienki Królewskie wraz z ogrodami odwiedziło w 2022 roku ponad 5,2 mln osób (https://www.pot.gov.pl/). Park zajmuje powierzchnię 74 hektarów i występują w nim 92 gatunki drzew i krzewów (Babińska-Werka i Żółw 2008). Około 20% drzew ma ponad 150 lat (Babińska-Werka i Żółw 2008). Występujące drzewa to przede wszystkim gatunki liściaste: grab (*Carpinus betulus*), dąb szypułkowy (*Quercus robur*), buk (*Fagus sylvatica*) jak również leszczyna (*Corylus avellana*), orzech włoski (*Juglans regia*) oraz orzech czarny (*Juglans nigra*) (Babińska-Werka i Żółw 2008). Poza naturalną bazą pokarmową dostępną dla zwierząt, ptaki i wiewiórki są także dokarmiane zarówno przez pracowników parku jak również odwiedzających. Zwierzętom podawane są przede wszystkim nasiona, w tym słonecznika oraz orzechy (włoskie i laskowe).

Rezerwat przyrody Las Natoliński znajduje się na obrzeżach miasta, w odległości około 10 km od centrum Warszawy. Rezerwat został utworzony w 1991 roku, a jego powierzchnia to 105 hektarów. Obszar jest objęty ochroną oraz wstęp do niego jest bardzo ograniczony (do wejścia niezbędna jest przepustka wydawana przez Regionalną Dyrekcję Ochrony Środowiska). Obecnie praktycznie cały teren rezerwatu jest zalesiony. Najstarsze drzewa mają ponad 250 lat, martwe są pozostawiane do naturalnego składu. Bezpośredni wpływ ludzi jest bardzo ograniczony. Teren stanowi ostoję przyrody, gdzie wpływ antropopresji jest niższy niż na obszarach otaczających. Drzewa są głównie liściaste, występuje między innymi grab, dąb szypułkowy, jesion wyniosły (*Fraxinus excelsior*), wiąz pospolity (*Ulmus minor*), leszczyna oraz olsza czarna (*Alnus glutinosa*). Wiewiórki na tym terenie nie są w żaden sposób dokarmiane.

Na obu terenach obecne są drapieżniki, w tym puszczyki (*Strix aluco*, Gryz, Krauze i Goszczyński 2008) oraz krogulce (*Accipiter nissus*, Gryz J. os. obs.). Stwierdzono również gniazdujące jastrzębie *Accipiter genitilis* na terenie rezerwatu Las Natoliński (Gryz J. os. obs.). Z kolei na terenie parku liczne są ptaki krukowate, które mogą również polować na wiewiórki: wrony siwe (*Corvus corone*) oraz gawrony (*Corvus frugilegus*)

(Beliniak i Krauze-Gryz 2024). Na obu terenach można spotkać ssaki drapieżne: lisa pospolitego (*Vulpes vulpes*) (Jackowiak i in. 2021) kunę domową (*Martes foina*) (Krauze-Gryz D., os. obs.) oraz dziko żyjące koty, szczególnie często obecne na terenie parku (Jankowska 2021).

Według poprzednich badań prowadzonych na obu obszarach zagęszczenie wiewiórek zostało oszacowane w parku na więcej niż 2 os./ha oraz na 0,29 os./ha na terenie rezerwatu (Krauze-Gryz i in. 2021*a*).

Na obszarze parku miejskiego i rezerwatu leśnego prowadzone były odłowy wiewiórek oraz obserwacje za pomocą fotopułapek. Odłowy były prowadzone od lipca 2018 do grudnia 2020 roku. Do badań wykorzystywane były pułapki żywołowne (51x15x15 cm) (“Jerzyk” Jerzy Chylarecki, Białowieża, Polska). Na terenie parku miejskiego użyto 30 pułapek, a w rezerwacie leśnym 40 pułapek. Pułapki umieszczone były na ziemi lub na drewnianych półkach przykrytych folią i częściowo zakryte plastikową folią, która chroniła przed deszczem i śniegiem. W celu zachęcenia i nauczenia wiewiórek wchodzenia do pułapek, przez 7 dni przed odłowami do środka wrzucane były orzechy laskowe i włoskie.. Po tym tygodniowym okresie nęcenia, przeprowadzania była sesja połowowa trwająca średnio cztery dni (od czterech do dziewięciu). Odłowy były prowadzone w tym samym miesiącu na obu terenach. Pułapki nastawiane były około godziny 6-7 rano, sprawdzane po 2-4 godzinach, a następnie zabezpieczane przed zamknięciem. Po złapaniu, wiewiórka była przekładana do jutowego worka, gdzie mierzony był wskaźnik zaniepokojenia (“struggle rate”). Przez 30 sekund mierzono czas, w którym wiewiórka poruszała się niespokojnie w worku. Następnie wiewiórka była przekładana się do zamkniętego z jednej strony drucianego cylindra, gdzie dokonywano pomiaru liczby oddechów w ciągu 20 sekund oraz gdzie oceniana była wokalizacja w czterostopniowej skali. Każda nowo złapana wiewiórka miała zakładany kolczyk (2x8 mm, National Tag&Band, Newport, KY, USA). Mierzona była masa ciała z dokładnością do 10 g (waga sprężynowa Pesola). Dodatkowo, za pomocą miarki, mierzona była prawa tylna stopa (bez pazurów) z dokładnością do 1 mm. Kondycja ciała była oceniana na podstawie porównania długości stopy oraz masy ciała (Wauters i Dhondt 1995). Oceniana była również płeć i status płciowy samic: nieaktywne płciowo, aktywne płciowo i w ciąży lub w trakcie laktacji (według Santicchia i in. 2018). Określany był również wiek osobników, przy podziale na osobniki dorosłe i młodociane (Wauters i Dhondt 1993). W trakcie pomiarów pobierano próbki sierści, które wycinano nożyczkami z ogona, a następnie umieszczano w torebkach strunowych. Zbierane były również odchody pozostawione w klatce, które następnie były umieszczane w probówkach typu Eppendorf i transportowane w lodówce do laboratorium. Po każdym odłowie zmieniano folię pod klatką, co pozwalało na zbieranie jedynie świeżych odchodów. Sierść i odchody zostały następnie przeanalizowane w laboratorium w celu oceny zawartości metabolitów tzw. hormonów stresu.

Fotopułapki zostały użyte do monitorowania dobowej aktywności wiewiórek, wyrażonej jako liczba obserwacji wiewiórek na godzinę. Fotopułapki (LTL – Acorn 6210MC/MG, USA) używano w okresie od września 2018 do lipca 2019 roku. Fotopułapki umieszczone były na drzewach przed pułapkami żywołownymi około 20-150 cm nad ziemią. Na każdym terenie badań wykorzystane było 5 fotopułapek w odległości minimum 100 metrów od siebie. Liczba pułapkodni w rezerwacie leśnym wynosiła 432, w parku 482.

Pozwolenie na wstęp do Rezerwatu Przyrody Las Natolioński oraz na łapanie wiewiórek zostało wydane przez Regionalną Dyrekcję Ochrony Środowiska (WPN-I.6205.124.2018.AS i WPN-I.6401.208.2018.PF). Procedura łapania wiewiórek była przeprowadzona zgodnie z obowiązującymi zasadami oraz za zgodą Lokalnej Komisji Etycznej (WAW2/072/2018).

Badania zostały częściowo dofinansowane przez Wydział Leśny SGGW w Warszawie z grantu dla młodych naukowców oraz Instytut Ochrony Środowiska – Państwowy Instytut Badawczy.

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Publikacja artykułu: Beliniak A., Gryz J., Klich D., Łopucki R., Sadok I., Ożga K., Jasińska K.D., Ścibior A., Gołębiowska D., Krauze-Gryz D. 2024. Long-term, medium-term and acute stress response of urban populations of Eurasian red squirrels affected by different levels of human disturbance. PLoS ONE. https://doi.org/10.1371/journal.pone.0302933. była możliwa dzięki finansowaniu przez Ministerstwo Nauki i Szkolnictwa Wyższego ze środków Katolickiego Uniwersytetu Lubelskiego Jana Pawła II.

7. Streszczenia rozdziałów

Rozdział 1.

Różnice we wzorcach dziennej aktywności wiewiórek pospolitych zamieszkujących miejski park oraz rezerwat leśny

Beliniak A., Krauze-Gryz K., Jasińska K., Jankowska K., Gryz J. 2021. Contrast in daily activity patterns of red squirrels inhabiting urban park and urban forest. Hystrix, the Italian Journal of Mammalogy, 32(2):159–164. https://doi.org/10.4404/hystrix-00476-2021.

W pierwszym artykule porównano aktywność dobową i sezonową wiewiórek przy użyciu zdjęć z fotopułapek. Badania pilotażowe prowadzone w poprzednich latach wykazały zmianę zachowania wiewiórek w parku miejskim w odpowiedzi na obecność człowieka, m.in. spędzały one więcej czasu na ziemi (Krauze-Gryz i in. 2021*a*). Na tej podstawie założono, że dobowy i sezonowy wzorzec aktywności wiewiórek na dwóch terenach będzie się różnił – w rezerwacie leśnym będzie on typowy dla gatunku w warunkach naturalnych, a w parku będzie on zmieniony pod wpływem obecności odwiedzających. By to sprawdzić użyto po pięć fotopułapek na obu terenach badań (w sumie 10 fotopułapek), które działały przez całą dobę od września 2018 do lipca 2019 roku (okres badawczy objął wszystkie pory roku). W sumie liczba pułapkodni dla rezerwatu wynosiła 432 i 482 dla parku, a wiewiórki zaobserwowano 323 razy w rezerwacie i 604 razy w parku miejskim. Na podstawie zdjęć z fotopułapek wykazano, że wzorzec aktywności dobowej różnił się w zależności od pory roku oraz terenu badań. Wiewiórki w rezerwacie prezentowały bimodalny wzorzec aktywności, typowy dla pozamiejskich populacji, tzn. widoczne były dwa szczyty aktywności – jeden po wschodzie słońca, a drugi przed zachodem (wiewiórki podczas najcieplejszych godzin w ciągu dnia odpoczywają w gniazdach) (Tonkin 1983; Wauters i Dhondt 1987, 1992). Osobniki z parku natomiast prezentowały nietypowy, pojedynczy szczyt aktywności, który zaczynał się kilka godzin po wschodzie słońca i trwał do południa/wczesnego popołudnia (w zależności od pory roku). Przypuszczalnie wiewiórki zamieszkujące park zmieniły swój wzorzec aktywności tak, by pokrywał się on ze zwiększoną obecnością ludzi odwiedzających park, co zwiększa szanse na zdobycie dodatkowego pokarmu antropogenicznego. Wiewiórki to zwierzęta typowo dzienne, początek ich aktywności jest ściśle związany ze wschodem słońca (Wauters i Dhondt 1992), jednak gdy dni są krótkie, zwierzęta te mogą być aktywne już przed wschodem a nawet po zachodzie słońca (Wauters i Dhondt 1987). Potwierdzono to również w przypadku wiewiórek z rezerwatu, które rejestrowane były zarówno przed wschodem słońca oraz po jego zajściu. Inaczej było w przypadku wiewiórek z parku, które aktywne były prawie wyłącznie w ciągu dnia. Co zaskakujące, w parku częstość rejestracji wiewiórek była najmniejsza latem, a najwyższa zimą. Taka wysoka aktywność wiewiórek zimą jest sprzeczna ze znanym z literatury wzorcem aktywności sezonowej; w czasie niskich temperatur wiewiórki ograniczają swoją aktywność i odpoczywają w gniazdach by unikać strat energii (Wauters i Dhondt 1987). Przypuszczalnie wiewiórki z parku były w stanie utrzymać wysoką aktywność zimą dzięki obecności pokarmu antropogenicznego w ciągu całego roku. Podsumowując, w artykule wykazano odmienne wzorce aktywności wiewiórek z dwóch populacji, co może być spowodowane m.in. całorocznym dokarmianiem przez ludzi.

Słowa kluczowe: dokarmianie, fotopułapki, *Sciurus vulgaris*, zaburzenia środowiskowe

Rozdział 2.

Kondycja ciała i aktywność płciowa miejskich wiewiórek pospolitych: porównanie dwóch populacji będących pod wpływem odmiennej urbanizacji

Beliniak A., Gryz J., Klich D., Jasińska K., Krauze-Gryz D. 2022. Body Condition and Breeding of Urban Red Squirrels: Comparison of Two Populations Affected by Different Levels of Urbanization. Animals, 12(23): 3246. https://doi.org/10.3390/ani12233246.

W drugim artykule została porównana masa ciała, kondycja ciała oraz aktywność płciowa wiewiórek z rezerwatu leśnego oraz parku miejskiego. Wiedząc, że populacja wiewiórek z rezerwatu nie jest dokarmiana (ponieważ teren ten jest zamknięty), założyliśmy, że wiewiórki z parku miejskiego, które przez cały rok mają dostęp do dodatkowych źródeł pokarmu, będą miały lepszą kondycję i większą masę ciała. W celu sprawdzenia tej hipotezy w okresie od lipca 2018 do grudnia 2020 roku prowadzono odłowy wiewiórek w pułapki żywołowne. W sumie wiewiórki łapano podczas trzynastu przeciętnie 4-dniowych sesji, które odbywały się co dwa miesiące, równolegle na obu terenach. W sumie zostało złapanych 36 osobników w rezerwacie leśnym (19 samic i 17 samców) oraz 106 wiewiórek w parku (48 samic i 58 samców). W rezerwacie leśnym łączna liczba złowień wyniosła 129 (średnio każdy osobnik łapał się 3,58 razy, minimum 1, maksimum 11), w parku miejskim natomiast 266 (średnio każdy osobnik łapał się 2,44 razy, minimum 1, maksimum 12). Złapane wiewiórki znakowano oraz określano ich masę ciała, długość stopy (potrzebną do oceny kondycji), wiek oraz aktywność płciową. Wbrew oczekiwaniom okazało się, że wiewiórki zamieszkujące rezerwat były cięższe oraz miały lepszą kondycję ciała niż wiewiórki z parku (wiewiórki z rezerwatu ważyły średnio 355 g, a wskaźnik kondycji wynosił 6,58, wiewiórki z parku ważyły średnio 337 g i wskaźnik kondycji wynosił 6,21). Niższa masa ciała wiewiórek zamieszkujących park może być z jednej strony spowodowana konkurencją międzyosobniczą, wynikającą z dużego zagęszczenia populacji (w parku zagęszczenie populacji wynosiło 1,05-1,89 os./ha, w rezerwacie 0,2-0,28 os./ha). Z drugiej strony, może być również efektem całorocznego dostępu do pokarmu, w związku z czym gromadzenie tkanki tłuszczowej przed zimą nie jest tak istotne. Prawdopodobnie dostępność wysokokalorycznych nasion (orzechów laskowych, orzeszków grabowych i żołędzi) na terenie rezerwatu pozwoliła

na osiągnięcie wystarczająco wysokiej masy ciała, co jest kluczowe dla przeżywalności poszczególnych osobników (Wauters i in. 2007). Otrzymane wyniki nawiązują do innych badań, gdzie masa ciała i kondycja nie były związane z dostępnością dodatkowego pokarmu (Klenner i Krebs 1991; Shuttleworth 2000; Magris i Gurnell 2002). Masa ciała jest szczególnie istotna dla samic, ponieważ odpowiednia ilość tłuszczu zapewnia stabilne źródło energii podczas laktacji i opieki nad młodymi (Wauters i Dhondt 1989). Sukces reprodukcyjny samic rośnie wraz z masą ciała i kondycją – wiewiórki cięższe dłużej żyją i mają więcej młodych (Wauters i Dhondt 1989, 1995; Wauters i in. 2007; Santicchia i in. 2018). Co ciekawe, mimo że wiewiórki w parku były generalnie w gorszej kondycji, udział aktywnych płciowo samic był wyższy (35%) w porównaniu do wiewiórek z rezerwatu (23%). Niezależnie od terenu badań, najwięcej samic aktywnych płciowo zaobserwowano wiosną, choć aktywne płciowo samice rejestrowano również zimą. Wcześniejsze przystąpienie do rozrodu jest możliwe dzięki większej dostępności pokarmu i wyższym temperaturom w mieście (Selonen i in. 2016). Samice mające młode odpowiednio wcześnie w roku mają odpowiednio dużo czasu, by mieć drugi miot w roku (Gurnell 1983) oraz większe są też szanse, że ich potomstwo znajdzie optymalne środowisko podczas dyspersji (Klenner i Krebs 1991). Uzyskane wyniki uzupełniają wykazane wcześniej różnice pomiędzy dwiema populacjami wiewiórek i dowodzą wysokiej plastyczności gatunku.

Słowa kluczowe: aktywność płciowa, łapanie w pułapki żywołowne, masa ciała, *Sciurus vulgaris*, zmiany sezonowe, ssaki miejskie

Rozdział 3.

Długoterminowa, średnioterminowa i nagła reakcja na stres miejskich populacji wiewiórek w różnym stopniu dotkniętych zaburzeniami antropogenicznymi

Beliniak A., Gryz J., Klich D., Łopucki R., Sadok I., Ożga K., Jasińska K.D., Ścibior A., Gołębiowska D., Krauze-Gryz D. 2024. Long-term, medium-term and acute stress response of urban populations of Eurasian red squirrels affected by different levels of human disturbance. PLoS ONE. https://doi.org/10.1371/journal.pone.0302933.

Tereny zurbanizowane, które różnią się od naturalnych, mogą potencjalnie wywoływać stres u dzikich zwierząt. Osobniki bytujące w takich środowiskach muszą sobie radzić w specyficznych warunkach, do których naturalnie nie były przystosowane. Celem badania było porównanie długoterminowej, średnioterminowej i nagłej reakcji na stres u wiewiórek. W badaniu przeanalizowano reakcje dwóch populacji wiewiórek pospolitych żyjących na terenach w różnym stopniu przekształconych antropogenicznie: wysokim (park miejski) oraz niskim (rezerwat leśny). Reakcje na potencjalnie czynniki stresowe oceniono w trzech perspektywach czasowych: długoterminowej, średnioterminowej i nagłej. Dane zbierane były w okresie od lipca 2018 do grudnia 2020 roku. Długoterminową reakcję na stres oceniono poprzez analizę poziomu metabolitów hormonów stresu (kortyzolu i kortyzonu) w sierści (N=93), średniofalową poprzez analizę poziomu metabolitów hormonu stresu (kortyzolu) w odchodach (N=112). Reakcję na nagły stres oceniono w oparciu o trzy wskaźniki mierzone tuż po złapaniu danego osobnika: 1) częstotliwość oddechów (N=333), 2) wskaźnik zaniepokojenia (ang. “struggle rate”, N=336) oraz 3) wokalizację (N=335). Nie wykazano różnic pomiędzy populacjami, płciami oraz aktywnością płciową (osobnik aktywny/nieaktywny) w odpowiedzi na stres długoterminowy i średnioterminowy. Poziomy metabolitów hormonu stresu wyraźnie zależały od pory roku – wyższe wartości odnotowano jesienią i zimą, niezależnie od terenu badań, płci i statusu rozrodczego. Poszczególne wskaźniki reakcji na nagły stres zależały od różnych czynników: 1) częstotliwość oddechu była wyższa u osobników z rezerwatu leśnego (średnio 28,661 oddechów w ciągu 20 sekund, w parku wynosiła średnio 25,597 oddechów w ciągu 20 sekund), 2) wskaźnik zaniepokojenia (“struggle rate”) był podobny na obu terenach (7,178 w rezerwacie i 7,956

w parku), choć zależał od pory roku i był najwyższy zimą, 3) wokalizacja nie różniła się pomiędzy populacjami (wskaźniki wynosiły odpowiednio 1,619 w rezerwacie i 1,820 w parku), ale była najmniej intensywna w przypadku pierwszego złapania danego osobnika. Podsumowując, populacje wiewiórek żyjące na dwóch terenach o różnym stopniu dotkniętych zaburzeniami antropogenicznymi różniły się wyraźnie jedynie częstotliwością oddechu (jednym ze wskaźników reakcji na nagły stres). Brak różnic w reakcji na długo- i średnioterminowy stres (tj. poziomach metabolitów hormonów stresu w sierści i odchodach) pomiędzy populacjami wskazuje na podobny wpływ czynników środowiskowych (tj. pory roku, warunków pogodowych). Odmienna reakcja wiewiórek z rezerwatu leśnego w przypadku nagłego stresu (wyższa częstotliwość oddechu) najprawdopodobniej była spowodowana ograniczonym kontaktem z ludźmi. Wiewiórki z parku miejskiego reagowały słabiej, ponieważ przyzwyczaiły się do bliskiej obecności człowieka. To również wskazuje, że częstotliwość oddechu może być traktowana jako najdokładniejszy wskaźnik reakcji na nagły stres w kontakcie z człowiekiem (w porównaniu do wskaźnika zaniepokojenia ("struggle rate") i wokalizacji). Powyższe wyniki wyraźnie wskazują, że zastosowanie szerszego zakresu wskaźników i różnych metod analitycznych, w tym analiz behawioralnych, jest najbardziej odpowiednie przy dokładnej ocenie wpływu antropogenicznych czynników stresogennych na zwierzęta. Takie podejście umożliwia kompleksowe zbadanie reakcji zwierząt na stres, a także identyfikację wskaźników odpowiednich do przyszłych badań nad wpływem urbanizacji na dziką przyrodę.

Słowa kluczowe: metabolity kortyzolu, *Sciurus vulgaris*, urbanizacja, wokalizacja, wskaźniki stresu

Rozdział 4.

Czynniki warunkujące bioakumulację rtęci u wiewiórek pospolitych w mieście

Jackowiak M., Krauze-Gryz D., Beliniak A., Jasińska K. D., Gryz J., Żyfka-Zagrodzińska E., Matracka A. The heavy burden of city life: factors affecting mercury bioaccumulation in urban red squirrels.

Manuskrypt w trakcie recenzji w Environmental Science and Pollution Research

W ostatnim artykule z cyklu publikacji zostało ocenione stężenie rtęci w organizmach wiewiórek, traktowane jako przykład zaburzenia środowiskowego. Do analiz zostały użyte próbki sierści pobrane podczas łapania wiewiórek na terenie rezerwatu leśnego oraz parku miejskiego w Warszawie. Zawartość rtęci została oceniona laboratoryjnie za pomocą absorpcyjnej spektrometrii atomowej. Średnia zawartość rtęci w sierści wiewiórek wynosiła 100,65 µg/kg. Zawartość rtęci u osobników młodocianych była niższa niż u dorosłych i wynosiła 35,27 µg/kg (u dorosłych 105,08 µg/kg). Zawartość rtęci zależała od terenu badań, płci oraz aktywności płciowej (osobniki aktywne/nieaktywne). Zawartość rtęci w sierści osobników z rezerwatu wynosiła średnio 60,21 µg/kg, w parku miejskim natomiast 135,13 µg/kg. Taka dwukrotna różnica może wynikać z zanieczyszczenia środowiska, jak również składu pokarmu, ponieważ metale ciężkie mogą być dostarczane do organizmu wraz z pożywieniem. Zawartość rtęci w sierści osobników młodocianych była wyraźnie niższa niż osobników dorosłych, ponieważ rtęć akumuluje się w tkankach, a dłuższy czas ekspozycji powoduje większą koncentrację tego pierwiastka. Dodatkowo stężenia rtęci były najwyższe u samic, które były w ciąży lub karmiły (średnio 165,63 µg/kg), co może być wynikiem większego zapotrzebowania na składniki odżywcze w tym okresie. Podsumowując, można uznać, że stężenie rtęci w sierści wiewiórek może być uznawane za wskaźnik zaburzeń środowiskowych oraz zanieczyszczenia środowiska. Poziom zanieczyszczenia metalami ciężkimi może być zależny m.in. od strategii żerowania i zachowania, ponieważ metale ciężkie mogą być dostarczane z pokarmem. Planowane są dalsze badania oceniające zawartości rtęci w glebie oraz roślinach, stanowiących pokarm wiewiórek.

Słowa kluczowe: aktywność płciowa oraz płeć, masa ciała, pierwiastki śladowe, próbki sierści, wiek, zanieczyszczenie

8. Rozdziały

Rozdział 1.

Beliniak A., Krauze-Gryz K., Jasińska K., Jankowska K., Gryz J. 2021. Contrast in daily activity patterns of red squirrels inhabiting urban park and urban forest. Hystrix, the Italian Journal of Mammalogy, 32(2):159–164. https://doi.org/10.4404/hystrix-00476-2021.

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Research Article

Contrast in daily activity patterns of red squirrels inhabiting urban park and urban forest

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Abstract

The Eurasian red squirrel is one of the species that has well adapted to urban habitats and is habituated to human presence. Its urban populations differ from those in rural habitats in terms of e.g. abundance, spatial organisation and behaviour. Food availability affects the ecology of red squirrels, so in this study we hypothesise that in the urban park with supplemental food, red squirrels will alter their activity rhythms to benefit from human presence. We therefore compared seasonal changes in the daily activity patterns of two red squirrel populations, inhabiting two different areas in Warsaw: a busy urban park (with plentiful supplemental feeding) and an urban forest reserve (closed for public). Between September 2018 and July 2019 we used camera traps to monitor red squirrels activity round-the-clock. In the park (contrarily to the forest), the number of records of red squirrels per 100 trap days was lowest in summer and highest in winter. Probably squirrels in the urban park, were able to maintain high activity during winter thanks to all-year-round availability of supplementary food. Daily activity patterns differed seasonally and between the study sites. In the forest they resembled those recorded in natural habitats, i.e. two activity peaks, one after the sunrise and second before the sunset. In contrast, park squirrels showed mostly one activity peak, beginning some hours after the sunrise and lasting until noon/early afternoon (depending on the season). Park squirrels were almost exclusively day-active, while forest squirrels were also recorded before sunrise and after sunset. We suggest that park squirrels shifted their activity to times with higher visitor frequency, which, assumingly, increased chances to obtain supplemental food.

Introduction

Historically, urban habitats have been ignored as potentially suitable wildlife habitats (McCleery et al., 2014). However, many animal species may adapt to these specific conditions (Jackowiak et al., 2021; Jasińska et al., 2021; Lesińskiet al., 2021; Gryz and Krauze-Gryz, 2018; Francis and Chadwick, 2012; Alvey, 2006), by changing e.g. their diet preference, home range size, behaviour, including vigilance and activity budget (Ritzel and Gallo , 2020). Wildlife species exhibit a variety of responses to human presence, ranging from attraction to habituation to avoidance (review in Patten and Burger, 2018). In example, bobcats (*Lynx rufus*) and coyotes (*Canis latrans*) clearly avoided humans by becoming more nocturnal (George and Crooks , 2006), while striped-field mouse (*Apodemus agrarius*), which is strictly nocturnal in nonurban habitats, became diurnal in town parks, despite the constant presence and high levels of human activity (Gliwicz et al., 1994). Understanding impacts of increasing urbanisation on wildlife is crucial for conservation of animal species (McCleery et al., 2014).

Eurasian red squirrel *Sciurus vulgaris* is one of the species that has adjusted to urban habitats and is habituated to human presence (Krauze-Gryz et al., 2021a,b; Kostrzewa and Krauze-Gryz, 2020; Uchida et al., 2019; Fay et al., 2016; Reher et al., 2016; Rézouki et al., 2014; Babińska-Werka and Żółw, 2008). In the past few years, research interest in urban populations of red squirrel has increased (reviewed in Fingland et al., 2021), and they were shown to differ from rural habitats, e.g. abundance of this species increased with human population density (Jokimäki et al., 2017), individuals occupied smaller home ranges (Krauze-Gryz et al., 2021a; Hämäläinen et al., 2018), and utilized various urban structures (Hämäläinen et al., 2018). Animals spent more time on the ground and frequently interacted with humans (Krauze-Gryz et al., 2021a,b), and an increasing boldness was one of the most prevalent behavioural modifications (Uchida et al., 2016, 2019). Surprisingly, little is known about activity pattern of red squirrels inhabiting cities. Yet, in Hamburg it was noted that red squirrels inhibiting city centre were less active throughout the day than their conspecifics in semi-natural habitat (Thomas et al., 2018). Nevertheless, that study was based only on late-winter/early-spring data.

Readily available food in urban environments is believed to be one of the reasons why numerous animal species are thriving in cities around the world (review in Spelt et al. 2021). In urban conditions, red squirrels are offered various sources of supplementary foods including the gathering of food from bird feeders as well as the provision of nuts offered directly by park visitors (Krauze-Gryz et al., 2021a; Kostrzewa and Krauze-Gryz, 2020; Krauze-Gryz and Gryz, 2015; Bosh and Lurz, 2012). It has been shown that supplemental food is a crucial factor that may attract squirrels in urban environments (Jokimäki et al., 2017), and this extra food is most important when natural food availability is low (Magris and Gurnell, 2006). Because food availability (i.e. tree seeds) affects the ecology of red squirrels (Jokimäki et al., 2017; Selonen et al., 2016; Wauters et al., 2007, 2008), we can hypothesise that in the urban park with plentiful supplemental food, red squirrels will alter their activity rhythms to benefit from human presence. Indeed, food provided by humans to wildlife in urban areas may lead to changes in behaviour of animals that use this resource (Sol et al., 2013; Lowry et al., 2013). For example chipmunks (*Tamias striatus*), in urban environment altered their seasonal behaviour patterns, i.e. there was no reduced activity levels, normally observed in rural habitats, because

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of human-suppled food resources (Ryan and Larson, 1976). Coypus (*Myocastor coypus*) in the urban habitat (differently that in non-urban areas) were active during the day and early evening and this reversal of activity patterns was attributable to deposition of human food in the city area during the daytime (Meyer et al., 2005). Finally, gulls adapted their foraging behaviour to human time schedules when beneficial (Spelt et al., 2021).

The aim of a study was to compare seasonal changes in the daily activity patterns of the two red squirrel populations inhabiting the same city but two utterly different areas: a busy urban park and an urban forest. Both green spaces are within city districts. Nevertheless, one is expected to be highly influenced by the human presence and supplementary food from park visitors, whereas the effect of human disturbance on the other population should be negligible. Indeed, an earlier study showed that park squirrels significantly altered their behaviour in response to human presence by spending more time on the ground and tolerating close contact. Moreover human-delivered nuts made the bulk of a diet (Krauze-Gryz et al., 2021a). We hypothesise that daily activity patterns of squirrels inhabiting urban park will also differ from those observed for forest squirrels as they may adjust their activity to benefit from supplemental feeding.

Materials and methods

Study area

We conducted our study in Warsaw (52°14′13.37″ N, 21°1′13.1″ E), Poland, a large city with approximately two million inhabitants. It is located in the central part of the country, a region that is affected by both the mild oceanic climate of Western Europe, and the harsh and dry continental climate of Eastern Europe and Asia. The duration of the growing season is approximately 210 days, the total precipitation measures 600 mm per year, and the mean ambient temperature ranges from -4° in January to 18° in July. Nevertheless, the minimum temperature may drop below -30° and the maximum temperature may rise above 35°.

This research was conducted in two green areas. The first was an urban park (together with historical buildings) named Łazienki Royal Museum (52°12′27.60″ N, 21°1′34.80″ E). The park was formed in the 17th century, is located in the city centre and is very popular among visitors and local inhabitants: every year the park is visited by more than 2 million people (Kruczek, 2015). Squirrels are often fed nuts by visitors (Krauze-Gryz et al., 2021a,b; Krauze-Gryz and Gryz, 2015). The park covers 76 ha and it has 92 species of trees and shrubs. Tree cover is about 70% and about 20% of trees reach more than 140 years (Babińska-Werka and Żółw, 2008). Tree species are mostly deciduous, e.g. hornbeam (*Carpinus betulus*), common oak (*Quercus robur*), beech (*Fagus sylvatica*), as well as hazel (*Corylus avellana*), walnut (*Juglans regia*) and North American walnut (*Juglans nigra*) (Babińska-Werka and Żółw, 2008).

The second study site was Natolin Forest Reserve (52°8′20″ N, 21°4′25″ E), which is located about 10 km from the city centre (we assumed the Palace of Culture and Science to be the most central point of the city). An area of the reserve measuring 105 ha has been protected since 1991, the reserve is closed to the public and a permission is needed for entrance. Although this land was formerly parkland, spontaneous regenerations of woodland occurred during the post-war period, nowadays the whole area of the reserve is tree covered. The oldest stands are more than 250 years old, dead or fallen trees are left for natural decomposition, only natural regeneration occurs, in practice very little human intervention is allowed. Trees are mostly deciduous, e.g. hornbeam, common oak, ash (*Fraxinus excelsior*), elms (*Ulmus* spp.), hazel and black alder (*Alnus glutinosa*).

In both areas avian predators were present, this included tawny owl *Strix aluco* (Gryz et al., 2008), and sparrowhawk *Accipiter nissus* (Gryz J., pers. observ.). One pair of the Northern goshawk *Accipiter genitilis* nested in the Natolin reserve (Gryz J., pers. observ.), while corvids (mainly hooded crows *Corvus corone* and rooks *Corvus frugilegus*) were numerous in the Łazienki park (Beliniak A. pers. obs.). As for mammals, red fox *Vulpes vulpes* (Jackowiak et al., 2021), stone marten *Martes foina* (Krauze-Gryz D., pers. obs.), and free-ranging domestic cat (*Felis catus*) were present in both areas, with the last species being recorded very often in the urban park (Krauze-Gryz D., pers. obs.). In the Łazienki Park, squirrel density was estimated to be more than 2 ind./ha (Krauze-Gryz et al., 2021a; Babińska-Werka and Żółw, 2008) whereas in the Natolin Reserve it was 0.29 ind./ha ((Krauze-Gryz et al., 2021a).

Camera-trap data collection

In this study, we used camera traps to monitor red squirrels activity, i.e. the number of squirrel observations per hour of a day (e.g. Andreoni et al., 2011; Jasińska et al., 2021; Cerbo and Biancardi, 2013, please see below for more details), and round-the-clock. The study started in September 2018 and ended in July 2019. We used camera traps (LTL - Acorn 6210MC/MG, USA), which recorded date of the observation, time (24 h record) in Central European Time (CET). Each camera trap captured three still images, and a 5-sec video, shortly one after another (no interval was set). It also recorded date and hour. Cameras were located in front of the live-traps for squirrels (squirrels were live-trapped for another study, this data is not analysed here but the study design was similar to Krauze-Gryz et al. (2021a,b), about 20-150 cm above ground (depending if a trap was located on a ground or in a tree on a wooden platform). Traps were pre-baited with unshelled hazelnuts and walnuts for seven days (mostly around 8-10 AM), and then baited and set for five to nine days. They were set at dawn and checked after approximately 2–4 h, depending on the temperature, and blocked open for the night (with nuts left inside). After every checking and securing traps, bait was provided, regular baiting ensured the presence of food at all times (including night). Five camera traps, located at least 100 meters apart, were placed in each study site. In the forest, the camera traps were set at one of trapping points (i.e. locations of live-traps) that were distributed on a regular grid. In the park, due to its representational character and high-visitor frequency, the traps were hidden and placed in three trapping areas distributed in different park zones and separated by 200-400 m (Krauze-Gryz et al., 2021a). Camera traps were set in each of these locations. In total, data was collected in six months: September, November 2018 and January, March, May, July 2019 in both study areas, for a total of 76 days in the forest and 104 days in the park (Table S1). Trap-days, an index of the trapping effort, were calculated as the number of devices multiplied by the number of operational days. Number of trap-days was 432 in the forest and 482 days in the park, the trapping effort depended on the number of trapping sessions (i.e. months in which trapping was done) in a given season (Tab. 1). Every photo was checked for presence of animals, the species and number or individuals were recoded (if present together on the same photo). We recorded each squirrel appearing in the images without distinguishing between individuals. A new observation was considered if a minimum of 15 minutes elapsed between subsequent photos or series of photos showing an animal/animals. This rule was abandoned only when an animal in the photo was different in plumage, age class or in other characteristics (i.e. had a radio-collar, ear-tag), indicating clearly that the animal in the photo was a different one than the previously registered. The intermission length ranged from 0 to 30 min in other studies (review in Cerbo and Biancardi, 2013). In our case, there were mostly cumulated observations of one squirrel taking bait, which were treated as a single observation; new observations took place after a few/several hours or on the other day. Thus, we believe 15 minutes break allowed to avoid pseudo-replication. The year was divided into four astronomical seasons: spring (1 March–31 May), summer (1 June–31 August), autumn (1 September–30 November) and winter (1 December–28 February). Squirrel records were attributed to one of four parts of 24-hours day: dawn (one hour before sunrise), day (the time between sunrise and sunset), dusk (one hour after sunset) and night (the time between one hour after sunset and one hour before sunrise) (Jasińska et al., 2021). Time of sunrise and sunset was downloaded for Warsaw from https://www.timeanddate.com and converted to CET.

Table 1 – Number of trap-days, squirrel records in total and records per 100 trap days as recorded by camera traps by season in the two study sites in Warsaw: park and forest.

	Park			Forest		
Season	**Trap days**	**Squirrel records**	**Records per 100 trap days**	**Trap days**	**Squirrel records**	**Records per 100 trap days**
Spring	102	108	105.9	176	139	79.0
Summer	51	19	37.3	72	98	136.1
Autumn	135	153	113.3	100	61	61.0
Winter	194	324	167.0	84	25	29.8
Total	482	604	-	432	323	-

Access to the Natolin Forest Reserve was allowed with the permission issued by the Regional Directorates for Environmental Protection.

Statistical analysis

Differences between the frequencies of records of red squirrels in the two study sites and in subsequent seasons, subsequent hours and times of a day were compared with chi-square test. For observation of squirrels that took place before 12 PM, we calculated time that elapsed from the sunrise, for observations after 12 PM, we measured time between the observation and the sunset. Next, to compare the number of hours after the sunrise and before the sunset and the time when squirrels were recorded, Kruskal-Wallis test was used, with Mann-Whitney pairwise test for post hoc analysis (Bonferroni-corrected *p*). Normality of data distribution was checked with Shapiro-Wilk W test ($p < 0.05$). The analyses were done in Past 4.05 (Hammer et al., 2001) software.

Results

Number of records of red squirrels per 100 trap days in a given season (Tab. 1) differed between study areas (Chi-square test: $\chi^2 = 158.3$, DF=3, $p<0.0001$). In the park, the number of records per 100 trap-days was lowest in summer and highest in winter. In the forest the number of records was lowest in winter and highest in summer (Tab. 1). Number of observations of squirrels recorded per hours of a day varied between the two study sites in all seasons: in spring (Chi-square test: $\chi^2 = 53.4$, DF=15, $p<0.0001$), summer ($\chi^2 = 28.8$, DF=15, $p<0.05$), autumn ($\chi^2 = 66.8$, DF=11 , $p<0.0001$), and winter ($\chi^2 = 160.3$, DF=10, $p<0.0001$) (Fig. 1).

Activity patterns of squirrels in the two study areas seemed to be different (Fig. 1). In spring, forest squirrels were mostly recorded in the early morning (6–7 AM), after sunrise, and then in the afternoon (3–5 PM), two peaks of activity were visible (Fig. 1). On the other hand, park squirrels were mostly recorded between 6 and 11 AM, with no afternoon activity peak. In summer, most records of forest squirrels were taken between 4 and 8 AM, with some observations recorded also in other day hours (till 8 PM). Park squirrels were also recorded in high proportion in the morning (4–8 AM), but bigger share of observations were from noon and some afternoon hours (i.e. 2 and 5 PM). In autumn, activity of park squirrels was clearly unimodal, with biggest share of observations between 9 AM to 1 PM and very few observations afterwards. In contrast, forest squirrels showed bimodal activity pattern, i.e. first between 6-8 AM, and later 2-3 PM, clearly connected to the sunrise and the sunset. This difference was even clearer in winter. Park squirrels were recorded almost exclusively between 7 and 11 AM, while forest squirrels had two activity peaks, i.e. between 6-8 AM and 1–4 PM (Fig. 1).

Observations of forest squirrels were distributed across all defined times of a day, while park squirrels were almost exclusively active during day (Chi-square test: $\chi^2 = 333.6$, DF=21, $p<0.0001$) (Fig. 2). No more than 1% of observations of park squirrels came from dawn (one record in spring and autumn, four records in winter), with 100% of diurnal observations in summer. In all seasons, there were no observations of park squirrels at dusk or at night. Forest squirrels were also recorded mostly during day in most seasons, but dawn and dusk observations were always recorded. Only in winter, half of records came from dawn, around one third came from day while 12 and 8% of observations came from dusk and night, respectively.

For all observations of squirrels recorded before noon we calculated time (n of hours) that elapsed from the sunrise, these values (2.4 and 2.5 for park and forest, respectively) did not differ between the two study sites (Kruskal-Wallis test, H=0.055, $p>0.05$). Nevertheless, in both study sites (Fig. 3), differences were found between seasons (Kruskal-Wallis test, park: H=45.22, $p<0.001$, forest: H=50.61, $p<0.001$). In the park, in winter, squirrels were recorded earlier than in spring or autumn, as shown by Mann-Whitney post hoc test ($p<0.05$), i.e. in winter squirrels were recorded on average two hours after the sunrise, while in other seasons approximately three hours after the sunrise. In the forest, red squirrels were recorded approximately three hours after the sunrise in spring and autumn, less than two hours in autumn while in winter they were observed mostly before the sunrise (mean=-0.48) (Fig. 3). In this case, Mann-Whitney post hoc test showed differences ($p<0.05$) between autumn and winter and the remaining seasons.

Similarly, for all observations of squirrels recorded after noon we calculated time (n of hours) before the sunset. On average, observations of squirrels in the forest were recorded closer to the sunset (on average 2.4 hours before the sunset) than those in the park (3.5 hours before the sunset) (Kruskal-Wallis test, H=18.04, $p<0.001$). Again, for both study sites (Fig. 4), seasonal differences were found (Kruskal-Wallis test, park: H=36.00, $p<0.001$, forest: H=17.12, $p<0.001$). In the park, red squirrels were recorded shortest before the sunset in autumn (2.7 hours before sunset) as compared to other seasons (i.e. in spring and summer this was approximately 5 hours before the sunset). Differences were also found between winter and spring (Mann-Whitney test for post hoc-comparisons, $p<0.05$). In the forest, in winter, red squirrels were on average recorded almost at the sunset, i.e. 0.4 hours before the sunset, while in spring and summer this was approximately three hours before the sunset (Mann-Whitney test for post-hoc comparisons showed differences between spring and autumn and winter, $p<0.05$).

Discussion

In this study we compared seasonal changes in the activity patterns of red squirrels based on camera traps observations in two study areas: a busy urban park (with plentiful supplemental feeding) and an urban forest reserve (closed for public). These two habitats were located within city districts of Warsaw, and the study was conducted in the same months in both areas, which was supposed to minimise an influence of abiotic cues such as photoperiod and temperature. Daily activity patterns differed seasonally and between the study sites. In the forest, they resembled those recorded in natural habitats, i.e. two activity peaks, one around the sunrise and second around the sunset, while park squirrels were almost exclusively day-active. We suggest that park squirrels shifted their activity to times with higher visitor frequency, which, assumingly, increased chances of obtaining supplementary food and may allow for limiting time devoted to food search. This is the first paper that shows changes in the activity patterns of red squirrels in relation to urbanization round-the-year.

In the park, the number of records per 100 trap days was lowest in summer and highest in winter. This high activity level in winter seems counterintuitive because at low temperatures, resting in the nest is an important behavioural response, which decreases the energy costs of thermoregulation (Wauters and Dhondt, 1987). Moreover, the effect of temperature is more distinct in deciduous habitats, which do not provide much shelter for squirrels in winter (Wauters et al., 1992) and deciduous trees dominated in this areas. In contrast, in the forest the

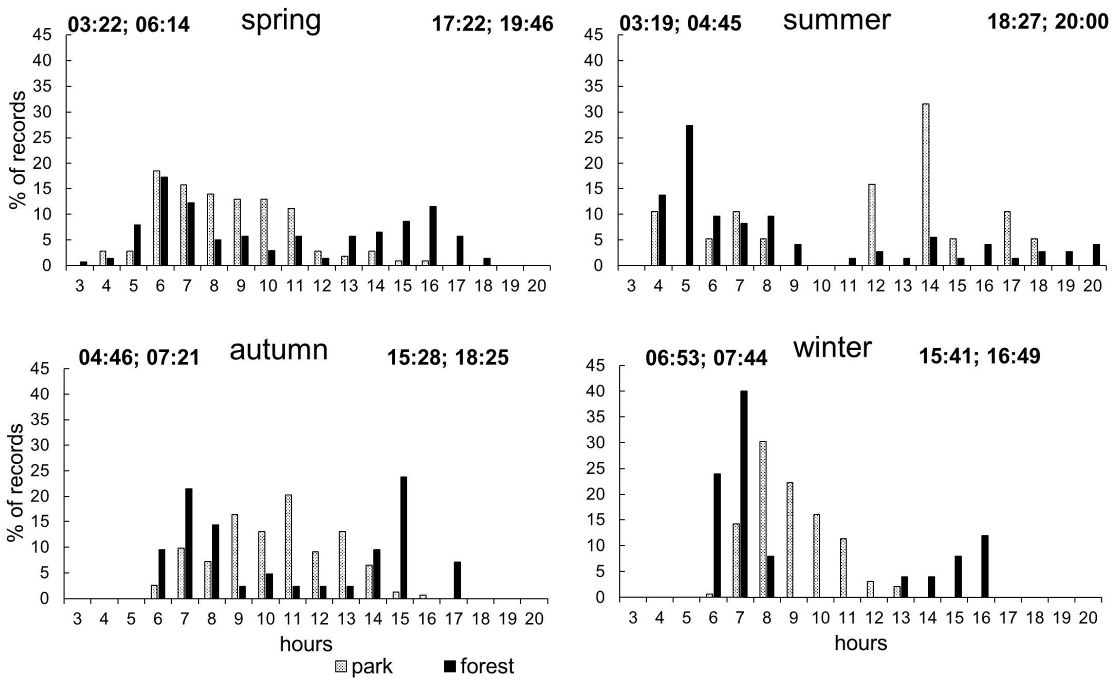

Figure 1 – Percentage of records of red squirrels in hours of a day in subsequent seasons in the two study sites in Warsaw: park and forest, as recorded by camera traps. Hours of the earliest and latest times of sunrise or sunset (converted to CET) are given above the graphs. The total number of records of squirrels in each season was in the park: spring 108, summer 19, autumn 153, winter 324; and in the forest: spring – 139, summer 73, autumn 42, winter 25 .

number of records was lowest in winter and highest in summer, which reflects typical decrease in the activity level of red squirrels with low ambient temperatures (Bosh and Lurz, 2012; Wauters et al., 1992) because of the high thermoregulatory cost of foraging at low ambient temperature and shorter day-length (Bosh and Lurz, 2012; Wauters et al., 1992; Pullianen, 1973). Abundant and predictable food sources in urban habitats can result in minimal physiological responses to the seasons through small changes in metabolism and conductance. In one study in Germany, squirrels were able to maintain a high body mass and positive energy balance year-round despite living in a climate with large seasonal changes in temperature. This was because of the presence of diverse food trees, and artificial food supplemented by cemetery visitors, as well as the modification of activity patterns (Turner et al., 2017). Probably also in our case, red squirrels in the urban park were able to maintain high activity during winter thanks to all-year-round availability of supplemental food. Also, higher temperature in the city centre (Kim, 1992) might have allowed for high activity in winter. The lowest number of observations in summer may result from high availability of natural food combined with a very high frequency of park visitors (including tourists) during holidays. Squirrels are offered so much food they assumingly search for alternate food sources less often.

In spring and autumn, forest squirrels presented crepuscular activity, similarly to what was recorded in other rural habitats, both in coniferous and deciduous habitats (Wauters et al., 1992). In turn, park squirrels were mostly recorded in the morning, between the sunrise and till noon, with no afternoon activity peak. In warm months, red squirrels in rural habitats show bimodal activity pattern, with a rest period in the warmest hours of the day (Wauters et al., 1992; Wauters and Dhondt, 1987; Tonkin, 1983). In our study, no clear rest period was visible, possibly also due to small sample collected in the summer. In winter, we

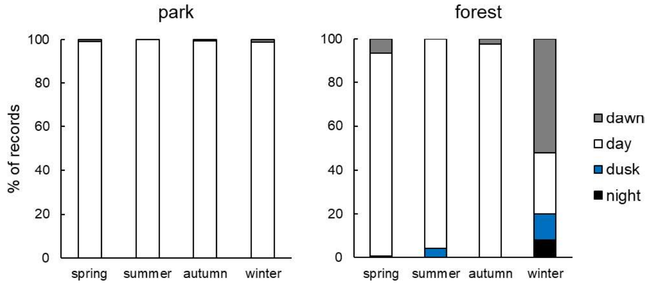

Figure 2 – Seasonal changes in the share of records of red squirrels in four parts of 24-hour day: dawn (one hour before sunrise), day, dusk (one hour after sunset) and night as recorded by camera traps, in the two study sites in Warsaw: park and forest. The total number of records of squirrels in each season was in the park: spring 108, summer 19, autumn 153, winter 324; and in the forest: spring 139, summer 73, autumn 42, winter 25 .

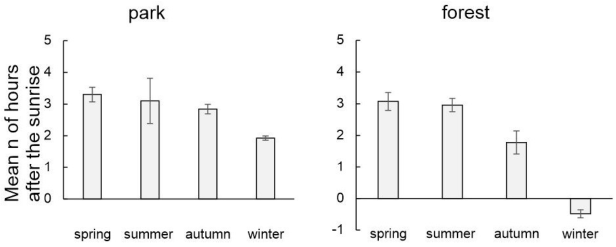

Figure 3 – Seasonal changes in the mean (±SE) number of hours than passed from the sunrise to time of observations of red squirrels as recorded by camera traps in the two study sites in Warsaw: park and forest. Only observations that were recoded before noon (CET) were taken into analysis. The number of records of squirrels in each season was in the park: spring 95, summer 6, autumn 106, winter 303; and in the forest: spring 82, summer 54, autumn 23, winter 18.

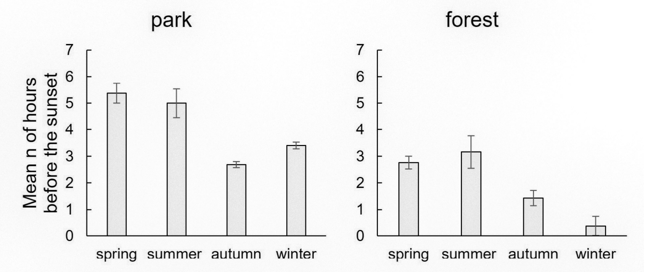

Figure 4 – Seasonal changes in the mean (±SE) number of hours before the sunset to time of observations of red squirrels as recorded by camera traps in the two study sites in Warsaw: park and forest. Only observations that were recoded after noon were taken into analysis. The number of records of squirrels in each season was in the park: spring 10, summer 13, autumn 47, winter 17; and in the forest: spring 57, summer 19, autumn 19, winter 7.

noticed the clearest difference between the two populations: activity of park squirrels was unimodal, i.e. they were recorded almost exclusively after the sunrise and till noon. In turn, forest squirrels showed crepuscular activity. As shown by earlier studies, winter activity of squirrels was unimodal in coniferous forest (Steen and Barmoen, 2017; Wauters et al., 1992; Wauters and Dhondt, 1987), and bimodal in deciduous forest (Wauters et al., 1992). In our case, both study sides were deciduous, so other factors should have affected this result. Consequently, forest squirrels were observed across all defined times of a 24-hour day (e.g. night, dusk, day and dawn), while in the park squirrels were almost only active during a day. In general, red squirrels are exclusively day-active, and the start of activity is closely related to sunrise (Wauters et al., 1992). Yet, when length of day is shortest, squirrels can be active soon after the first light, thus somewhat before sunrise and after sunset (Wauters et al., 1992; Wauters and Dhondt, 1987). Indeed, in a study conducted in South Korea, which also involved camera traps, about 11% of photos were captured in night time photo rate (Lee et al., 2019). In the urban park we did not record any images of active squirrels before the sunrise and after the sunset and very few observations came from dawn (with none from dusk or night). It seems that squirrels in urban forest behaved more like their conspecifics in natural habitats and park squirrels changed their activity rhythm, i.e. their activity concentrated shortly after sunrise (as in natural habitats) and in the subsequent hours. We may assume that one of the possible factors driving this difference was the presence of park visitors and supplementary food supplies provided by people (Krauze-Gryz et al., 2021a; Kostrzewa and Krauze-Gryz, 2020). Red squirrels can reduce activity and foraging time if food items are energy-rich and easy to handle (Wauters et al., 1992) and food availability affects duration of activity during a day (Wauters and Dhondt, 1987; Tonkin, 1983). Availability of anthropogenic food increases the habitat quality, especially in urban habitats (Fingland et al. 2021). Consequently, activity of red squirrels may be altered by food provisioning in urban parks (Thomas et al., 2018), which may also help animals to maintain a positive energy balance (Turner et al., 2017). It was previously suggested that food provided by people may be more important than abiotic factors in urban habitats (Thomas et al., 2018). The primary abiotic environmental cues influencing red squirrel activity timing appear to be temperature (Wauters et al., 1992), photoperiod (i.e., day length,Wauters and Dhondt, 1987; Tonkin, 1983) or a combination of both (Holm, 2018). In our study, in both study sites, we compared data collected during the same month, with similar weather conditions (recordings were conducted at the same time or maximum seven days apart). We may thus assume that differences in activity patterns between urban and forest squirrels were not driven by those abiotic factors. We suggest that park squirrels shifted their activity to times with higher visitor frequency. Indeed, they also changed their behaviour in a way that made receiving food from people more likely, i.e. squirrel spent much time on the ground and often approached people and begged for food (Krauze-Gryz et al., 2021a). This was different from population of squirrels inhabiting wildlife park in Ireland, which altered their activity to avoid human encounters, were observed to concentrate their activity in non-public areas and moved into public areas when the human disturbance was lower (Haigh et al., 2017). What is more, squirrels inhibiting city centre were less active throughout the day than their conspecifics in semi-natural habitat in urban areas of Hamburg (Thomas et al., 2018). This shows that, in our case, urban park squirrels adjusted to human presence. Indeed, humans are important source of disturbance for animals (Sol et al., 2013), yet, the flight initiation distance (FIDs), is often shortened in urban habitats (Uchida et al., 2016, 2019; Sol et al., 2013).

Nevertheless, other abiotic factors that may differ between the two study sites need to be considered, i.e. both artificial light at night and anthropogenic noise can drive changes in activity patterns of animals (Dominoni et al., 2020). In example, urban birds sing earlier during the day, have similar singing effort in the dawn chorus, but sing less than rural birds during the rest of the day (Bermúdez-Cuamatzin et al., 2020). Indeed, anthropogenic noise in the urban park, located in the city centre, was surely higher than in the urban forest. However, being a big park, this was rather a quiet zone, which was not close (at least our study plot) to any busy road, no vehicles were allowed there and gardeners used only electric cars. Also, times with highest noise (i.e. rush hours) did not seem to affect activity of squirrels, i.e. animals were mostly active in the morning but had a rest in the afternoon.

Although in this study we did not test directly an influence of supplemental feeding on shifts in the activity patterns in the population of urban squirrels, we suggest this factor played the crucial role. Squirrels in this park were shown to differ in their behaviour, i.e. some squirrels spent most of their time on the ground and reacted positively to people (approached them and/or begged for food), while others were mainly arboreal and reacted to humans with alert or escape behaviours (Krauze-Gryz et al., 2021b). Assumingly, activity patterns of certain individuals could also differ, which should be investigated in future studies.

To sum up, this study showed differences in daily activity patterns of the two urban populations, inhabiting the same city but different green areas with various levels of human disturbance. Urban forest squirrels showed mostly typical crepuscular activity, while park squirrels were active from the morning and until midday. We suggest a reason for these differences could have been a year-round access to supplementary food in the urban park, i.e. squirrels stayed active at times when park visitors were present and often provided food. This difference complete our earlier findings on how far urban park squirrels differed from their forest counterparts in other aspects of ecology (i.e. space use, food choice and interactions with people) and points to very high plasticity of this species, inhabiting human-dominated landscapes. Nevertheless, as we compared only two study sites located in one city, and we did not have independent replicates for the two populations, we cannot exclude other factors that could have played a role. Thus, more studies are needed to confirm that red squirrels changed their activity rhythms to benefit from human presence.

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 Number of days of trapping in the two study sites.

Supplementary Information

Contrast in daily activity patterns of red squirrels inhabiting urban park and urban forest

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Table S1. Number of days of trapping squirrels in seasons and months in two study sites: urban park and urban forest.

Season	Month	Number of days of trapping squirrels	
		Park	Forest
Spring			
	March	12	15
	May	16	12
Summer			
	July	12	12
Autumn			
	September	12	12
	November	16	13
Winter			
	January	36	12
Total		104	76

Rozdział 2.

Beliniak A., Gryz J., Klich D., Jasińska K., Krauze-Gryz D. 2022. Body Condition and Breeding of Urban Red Squirrels: Comparison of Two Populations Affected by Different Levels of Urbanization. Animals, 12(23): 3246. https://doi.org/10.3390/ani12233246.

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Article

Body Condition and Breeding of Urban Red Squirrels: Comparison of Two Populations Affected by Different Levels of Urbanization

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Simple Summary: The red squirrel is among the mammals that have adjusted well to urban habitats. We compared two populations inhabiting Warsaw: in a park (with plentiful supplemental feeding) and in a forest (with no feeding and restricted visitor access). In previous studies, we showed that squirrels in the park differed from forest squirrels in space use, food choice, activity patterns and interactions with people. Now, we predicted that the supplemental feeding of park squirrels would result in their higher body mass, better body condition, a higher share of breeding females and extended breeding season. Contrary to our assumptions, forest squirrels were heavier and had better body conditions. Yet, in both populations, better body conditions increased chances for breeding. There were more breeding females and more young squirrels in the park. Squirrels from both areas bred mostly in spring but also in winter. The lower body mass/condition of park squirrels may have been due to competition in a high-density population, or may suggest that with year-round access to food, accumulating fat was not crucial. The extended breeding season may have been thanks to good feeding conditions and/or mild winters in the city. Again, we showed the high plasticity of red squirrels living in human-transformed habitats.

Abstract: The red squirrel is among the mammals that have adjusted well to urban habitats. Here, we focused on the two populations inhabiting Warsaw: in a park (with year-round supplemental feeding) and in an urban forest. We hypothesised that park squirrels would have higher body mass (and better body condition), being more stable over the year, and would have a higher breeding rate (i.e., the share of breeding females). Contrary to our hypothesis, forest squirrels were heavier and had better body condition than park squirrels. The body masses of squirrels from both areas were quite stable (with the highest values obtained in spring). Females in better body conditions were more likely to breed. More breeding females and sub-adults were trapped in the park. Regardless of the study site, the highest share of breeding females was in spring, but they also bred in winter and in the remaining seasons. The lower body mass/condition of park squirrels may be possibly explained by high intraspecific competition, or by stable food (and thermal as typical for the city) conditions, in which accumulating fat was not crucial. Mild winter conditions may have also enabled squirrels to breed early. This study showed the high plasticity of red squirrels living in human-transformed habitats.

Keywords: body mass; live-trapping; seasonal changes; breeding activity; *Sciurus vulgaris*; urban mammals

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1. Introduction

Human populations have become increasingly urbanised and, as an effect, cites and their infrastructures are rapidly expanding [1]. These highly modified landscapes, however, may offer novel habitats for wildlife [2–4]. Anthropogenic changes in landscape can impose rapid evolutionary change [5]. Urban habitats differ from natural environments by their altered food availability, acoustic and light pollution, thermal conditions, or different sources and rates of mortality [4,6,7]. Nevertheless, many animal species adjust to urban conditions (e.g., [2,3,8–11]) by changing their diet preference, home range size, or behaviour [6].

The high availability of food in urban environments is thought to be among the reasons why many species of animals are thriving in cities (review in: [12]). Predictable anthropogenic food subsidies affect the body conditions and breeding parameters of individuals [13]. The effect of anthropogenic food sources may vary; urban vertebrates can benefit from increased food access and be in good body condition. Alternately, high food predictability can result in reduced body mass thanks to the limited need to accumulate body reserves. Finally, urban diet may lead to poor body condition in urban individuals due to low food quality (review in: [14]). In general, increased body mass and fertility are observed, yet this effect may be sex-dependent [13]. For example, male silver gulls (*Larus novaehollandiae* Stephens, 1826) from urban environments with access to an anthropogenic diet were heavier and had better body condition than non-urban male gulls, but no differences were detected between females [15]. On the other hand, sparrows (*Passer domesticus* L., 1758) in more urbanised habitats had reduced body size and body mass compared to their rural conspecifics but with no differences in condition indices [14]. In American red squirrel (*Tamiasciurus hudsonicus* Erxleben, 1777), there were no or very small differences in the body mass between experimentally fed and control populations, although males tended to be heavier in a fed population in winter [16], while urban female eastern chipmunks (*Tamias striatus* L., 1758) were in better body condition than their non-urban conspecifics [17].

Increased year-round food resources in urban conditions results in changes to the onset and duration of breeding seasons, which was observed in numerous animals [18]. A striking example of such is seen in the tawny owl (*Strix aluco* L. 1758), which, in cities starts laying eggs a few weeks earlier even than in non-urban populations, potentially due to, among other reasons, a stable food base [11,19]. Indeed, a case of American red squirrel showed that the fed population had a longer breeding season, with some females having more than one litter [16], which (also thanks to an increased survival) led to a ten-fold increase in the density of the population [20].

The Eurasian red squirrel (*Sciurus vulgaris* L. 1758) has adjusted well to urban habitats [21–27]. This species is strongly habituated to human presence, and its behavioural flexibility helps it to adjust to these specific conditions [28]. Urban habitats serve as suitable refugia for red squirrels [28,29], who are known to utilise urban structures [30] and move through built-up landscapes [31]. The abundance of red squirrels increases with human population density [29] and can be higher in cities than in rural areas [23,32,33]. Urban red squirrels can change their activity pattern [34], interact more with humans [23,24] and be bolder than their non-urban counterparts [35]. Nevertheless, little is known about the body mass, condition and breeding of typically urban populations of red squirrels. In urban conditions, red squirrels are offered various sources of supplementary food, including the opportunity to gather food from bird feeders, as well as the provision of nuts offered directly by park visitors [22,23,25,36–38]. It has been already shown that supplemental food is a key factor that may attract squirrels, and this extra food is most important when natural food is scarce [29,39]. Indeed, in Hamburg, in a supplementarily fed population, red squirrels maintained stable body mass over the course of a year, likely indicating that they were never food-stressed [25,38]. Moreover, individuals whose core areas were located closer to high-energy anthropogenic food sources were heavier, while natural food sources did not affect body mass [25].

The aim of this study was to compare two red squirrel populations inhabiting the same city but two different areas: an urban park and an urban forest, both placed within city districts. Nevertheless, one is expected to be highly affected by human and plentiful supplementary food from park visitors, whereas the effect of human disturbance on the other population should be inconsiderable. Indeed, earlier studies showed that park squirrels lived at higher densities and occupied smaller home ranges. They also changed their behaviour and feeding strategies in response to human presence: they spent more time on the ground, tolerated close contact, and human-delivered nuts made up the bulk of their diet [23,24]. They also changed their activity pattern, making the most of human presence [23,24,34]. In this work, we aimed to compare the body mass and body condition of both sexes and the reproductive activity of females between the two populations. Because food availability affects the ecology of red squirrels [29,31,40,41], we can hypothesise that park squirrels, with access to abundant supplemental food and a rich natural food base, will have higher body mass (and better body condition), which remains more stable over the year, and females will start breeding earlier and prolong their breading season in comparison to the urban forest squirrels.

2. Materials and Methods

2.1. Study Area

The study was conducted in two sites (Figure 1), both located in Warsaw (52°14′13.37″ N, 21°1′3.11″ E), the capital city of Poland. The city is populated by approximately two million people and is placed in the central part of the country. This region is affected by both the harsh and dry continental climate of Eastern Europe and Asia and the mild oceanic climate of Western Europe. Growing season lasts for about 210 days, and the total precipitation measures 600 mm per year. The mean ambient temperature is from −4 °C in January to 18 °C in July.

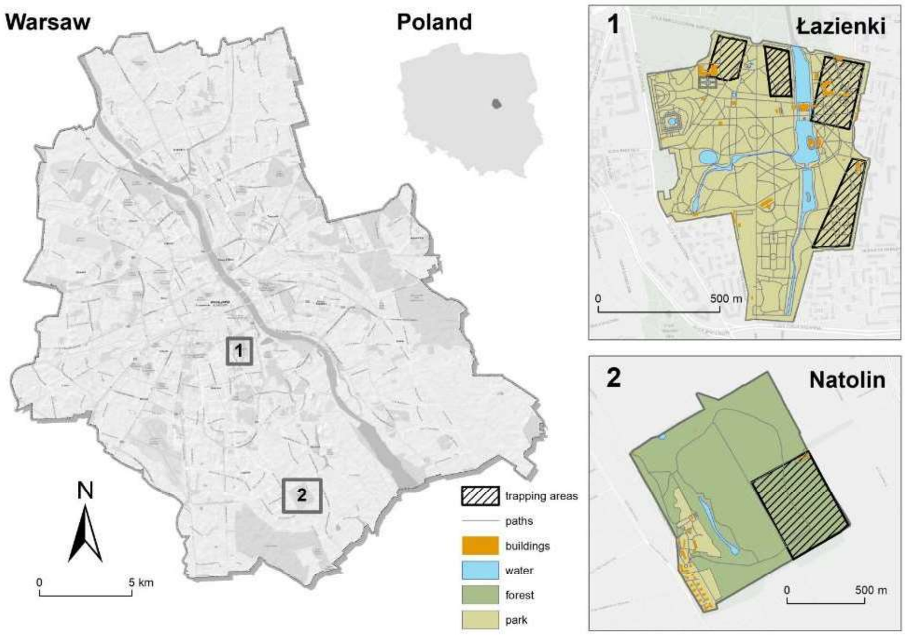

Figure 1. Study areas: 1. Royal Łazienki Museum (an urban park) and 2. Natolin Forest Reserve (an urban forest) in Warsaw, where red squirrels were live-trapped. Approximate locations of trapping areas are shown.

The first study site was Royal Łazienki Museum, a park located in the city centre (hereafter 'urban park') (Figure 1). This park is very popular among local inhabitants and visitors. It is difficult to estimate how many people visit the park because no entrance ticket is required, and the number of people passing through the gates is not monitored. According to an annual report made by the Warsaw Tourism Organization, the Royal Łazienki Museum park was visited by 3.5 million people in 2018, in 2019, by almost 5 million, and in 2020, by more than 4 million [42]. The urban park covers 76 ha, and it is surrounded by busy streets and built-up areas. The park has more than 90 species of trees and shrubs, both native and foreign species. Deciduous trees are most numerous, e.g., common hornbeam (*Carpinus betulus* L.), common oak (*Quercus robur* L.), common beech (*Fagus sylvatica* L.), as well as common hazel (*Corylus avellana* L.), English walnut (*Juglans regia* L.) and North American walnut (*Juglans nigra* L.). Tree stands can reach more than 150 years old, and numerous old trees provide a rich natural food base for animals [32]. Squirrels here are fed by park visitors every day; some individuals even deliver food for animals on an everyday basis [43]. According to our radio telemetry data and direct observations of tagged squirrels, squirrels took food from humans on average 0.89 times per hour [44]. Most (almost 90%) of the recorded food items eaten by red squirrels in the park were nuts, and most of those (66%) were provided by park visitors [23]. Moreover, there are about 10 feeders located in the park (and the presence of feeders is known to attract squirrels and to be able to increase their abundance [45]), which are stocked with seeds and nuts by park employees during the period September–March. However, feeders stay in the park all year round, and visitors leave food in them for animals throughout the remainder of the year, too.

The second study site was Natolin Forest Reserve, located approximately 10 km from the city centre (hereafter 'urban forest') (Figure 1). This land was formerly parkland that extended around the residences of Polish magnates. The spontaneous regeneration of woodland took place after the Second World War, so the reserve area is wholly tree-covered these days. The oldest stands are over 250 years old, and only natural regeneration occurs. Dead and fallen trees are left for natural decomposition. The reserve covers 105 ha, and it has been protected since 1991. Since then, it has been closed to the public. To the west and north of the reserve are built-up areas, whereas on the other sides, it is surrounded by patches of farmland (mostly set-asides), which are successively built-up. Trees are mostly deciduous, e.g., common hornbeam, common oak, ash (*Fraxinus excelsior* L.), elms (*Ulmus* spp.), common hazel and black alder (*Alnus glutinosa* L. (GAERTN.)).

No food base availability assessment was carried out for this study. Nevertheless, tree inventory was previously conducted in 400 m^2 patches, which were placed exactly in our trapping areas [46]. It was found that the density of trees and shrubs was higher in the urban forest (44/400 m^2) than in the park (19/400 m^2); however, trees over 30 years old (which are assumed to be seed-bearing) were more numerous in the urban park than in the forest (40% vs. 11%) [46].

In the past studies, the density of the urban park red squirrels was estimated to be more than 2 individuals/ha [23,32], whereas in the urban forest, it was 0.29 ind./ha [23].

2.2. Live-Trapping

The study started in July 2018 and ended in December 2020. Squirrels were live-trapped with 30 traps in the urban park and 40 traps in the urban forest. We used standard wire mesh live traps (51 × 15 × 15 cm) (manufactured by "Jerzyk" Jerzy Chilecki, Białowieża, Poland). The traps were partly covered by dark plastic to provide shelter from rain and snow and were located on the ground or in trees on a wooden platform. Live traps were pre-baited with hazelnuts and English walnuts for seven days, then baited and set for four (in most cases) to nine days. In both areas, we trapped in the same month, during thirteen trapping sessions in total (i.e., in 2018: Jul, Sept, Nov; in 2019: Jan, Mar, May, Jul, Sept; in 2020: Mar, May, Jul, Oct, Dec). The traps were set in the morning (around 6–7 a.m., depending on the time of dawn), checked after 2–4 h and secured for the night in a manner which prevented them from being closed. We flushed every trapped squirrel into a wire mesh handling cone [47] to minimise stress during handling. Each newly trapped squirrel was individually marked with numbered ear-tags 2 × 8 mm (National Tag&Band, Newport, KY, USA). Squirrels were weighed to the nearest 10 g (Pesola spring balance), and the right hind foot, excluding the claw, was measured with tape measure. Body condition was calculated based on residuals of log body mass and foot length [48]. A linear regression model was built using data on 50 adult individuals (log body mass = 2.211 (±0.074) + 0.006 (±0.001) foot length, $R^2 = 0.29$, $p < 0.001$). Then, the regression model was used to calculate the residuals of body mass for all individuals. We also defined the sex and reproductive status of females. Females were defined as non-breeding (anoestrous, small vulva, no longitudinal opening), postoestrous and pregnant (swollen vulva with longitudinal opening, enlarged belly during pregnancy) or lactating (large nipples, milk excretion could be stimulated) [49]. We also defined if squirrels were adult or sub-adult. Sub-adult males had small scrotum and abdominal testes, and females had a very small vulva and the nipples were still invisible. Older animals were considered as adult [50].

We used the minimum number of animals known to be alive (MNA) (e.g., [41,47,51,52]) from the trapping during period October 2018–September 2019. We estimated squirrel density using edge-correction using the average female range radius in study sites [41]. According to a previous study [23], the average radius for females in the urban park was 75 m, while in the urban forest, it was 135 m. MNA estimates assessed during the first year of this study confirmed this difference in density between the two populations: the value obtained for the urban park squirrels ranged from 1.05 to 1.89 ind./ha and from 0.2 to 0.28 ind./ha for forest squirrels (Figure 2).

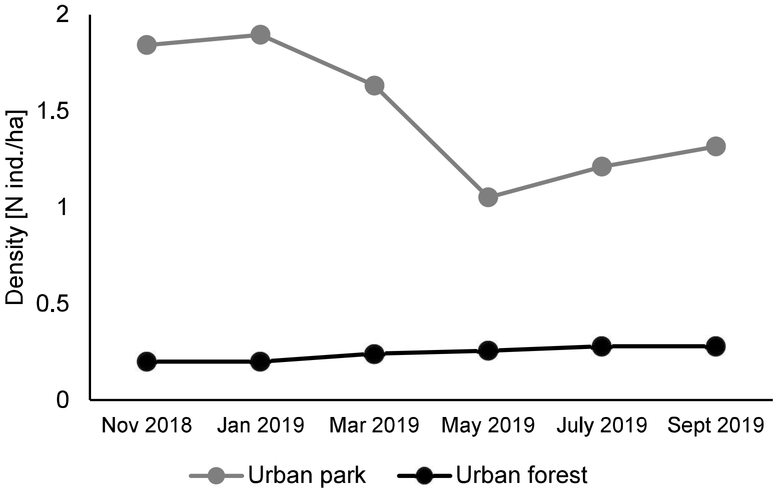

Figure 2. Density of the two studied populations of red squirrels in Warsaw: urban park and urban forest, assessed on the basis of live-trapping and MNA estimates.

Access to the Natolin Forest Reserve and red squirrel capture was allowed with the permission issued by the General and Regional Directorates for Environmental Protection. The trapping and handling of squirrels complied with current laws on animal research in Poland and were carried out with a permit from the Local Ethical Committee (WAW2/072/2018).

2.3. Statistical Analysis

We compared the sex proportion and age proportion of all individuals across both sites (urban park vs. urban forest) with the Chi-squared tests. We also analysed differences in the body mass and body condition of adult individuals and the reproductive status of adult females between sites (including other factors). We used three models: two linear mixed models (for body mass and body condition) and one generalised linear mixed binary model (logit regression) for reproductive status, where we included all adult females (including recaptures). In the first LMM, the dependent variable was body mass. In this model, we tested the effect of foot length (FOOT), sex (SEX), site (SITE), season (SEASON) and the interaction of site with other two variables: SITE*SEX and SITE*SEASON. SITE was a grouping variable of the two study sites: urban park (Łazienki) and urban forest (Natolin). SEASON was a grouping variable of four astronomical seasons: spring (1 March–31 May), summer (1 June–31 August), autumn (1 September–30 November) and winter (1 December–31 February). In the second LMM, the dependent variable was body condition, and a similar set of variables apart from foot length (FOOT) and interactions between variables was included. In the third model, the reproductive status of adult females was analysed. In this model, all breeding female squirrels (i.e., postoestrous and pregnant or lactating) were marked as 1, and all non-breeding squirrels were marked as 0. In this model, the independent variables were: site (SITE), season (SEASON), body condition (CONDITION) and interaction of site and season (SITE*SEASON). We did not include the body mass as it was highly correlated with the body condition (Pearson's r = 0.849). The IDs of squirrels were set as a random effect in both models to account for the repeated sampling of individual animals. Restricted maximum likelihood (REML) was used to estimate the parameters in the best model obtained. Model selection was based on *p*-values in stepwise backward selection [53]. Groups within variables included in the best model were compared with the LSD test.

All statistical analyses were performed with SPSS software (version 26.0, IBM, Armonk, NY, USA).

3. Results

In total, 36 squirrels (19♀, 17♂) were trapped in the forest, while 106 squirrels (48♀, 58♂) were trapped in the park. In the forest, squirrels were trapped in total 129 times (Mean = 3.58, Min = 1, Max = 11), and in the park, 266 times (Mean = 2.44, Min = 1, Max = 12).

The share of caught sub-adults in the forest did not exceed 2% of all caught individuals, while in the park, this age group constituted almost 10% (Figure 3A). The proportions were significantly different ($\chi^2 = 8.92$, $p = 0.003$). The proportions of males and females of all individuals did not statistically differ between the areas ($\chi^2 = 2.79$, $p = 0.095$) (Figure 3B).

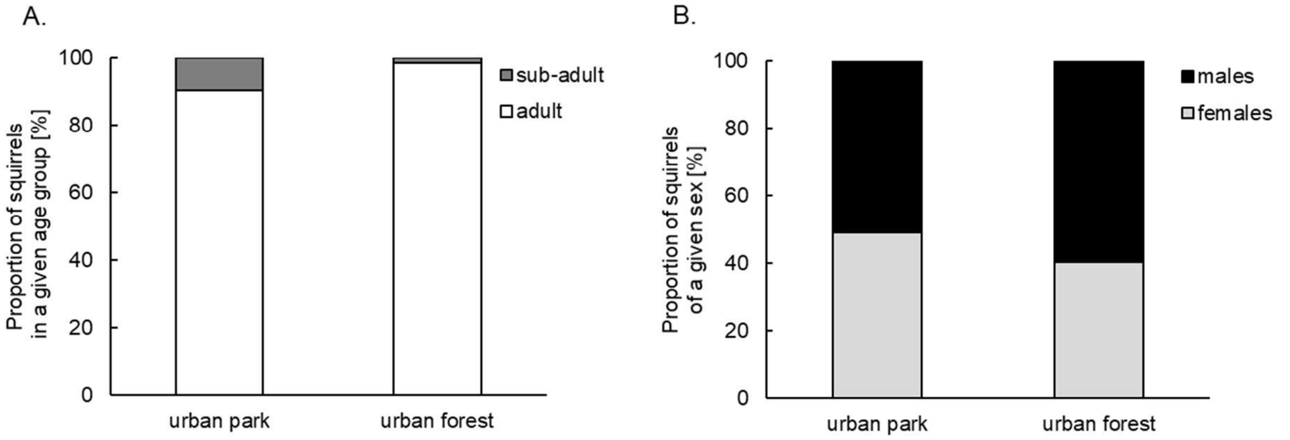

Figure 3. Proportion of red squirrels of given (**A**) age and (**B**) sex groups in the urban park and urban forest.

Adult red squirrels in the forest had higher body masses (Mean = 355 g, ±SD = 29) than those in the park (Mean = 337.2 g, ±SD = 33.2) (Figure 4B). The analysis of body mass showed that the model, after selection, contained three explanatory variables: FOOT, SITE and SEASON (Table S1). Regarding seasonal differences in the body mass, only spring differed significantly from other seasons (summer $p = 0.044$, autumn $p < 0.001$, winter $p = 0.007$) (Figure 4A).

The squirrels in the urban forest had better body conditions than those in the urban park (Mean = 6.58, ±SD = 0.53 and Mean = 6.21, ±SD = 0.63, respectively), and the difference was statistically significant ($p = 0.002$) (Figure 5B). Furthermore, body condition was higher in spring compared to autumn and winter ($p = 0.001$, and $p = 0.043$, respectively) (Figure 5A). Body condition did not differ with regard to sex or subgroups in the interactions and was excluded during model selection (Table S2).

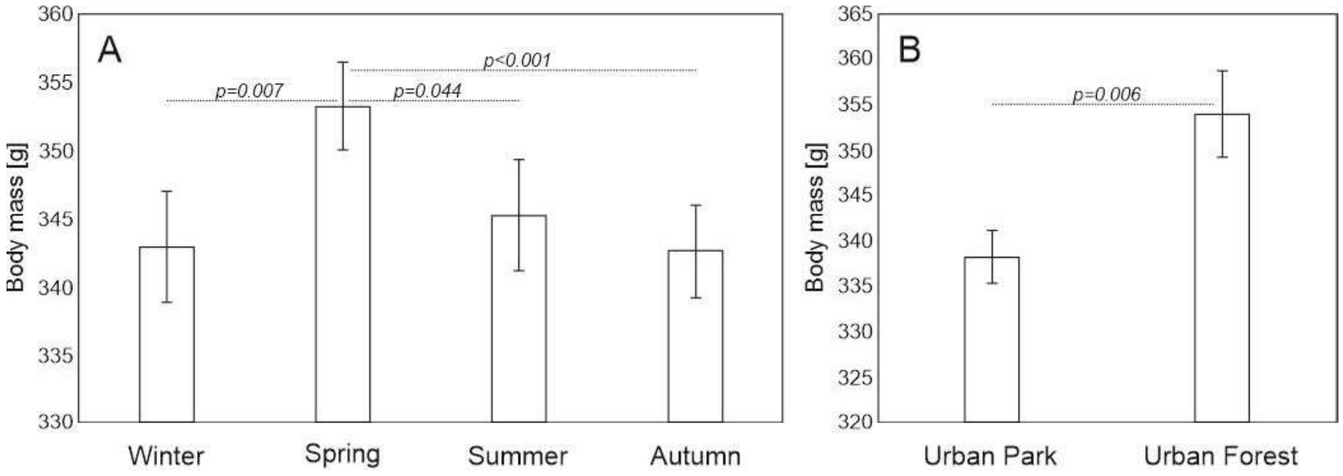

Figure 4. Mean (±SE) body mass of adult red squirrels with regard to (**A**) season and (**B**) study site, and pairwise comparison with Least Significant Difference test in LMM (significant differences presented above the bars). Please note that Y axis does not start from 0 value.

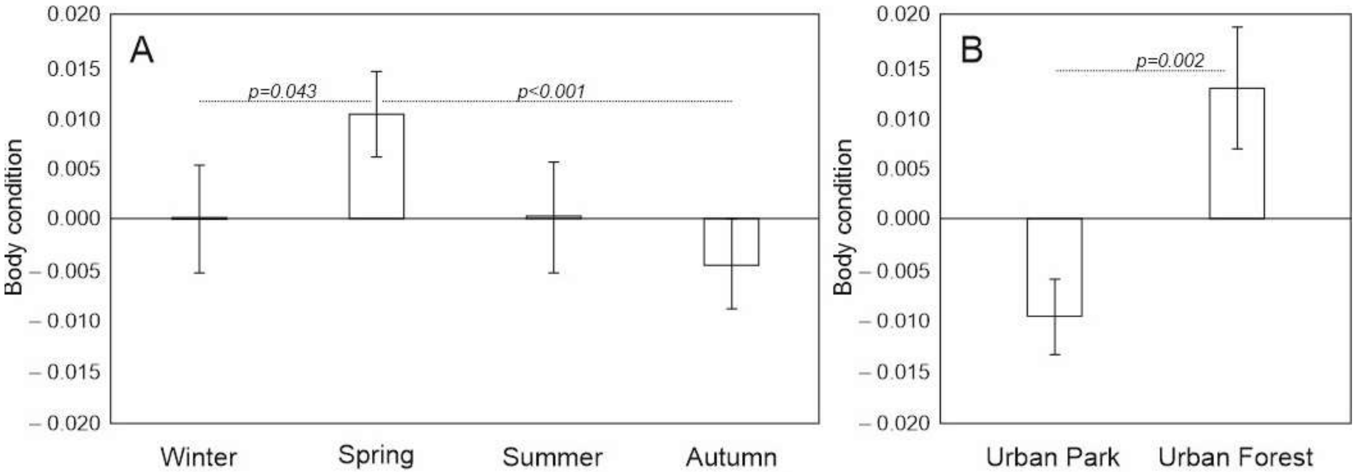

Figure 5. Mean (±SE) body condition of adult red squirrels with regard to (**A**) season and (**B**) study site and pairwise comparison with Least Significant Difference test in LMM (significant differences presented above the bars).

During the whole study period, the proportion of sexually active adult females in the urban forest was lower than in the urban park (23% and 35%, respectively). The best fit model contained all variables: study site, season and condition of animals (Table S3). The probability of being sexually active (i.e., breeding) increased with the body condition of females ($\beta = 9.38$, SE = 4.37, $p = 0.033$). The highest frequency of sexually active squirrels was observed in spring and summer, but sexually active individuals were also found in winter (Figure 6A). A higher frequency of sexually active adult squirrels was observed in an urban park (Figure 6B). No statistically significant differences were stated in pairwise comparison. The minimum body mass of breeding females was 350 g in the forest and 300 g in the park.

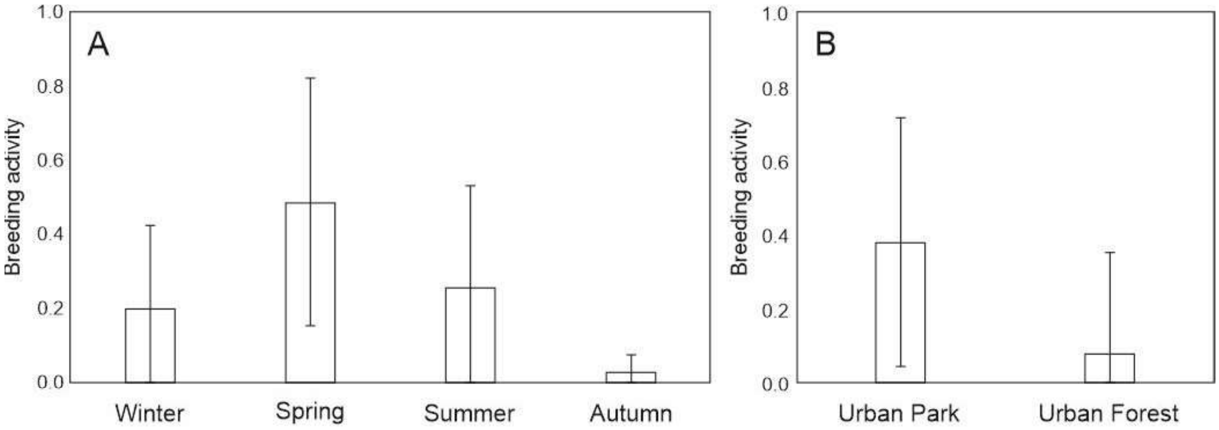

Figure 6. Frequency (±SE) of breeding (i.e., postoestrous and pregnant, lactating) adult female red squirrels with regard to (**A**) season and (**B**) study site. No statistically significant differences in pairwise comparisons using the Least Significant Difference test.

4. Discussion

As we predicted, the two populations of squirrels, one living in a busy urban park, and the other from an urban forest, differed in terms of body mass, body condition and breeding activity.

First, forest squirrels were heavier and had better body conditions than those from the park. Differences in the body masses between squirrels of the same population can be caused by variation in their size or in the amount of fat under the skin [54]. Body condition and habitat quality are strongly linked [54], so we may have expected that the year-round supplementary feeding of park squirrels [22–24,34] would result in increased body condition [17]. However, surprisingly, forest squirrels were generally in better body condition than park squirrels, who had access to year-round supplemental feeding. Optimal body mass and a layer of fat is crucial for squirrels to ensure thermal insulation and energy during periods of food shortage [55]. Habitat and population density may affect body mass, too. In general, squirrels inhabiting deciduous habitats had lower body mass than squirrels living in conifer habitats [54]. In our case, both study sites were deciduous; however, in the urban park, population density was much higher than in the urban forest. It has been reported that squirrels of high social rank, both males and females, weigh more than squirrels of low rank, and body mass may be positively correlated with boldness [56] and aggressiveness [54,57]. In high-density populations, squirrels may be less aggressive towards conspecifics [58]. It may thus be argued that in this high-density population, more subdominant squirrels with lower body mass survived. In the forest, in turn, access to large nuts (in our case hazelnuts, hornbeam nuts, and acorns) allowed the population to reach a higher body mass, which is crucial for settlement success and local survival [40]. Our results are to some extent in line with other studies in which body mass and/or condition were not driven by supplementary feeding [39,59,60]. According to Magris and Gurnell [39], supplementary food can affect squirrel density and population biomass (which is the case of park squirrels in our study) but does not affect individual body mass and condition.

In general, the body mass of red squirrels may change seasonally, according to food availability, weather and reproductive status [54,61]. In deciduous habitats, body mass is typically highest in autumn [55,62], when squirrels feed mainly on high-energy tree seeds and accumulate fat for the winter period [54,62]. On the other hand, no autumn or winter increase in body weight was observed in coniferous habitat, which was explained by more predictable food supplies [61]. In winter, food availability remains high, but due to high thermoregulation costs, fat reserves may become depleted [55]. Next, during spring, fat reserves become further depleted because of the high energy cost of reproduction and increased activity [55]. Therefore, squirrels with higher body mass and larger reserves of fat are better equipped to cope with stressful conditions, which in turn increases their chances of survival [40,49,54]. In our study, a slight seasonal variation in the body mass of squirrels was found, but surprisingly, they were heaviest in spring, with no differences between other seasons. This pattern of changes in the body mass stands in contrast to published findings (see above). There was no contrast between summer–autumn–winter activity in our study. Our forest squirrels were seen to typically reduce their winter activity, which is a response to high thermoregulatory costs [36,63]. This may help them to retain high fat resources regardless of changing environmental conditions. Additionally, recent mild winters [64] together with an influence of an urban island heat effect, reaching as far as the outer districts of Warsaw [65], may be facilitative. As shown in a previous study, forest squirrels occupied relatively small home ranges [23], which suggested rich natural food supply [61]. In contrast, park squirrels were more active, assumingly trying to adjust activity to the reduced presence of park visitors in winter [34], which might help them to retain fat resources. Our park is located in the central part of the city, where the temperatures were the highest [65], so squirrels could spend more time outside their dreys. On the other hand, in spring, which normally is a time of low food availability [62,66], park visitors (i.e., food providers) became abundant, which assumingly helped squirrels to restore fat reserves quickly. Low variation in the body mass during other seasons in the case of park squirrels is in line with findings from Hamburg, where in a fed population, individual body mass was stable [25], assumingly due to high and stable food energy availability [67].

For females, body mass is especially important, as a fat layer provides a valuable energy store during lactation and nursing young [55]. The reproductive success of females increases with their body mass and body condition [54,55,68]—heavier squirrels grow older and produce more offspring per litter and more litters in their lifetime [40,48,49,54,55]. Additionally, in our study, females in better body condition were more likely to breed. What is interesting is that although females in the park had generally worse body condition, the overall proportion of sexually active females was higher compared to the forest squirrels. A minimum body mass is required to enter oestrus [39,54], and this value was lower for park than forest squirrels. Squirrels in the park are fed by people during all seasons [22,23]; thus, probably, squirrels do not need such large fat reserves to commence breeding and produce successfully. Our study only specified very simple breeding parameters and did not measure the breeding success of females. Nevertheless, more sub-adult individuals were trapped in the park than in the forest, which suggested a higher reproduction rate. This is consistent with a study on fox squirrels, which were more often reproductively active and presented higher juvenile survival and juvenile/adult ratios in the urban population than the rural one [69]. Additionally, juvenile and sub-adult squirrels appeared more frequently in an experimentally supplementary fed American red squirrel population as a result of a longer breeding period [16].

Red squirrels are seasonal breeders; females have one or two litters during a year: first in February–March and second in June–July [51,52,70,71]. Seed availability can affect, among other things, the length of the breeding season and the number of adults which produce two litters [68]. The frequency of breeding females was, regardless the study area, highest in spring, but squirrels also bred in winter. This early reproduction may have been possible thanks to good feeding conditions and higher winter temperatures in the city [72]. Females giving birth early in the year would have sufficient time to produce a second litter [68]. Additionally, by breeding early, females may be able to enhance the chances of their offspring to settle in an optimal home range as juveniles from early litters can find more vacant areas [59]. As shown by earlier studies, and in line with our findings, the availability of supplemental food had no significant effect on the length of breeding season [59], and early breeding (spring litters) was not affected by food availability [39].

5. Conclusions

As was reviewed by Boutin [73], individuals receiving supplemental food usually had higher body mass and advanced breeding relative to those in control areas. In our study, urban park squirrels that received supplemental feeding had lower body mass (and worse body condition) than those inhabiting the urban forest, and there was no difference in breeding time. This difference in the body mass/condition may be explained by high intraspecific competition in a very abundant squirrel population. On the other hand, stable food conditions (i.e., year-round supplemental feeding) combined with less demanding weather conditions typical for the city centre might mean that accumulating fat is not crucial for survival and breeding. Abundant supplemental food may also result in a higher share of breeding females. These differences between the two populations complete our earlier findings on how far urban park and urban forest squirrels differed in certain aspects of their ecology (i.e., space use, food choice, activity patterns and interactions with people) and proved the high plasticity of squirrels inhabiting urban landscapes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12233246/s1, Table S1: Effects of a site (SITE), season (SEASON) and foot length (FOOT) on body mass of squirrels in a general linear mixed model; Table S2: Effects of site (SITE) and season (SEASON) on body condition of squirrels in a general linear mixed model; Table S3: Effects of site (SITE), season (SEASON) and body condition (CONDITION) on sexual activity of red squirrels in generalised linear binary model.

Author Contributions: Conceptualisation, D.K.-G. and A.B.; methodology, D.K.-G. and A.B.; investigation, A.B., J.G., D.K.-G. and K.J.; database preparation, A.B.; statistical analysis, D.K.; writing—original draft preparation, A.B., D.K.-G. and D.K.; writing—review and editing, A.B., D.K.-G., D.K., K.J. and J.G.; visualisation, D.K.; supervision, D.K.-G.; project administration, A.B.; funding acquisition, A.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Access to the Natolin Forest Reserve and red squirrel capture was allowed with the permission issued by the General and Regional Directorates for Environmental Protection. The trapping and handling of squirrels complied with current laws on animal research in Poland and were carried out with a permit from the Local Ethical Committee (WAW2/072/2018).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Supplementary material 1

Table S1. Effects of a site (SITE), season (SEASON), and foot length (FOOT) on body mass of squirrels in a general linear mixed model, * reference category.

Source	***B***	***SE***	***t***	***p***	***Lower CI***	***Upper CI***
Intercept	261.18	27.76	9.41	<0.001*	206.57	315.78
SITE (Łazienki)	-15,68	5.60	-2.80	0.006*	-26.77	-4.58
SITE (Natolin)	0*					
SEASON (winter)	-10.28	3.80	-2.71	0.007*	-17.76	-2.80
SEASON (autumn)	-10.57	3.17	-3.33	<0.001*	-16,81	-4.33
SEASON (summer)	-7.98	3,95	-2.02	0.044*	-15.75	-0.22
SEASON (spring)	0*					
FOOT	1.84	0.51	3,64	<0.001*	0.85	2.84

Table S2. Effects of site (SITE) and season (SEASON) on body condition of squirrels in a general linear mixed model, * - reference category.

Source	***B***	***SE***	***t***	***p***	***Lower CI***	***Upper CI***
Intercept	0.02	0.01	3.35	<0.001*	0.01	0.04
SITE (Łazienki)	-0,02	0.01	-3.17	0.002*	-0.03	-0.01
SITE (Natolin)	0*					
SEASON (winter)	-0.01	0.01	-2.02	0.043*	-0.02	0.00
SEASON (autumn)	-0.02	0.00	-3.45	<0.001*	-0.02	-0.01
SEASON (summer)	-0.01	0.01	-1.91	0.057	-0.02	0.00
SEASON (spring)	0*					

Table S3. Effects of site (SITE), season (SEASON) and body condition (CONDITION) on sexual activity of red squirrels in generalized linear binary model, * - interaction.

Source	***B***	***SE***	***t***	***p***	***Exp (B)***	***Lower CI***	***Upper CI***
Intercept	0.27	1.47	-0.19	0.853	1.31	0.73	23.42
SITE (Natolin)	-1,03	0.46	-2.24	0.026*	0.36	0.04	0.30
SITE (Łazienki)	0*						
SEASON (winter)	-0.49	0.38	-1.30	0.195	0.61	0.29	1.29
SEASON (autumn)	-2.75	0.46	-5.99	<0.001*	0.06	0.03	0.16
SEASON (summer)	-2.30	0.55	-4.14	<0.001*	0.10	0.03	0.30
SEASON (spring)	0*						
CONDITION	9.38	4.37	2,15	0.033*	$119*10^2$	2.18	$646*10^5$

Rozdział 3.

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PLOS ONE

RESEARCH ARTICLE

Long-term, medium-term and acute stress response of urban populations of Eurasian red squirrels affected by different levels of human disturbance

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Abstract

Animals in urban areas often encounter novel and potentially stressful conditions. It is important to understand how wildlife cope with anthropogenic disturbance. To investigate this specific adaptation we live-trapped squirrels in two study sites in Warsaw: a forest reserve and an urban park and we estimated stress responses at three levels: long-term and medium-term stress (the level of stress hormones, i.e. cortisol and cortisone concentrations, in hair and feces) and acute reaction to human-induced stress (measured during handling with the aid of the three indices: breath rate, struggle rate, and vocalization). According to GLMM models no difference in the stress hormones level was found between the two populations. The only differences in cortisol concentrations clearly depended on the season, i.e. being higher in autumn and winter comparying to other seasons. There was no influence of sex, or reproductive status on stress hormones. Forest squirrels had significantly higher breath rates, suggesting they were more stressed by handling. There was no difference in the struggle rate between study areas, this index was mostly affected by season (i.e. being highest in winter). First-trapped squirrels vocalized less than during the subsequent trappings. Assumingly, during the first, and more stressful trapping, squirrels used 'freezing' and/or little vocalization, while during next captures they used alarm calls to warn conspecifics. Overall, we showed that the two squirrel populations differed only in terms of their breath rate. This suggests that they did not differ in medium-term and long-term stress in general, but they can differ in acute response to handling. This also suggests that both populations were similarly affected by environmental factors. The lack of clear effects may also be due to population heterogeneity. Thus, in order to assess the effects of anthropogenic stressors a broader range of indicators and diverse analytical methods, including behavioral analyses, should be employed.

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Introduction

Urbanization is known to be one of the most drastic environmental changes caused by humans [1]. Animals living in urban areas often encounter novel conditions, which may be potentially stressful, like changes in predation pressure, altered food resources, and new species interactions [2] or increased disturbance from people [3]. However, some animals can adapt and survive or even thrive in cities [4–7]. Since cities and their infrastructure are rapidly expanding [4], it is increasingly important to understand how wildlife cope with anthropogenic disturbance [8].

Squirrels are one of the typical mammalian groups which have colonized urban areas and adapted to them successfully. This was observed for the Eurasian red squirrel (*Sciurus vulgaris*) [9–11], the grey squirrel (*S. carolinensis*) [12] and the fox squirrel (*S. niger*) [13]. Squirrels in urban habitats may exhibit higher population densities [9,12,14], higher aggressiveness [12], increased boldness [15] and different activity patterns than in a non-urban environment [16,17]. Red squirrels are commonly fed by people in parks and gardens [18–24] and may be considered an iconic species of coexistence between human and wildlife in urban areas [25].

Levels of glucocorticoid hormones, such as cortisol or corticosterone, provide information about the impact of environmental factors on wildlife [26,27]. Measuring hair cortisol and fecal cortisol metabolites as an indicator of adrenocortical activity in animals provides an insight into stress responses of animals over medium-term and long-term time scales, thus being a valuable tool in environmental studies [28,29]. Anthropogenic disturbances can be assumed as stressors to wildlife, and as a result, animals in contact with urban areas and humans are expected to have higher glucocorticoid levels compared to animals that are less exposed or not exposed at all [30–32]. It is often assumed that urban populations show higher baseline levels of glucocorticoid hormones because of chronic stress, however, results are often inconsistent (reviewed in Iglesias-Carrasco *et al.* [8]). It has also been suggested that animals living in urban habitats show reduction of HPA sensitivity through habituation [33], which may help animals to adapt to novel environments.

The analysis of hair and fecal glucocorticoid metabolites is considered less invasive compared to approaches that include blood sampling. For this reason, it has been applied to many wildlife species to monitor their stress responses to various disturbances [8,29,34,35]. Glucocorticoids concentrations in squirrels can depend on different factors like population density [36,37], sex [38–42], season [38,41], body mass [38,40] and condition [37,43]. To date there has been little agreement on how much urbanization influences stress levels in wildlife. In one study it was noted that levels of fecal cortisol metabolites were highest in areas where human disturbance was greatest [36]. On the other hand, chipmunks (*Tamias striatus*) inhabiting urban areas had lower concentrations of fecal cortisol than those from natural habitats [39]. The opposite effect was reported in a recent study in Japan, where levels of the fecal cortisol metabolites were not significantly different between the urban and rural squirrel populations [42,44].

In order to estimate acute stress responses to direct anthropogenic stressors, mainly behavioural indicators are used, such as: breath rate, struggle rate, and vocalisation, which show the response to handling [45–48]. Breath rate (i.e the frequency of respiratory acts) of an animal is assumed to be an index of the emotional and the stress response, similar to cardiovascular parameters, such as heart rate [46,49]. On the other hand, struggle rate, which is the amount of time animal moves around the bag after trapping, can be a measure of docility [50] and has been shown to correlate with aggressiveness and represent boldness [45–46]. Finally, the vocalization while handling can be measured. Squirrels use vocal communication to transfer vital information between conspecifics [51], which could be more difficult to obtain by using visual communication [52]. Tree squirrels produce different types of calls: alarm, agonistic, discomfort, mating, affiliative, and neonatal as well [51]. Alarm calls are the most frequently emitted and they typically serve as warning calls [53]. A call-type might also have one or several behavioral functions [51]. Here we assumed that vocalization during handling would show a reaction to potential danger and serve as an alarm call.

Behavioral traits may have fitness consequences [45,54] and intra-specific variation in capacity to cope with environmental challenges may buffer the species for strong fluctuations in the natural habitat [55]. Highly aggressive individuals adopt a proactive coping style whereas low levels of aggression indicate a more passive or reactive style of coping [55]. The two coping styles might explain a differential vulnerability to negative influence of stress due to the differential adaptive value of the two coping styles and the accompanying neuroendocrine differentiation [56]. A proactive coping animal may be adapted to stable environmental conditions. The reactive coping style may do better under variable and unpredictable environmental conditions [57]. Thus, they can determine how animals cope with environmental changes such as urbanization [58]. Therefore, in this study, the stress response of two urban red squirrel populations, which inhabit two different areas: an urban park and an urban forest reserve, both placed within city districts, was compared. The park squirrels live at very high densities [14], receiving plentiful supplementary food from park visitors [20,22], whereas the effect of human disturbance on the forest population should be considered negligible. We attempted to estimate stress responses at three levels: long-term and medium-term stress, revealed by the level of cortisol and cortisone concentrations in hair and feces, and acute reaction to human-induced stress, measured with the aid of the three indices: breath rate, struggle rate, and vocalization. We aimed to find out if squirrel behavior in respone to handling changed when they gained experience through multiple captures. We also hypothesized that in the urban forest, where squirrels live in lower density and generally maintain better body condition [14], the animals would exhibit lower concentrations of stress hormones. On the other hand, we supposed that the response to handling (vocalization, breath, and struggle rate) in the urban park (a site with higher human disturbance) would be less noticeable as park squirrels have direct and frequent encounters with humans [22,59].

Material and methods

Study area

We trapped squirrels in two study sites, both located in Warsaw, the capital of Poland. Warsaw is located in the central part of the country and has approximately two million inhabitants. This region is affected by both dry continental and harsh climates of Eastern Europe and Asia and the mild oceanic climate of Western Europe. Mean ambient temperature ranges from -4˚C in January to 18˚C in July. However, minimum temperature may be below -30˚C and the maximum temperature may rise above 35˚C.

One of the study sites was located in Royal Łazienki Museum, in a park in the central district of the city (hereafter 'urban park'). The park and its architectural attractions are very popular among visitors and local inhabitants. This 76-hectare green area is surrounded by busy streets and built-up areas. More than 90 species of trees and shrubs of both native and foreign species grow in the park [9]. About 20% of trees reach more than 150 years [9]. Tree species are mostly deciduous, e.g. common oak (*Quercus robur*), common beech (*Fagus sylvatica*), common hornbeam (*Carpinus betulus*), common hazel (*Corylus avellana*), English walnut (*Juglans regia*) and North American walnut (*Juglans nigra*). Apart from the natural food base, animals (birds and squirrels) are also fed by both park employees and visitors [14]. The most common supplemental food includes different kinds of seeds, e.g. sunflower (*Helliantus annuus*), hazelnut, and walnut [20,22].

The other study site was Natolin Forest Reserve (hereafter 'urban forest'), a forest located approximately 10 km from the city center. This area, which served as a royal hunting ground in the seventeenth century, was turned into a parkland that extended around the residences of Polish magnates in the eighteenth century. After the Second World War, the area was used as a government residence. Spontaneous regenerations of woodland occurred during the post-war period, nowadays the whole area of the reserve area is tree-covered. The reserve covers 105 ha and has been protected since 1991. It is closed to the public and permission is needed for an entrance. The oldest stands are more than 250 years old, dead or fallen trees are left for natural decomposition and only natural regeneration occurs. There are built-up areas to the west of the reserve, whereas on the other side it is surrounded by farmland. Trees are mostly deciduous like common hornbeam, common oak, ash (*Fraxinus excelsior*), elms (*Ulmus* spp.), common hazel, and black alder (*Alnus glutinosa*).

The two areas are approximately 10 km from each other and potentially interconnected via the the Warsaw Escarpment, which spans along the Warsaw section of the Vistula River. Nevertheless, this is intersected by roads and buildings so ecological connectivity between the two areas is highly disturbed. The urban park is much more affected by the urban heat island effect than the urban forest [60]. The two squirrel populations vary in density: the MNA (minimum number alive) value obtained in previous sudy for the urban park squirrels ranged from 1.05 to 1.89 ind./ha and from 0.2 to 0.28 ind./ha for forest squirrels. Adult red squirrels in the forest had higher body masses than those in the urban park (355 g and 337g respectivelly) [14]. Red squirrels in the forest had also significantly better conditions but were less sexulally active than these in the urban park (see details in Beliniak *et al.* [14]).

Live-trapping

Access to the Natolin Forest Reserve and red squirrel capture was allowed with permission issued by the General and Regional Directorates for Environmental Protection (WPN-I.6205.124.2018.AS and WPN-I.6401.208.2018.PF). Trapping and handling squirrels complied with current laws on animal research in Poland and was carried out with a permit from Local Ethical Committee (WAW2/072/2018).

Our study lasted from July 2018 to December 2020. We used standard wire mesh live traps (51×15×15 cm) (manufactured by "Jerzyk" Jerzy Chilecki, Białowieża, Poland). We live-trapped squirrels with 30 traps in the urban park and 40 traps in the urban forest. The traps were located on the ground or in trees on wooden platforms and were partly covered by black plastic to provide shelter from rain and snow. Before trapping, traps were pre-baited with hazelnuts and English walnut for seven days. After that we conducted trapping session: we baited and set traps for four (in most cases) to nine days. Trapping was conducted in both areas in the same month, in total during thirteen trapping sessions (i.e. in 2018: July, September, and November; in 2019: January, March, May, July, September; in 2020: March, May, July, October, December). Traps were set in the morning (around 6–7 a.m.), checked after 2–4 hours, and secured for the night (in a manner, which prevented them from being closed). Every trapped squirrel was flushed into a light hessian bag then we recorded the struggle rate for 30 sec.–the duration of time a squirrel kept moving in the bag. Then, to minimalize stress during handling, a squirrel was flushed into a wire mesh handling cone [61] and the breath rate estimated, i.e. the chest moves were counted during the 20 sec. Each newly trapped squirrel was marked with an individually numbered ear-tag (2x8 mm, National Tag&Band, Newport, KY, USA), weighted to the nearest 10 g (Pesola spring balance) and measured with tapmeasure right hind foot (without claws). Subsequently we defined sex and reproductive status of squirrels. Females were determined as non-breeding (anoestrous, small vulva, no longitudinal opening), or breeding. The latter category included: postoestrous and pregnant (swollen vulva with longitudinal opening, enlarged belly during pregnancy) or lactating (large nipples, milk excretion could be stimulated). Males were recorded as non-breeding (abdominal testes or semi-scrotal and scrotum small) or breeding (testes scrotal and scrotum large) [62]. We defined if a squirrel was sub-adult or adult. Sub-adult females had a very small vulva and the nipples were still invisible, males had small scrotum and abdominal testes. Older animals were considered adult [63]. Vocalization of squirrels was defined and ordered in increasing intensity from 1 to 4: 1 –none, 2 –growling, 3 –yelling, 4– screaming. At least two (usually three) people were involved in the trapping to make estimates as precise as possible. After handling we collected fresh feces left in the traps (not contaminated by the urine) to estimate fecal glucocorticoid metabolite levels. In Eurasian red squirrels, the fecal glucocorticoid metabolite levels from initial capture do not significantly differ from the hormone levels of at least 48 hours after the capture event [38]. Therefore, samples collected from recaptured squirrels were also analyzed if they were at least 48 hours apart. Fecal samples were collected into 2 ml centrifugal tubes, maximum 4 hours after setting a trap, immediately cooled in ice, and transported to the laboratory, where they were stored in a freezer at −20˚C until analysis. Additionally, during handling, a small hair sample (up to 50 mg) was collected. The hair was always sampled from the same region of the body (the middle part of the tail) and was cut as close as possible to the skin using fine scissors. Samples were stored in string plastic bags in the -24˚C freezer until analysis.

Laboratory analysis

Determination of hair steroids (cortisol and cortisone). In hair samples, cortisol and its metabolite—cortisone were determined using a validated ultra-high performance liquid chromatography coupled to electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) method. We decided to follow the concentration of two steroids in the hair because it has beed suggested that measuring multiple glucocorticoids simultaneously may provide more comprehensive information and more reliable interpretation of the acquired results [34]. The method of sample preparation and analysis was described in detail by Sadok *et al.* [34]. Briefly, hair samples were washed twice with isopropanol, dried overnight, cut into small pieces into a tube in an amount from 16 to 48 mg, and milled using steel beads. The steroids were extracted in the dark with 1 ml of methanol (LC-MS grade) fortified with internal standard (10 μg/ml of cortisol-D_4) for 24 hours. The supernatants were further purified by solid-phase extraction using the polymeric reversed phase Strata-X cartridges (30 mg/1 ml) supplied from Phenomenex as described previously (Sadok *et al.* [34]).

Samples were analyzed using a 1290 infinity UHPLC system coupled to a 6460 triple quadrupole mass spectrometer equipped with the Jet Stream electrospray ion (ESI) source from Agilent Technologies (Santa-Clara, CA, USA) and settings detailed in Sadok *et al.* [34]. The concentrations of steroids were determined in μg/ml from matrix-based calibration plots after signals normalization on the internal standard. The final results were expressed in pg/mg after conversion to the weight of hair samples subjected to extraction. An example of data obtained during the UHPLC-ESI-MS/MS analysis showing the presence of hair cortisol and cortisone in squirrel hair sample is presented in Fig 1.

Determination of fecal cortisol. The concentration of cortisol in the fecal samples was assessed with the ELISA method using a commercial kit with antibodies for this hormone (COR ELISA Kit No. EU0391, Wuhan Fine Biological Technology Co.). The analysis was performed according to the manufacturer's protocol described in detail previously [64,65] as follows: 100 mg samples of feces were weighed using an XA 100 3Y analytical balance (Radwag,

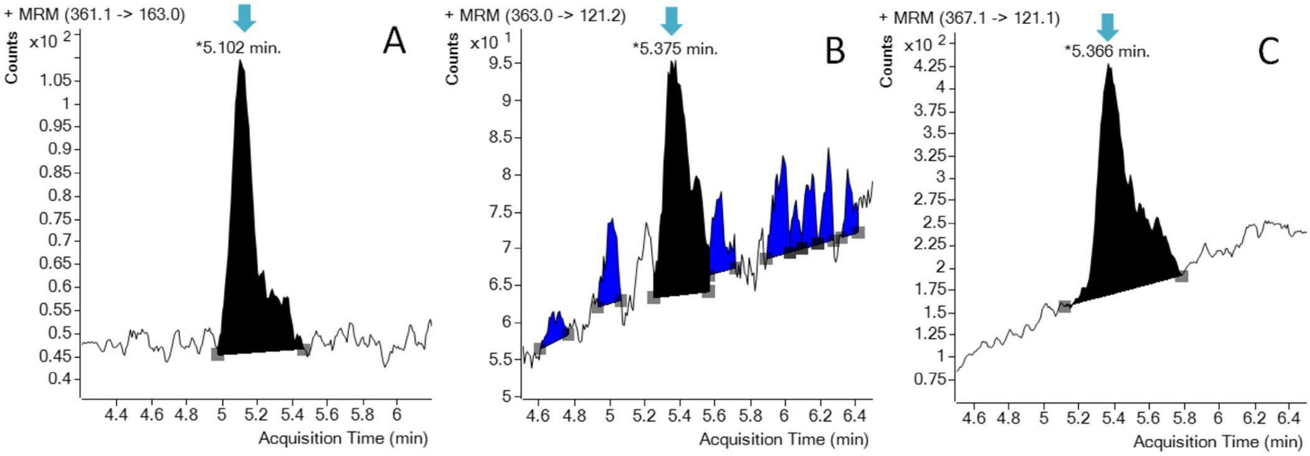

Fig 1. An example of UHPLC-ESI-MS/MS data obtained during the analysis of squirrel hair sample. Arrows indicate the position of signals of cortisone (A), cortisol (B) and cortisol-D4 used as isotopically labeled internal standard (C).

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Poland), mixed with 0.5 ml of Dulbecco's phosphate-buffered saline (DPBS, Thermo Fisher Scientific) without calcium and magnesium (pH = 7.0–7.3) in a centrifugal tube and shaken for 10 min using multivortex. Next, the suspension was centrifuged at room temperature (at 10000 rpm for 20 min) using a Heraeus Megafuge 11R centrifuge (Thermo Fisher Scientific, Germany). The obtained supernatants were immediately used for analysis using a Synergy 2 multi-mode microplate reader (BioTek Instruments, Inc. USA) equipped with an automated microplate strip washer (ELx50, BioTek Instruments, Inc. USA) and an ELMI DTS-4 digital thermostatic microplate shaker (Riga, Latvia). The concentration of cortisol in the samples was determined by comparing the optical density (OD) of the samples to the standard curve, the range of which was from 0.39 to 25 ng/ml. A separate calibration curve was made for each plate using eight different standard dilutions (0, 0.391, 0.781, 1.562, 3.125, 6.25, 12.5 and 25 ng/ml). Each dilution was duplicated. Intra- and inter-assay coefficients of variation were 10.6% and 12.8%, respectively. Samples with a concentration of cortisol above the upper limit of the curve were diluted and reassayed. We did not have any samples with a cortisol concentration below the lower range of the curve. The OD values of the samples were measured at 450 nm at room temperature. As in papers of Klich *et al.* [64,65], the concentrations of fecal cortisol were normalized per weight of feces and finally expressed as nanograms of cortisol per one gram of dry mass of feces (ng/g). We additionally used cortisone and corticosterone standards (Sigma-Aldrich, USA) at concentrations of 5 and 15 ng/ml to confirm the selectivity of the used ELISA kit (no reaction was observed).

Statistical analysis

To verify if the stress variables differentiate between the urban park and the urban forest squirrels, six generalized linear mixed models were run, of which three models with dependent variables related to long-term and medium-term stress: 1) hair cortisol concentration, 2) hair cortisone concentration, 3) fecal cortisol concentration, and three models with dependent variables indicated acute stress: 4) breath rate, 5) struggle rate, and 6) vocalization. There were six explanatory variables in each model, i.e.: SITE, SEASON, AGE, EXPERIENCE, REPRODUCTIVE STATUS, and CONDITION. SITE was a grouping variable of the two study sites: urban park (Łazienki) and urban forest (Natolin). SEASON was a grouping variable of four astronomical seasons: spring (1 March–31 May), summer (1 June–31 August), autumn (1

Table 1. Number of samples from squirrels first-trapped (FIRST) and re-trapped (RET) in a given study site.

Indicator	Urban forest		Urban park		Total
	FIRST	RET	FIRST	RET	
Hair cortisol concentration	11	20	22	40	93
Hair cortisone concentration	11	20	22	40	93
Fecal cortisol concentration	12	23	16	61	112
Breath rate	28	78	82	139	333
Struggle rate	28	86	82	140	336
Vocalization	28	85	89	133	335

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September–30 November), and winter (1 December–28 February). For hair hormone concentration winter was not included due to lack of hair samples from this period. AGE was a grouping variable of two age groups: adults and subadults. REPRODUCTIVE STATUS was a grouping variable dependent on sex, we distinguished two activity types for each sex: non-breeding males, non-breeding females, breeding males and breeding females. EXPERIENCE was a grouping variable of two squirrel groups: a) re-trapped–the squirrels which had been trapped before, and b) first-trapped–squirrels trapped for the first time. CONDITION was a covariate calculated based on residuals of log body mass and foot length (see: [14]). For Hair cortisol, Hair cortisone, Fecal cortisol concentrations and Struggle rate we used gamma distribution with log link function, for Breath rate and Vocalization we used negative binomial distribution with log link function. The IDs of squirrels were set as a random effect in the models to account for the repeated sampling of individual animals. Restricted maximum likelihood (REML) was used to estimate the parameters in the best model obtained. Model selection was based on the corrected Akaike information criterion (AICc) values in a multi-model selection procedure [66]. All possible model permutations were performed and, finally, the models were ranked according to their Akaike weights (ωi). The principle of model selection was lower AIC values. In total, a maximum of 336 red squirrel individuals were tested, however, due to limitations in the possibility of collecting material, the number of analyzed observations differed. For each model, the sample size was as follows: n = 93 for hair cortisol and cortisone concentrations, n = 112 for fecal cortisol concentration, n = 333 for breath rate, n = 336 for struggle rate, and n = 335 for vocalization. In general, twice as many individuals were examined in urban park as in forest and there were twice as many re-trapped squirrels as first-trapped (Table 1). All statistical analyses were performed with IBM SPSS v29.0 (Armonk, New York).

Results

Squirrels from urban forest presented lower levels of hair cortisol concentration, fecal cortisol concentration than squirrels front urban park. However, similar levels of hair cortisone concentration, struggle rate ans vocalization was observed. Slightly higher breath rate in forest squirrels was observed comparing to urban squirrels (Table 2).

Hair cortisol concentration

The highest ranked model contained AGE, SEASON, and CONDITION, but only season showed a statistically significant effect (Table 3). Squirrels presented higher hair cortisol concentration during spring, but lower during summer compared to the hair cortisol concentration in autumn (Table 3, Fig 2). CONDITION showed a nonsignificant, positive effect, squirrels with better condition presented higher levels of fecal cortisol concentration ($\beta = 1.418$, $p = 0.458$). AGE showed also nonsignificant effect, where adult squirrels presented

Table 2. Mean (±SE) levels of stress indicators observed in forest and urban squirrels (detailed data is presented in S1 Table).

Indicator	Urban forest		Urban park	
	Mean	SE	Mean	SE
Hair cortisol concentration [ug/g]	0.033	0.005	0.047	0.005
Hair cortisone concentration [ug/g]	0.046	0.003	0.048	0.002
Fecal cortisol concentration [ng/g]	29.209	4.603	62.976	8.483
Breath rate [chest moves/20 sec.]	28.661	0.451	25.597	0.248
Struggle rate [body moving time/30 sec.]	7.178	0.481	7.956	0.400
Vocalization [rank: 1–4]	1.619	0.086	1.820	0.076

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higher hair cortisol concentration (β = 0.505, p = 0.216). Other variables were excluded during the model selection, but within the ΔAICc = 2 there were five models, which also included SITE and EXPERIENCE. None of these models included REPRODUCTIVE STATUS (S2 Table).

Hair cortisone concentration

Only SEASON showed a statistically significant effect on hair cortisone concentration in squirrels, although also CONDITION was included in the model (Table 4). Squirrels in spring and summer presented lower hair cortisone concentrations than in autumn (Table 4, Fig 3). CONDITION showed nonsignificant, negative effect on hair cortisone concentration (β = -0.482, p = 0.367) (Table 4). Other variables were excluded during the model selection, but within the ΔAICc = 2 there were three models, which also included AGE (S2 Table).

Fecal cortisol concentration

The highest ranked model contained only SEASON and CONDITION. Nevertheless, within ΔAICc = 2 there were eight models which included also SITE, AGE, and EXPERIENCE. The highest ranked model presented much lower AICc values than the null model (ΔAICc = 18.6) (S2 Table). Cortisol presented significantly lower values in spring and summer compared to winter (Table 5, Fig 4). CONDITION showed nonsignificant, positive effect on fecal cortisol concentration (β = 0.583, p = 0.737) (Table 5).

Breath rate

For the breath rate the highest ranked model included SITE, EXPERIENCE, and CONDITION. The remaining predictors were excluded during model selection. The highest ranked

Table 3. Effect of AGE, SEASON, and CONDITION on hair cortisol concentration in squirrels in the highest ranked generalized linear mixed model (0*–reference category), REPRODUCTIVE STATUS, SITE, and EXPERIENCE were excluded during model selection (marginal R^2 = 0.144 and conditional R^2 = 0.690 for the highest ranked model).

Source	Beta	SE	t	p	Lower CI	Upper CI
Intercept	-3.454	0.8530	-5.323	<0.001	-6.236	-2.845
AGE (adult)	0.505	0.4051	1.246	0.216	-0.300	1.310
AGE (sub-adult)	0*					
SEASON (summer)	-0.489	0.2370	-2.064	0.042	-0.960	-0.018
SEASON (spring)	0.520	0.1875	2.775	0.007	0.148	0.893
SEASON (autumn)	0*					
CONDITION	1.418	1.9009	0.746	0.458	-2.360	5.195

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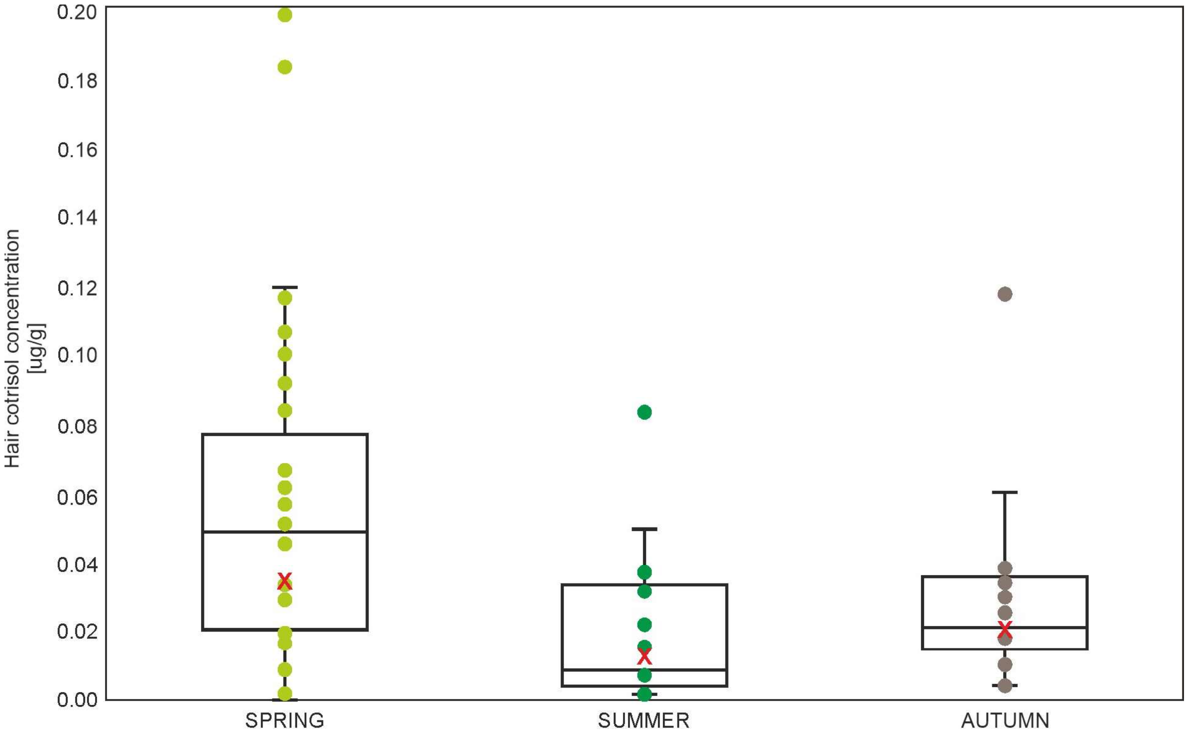

Fig 2. Boxplot with individual data points and mean (red X) hair cortisol concentration in squirrels with regard to SEASON (marginal means from generalized linear mixed model, S3 Table).

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model's AICc was lower only by 1.3 from the next ranked model with SITE and EXPERIENCE, but also 22.2 from the null model (S4 Table). Squirrels from the urban park (Łazienki) presented a lower breath rate ($\beta = -0.112$, $p < 0.001$) than squirrels from the urban forest site (Natolin) (Table 6, Fig 5A). A higher breath rate ($\beta = 0.067$, $p = 0.003$) was also observed in first-trapped squirrels in comparison to re-trapped squirrels (Table 6, Fig 5B). The CONDITION effect is also noticeable, although not statistically significant ($\beta = 0.334$, $p = 0.116$). The squirrels showed a higher breath rate as their body condition level increased (Table 6).

Struggle rate

A less pronounced effect of analyzed variables was found in the case of the struggle rate. The highest ranked model included only SEASON and CONDITION, but within the ΔAICc = 2 there was also a model which included SEASON, CONDITION, and EXPERIENCE

Table 4. Effect of SEASON and CONDITION on hair cortisone concentration in squirrels in the highest ranked generalized linear mixed model (0*–reference category), REPRODUCTIVE STATUS, AGE, SITE, and EXPERIENCE were excluded during model selection (marginal $R^2 = 0.074$ and conditional $R^2 = 0.480$ for the highest ranked model).

Source	Beta	SE	t	p	Lower CI	Upper CI
Intercept	-2.742	0.3022	-9.075	<0.001	-3.343	-2.142
SEASON (summer)	-0.331	0.0997	-3.321	0.001	-0.529	-0.133
SEASON (spring)	-0.214	0.0807	-2.650	0.010	-0.374	0.053
SEASON (autumn)	0*					
CONDITION	-0.482	0.5315	-0.907	0.367	-1.538	0.574

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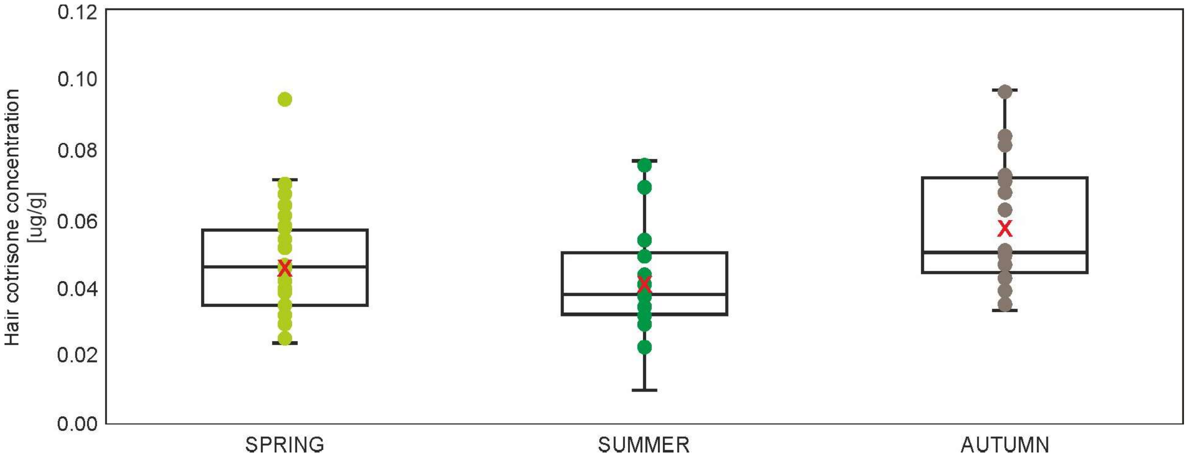

Fig 3. Boxplot with individual data points and mean (red X) hair cortisone concentration in squirrels with regard to SEASON (marginal means from generalized linear mixed model, S3 Table).

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(S4 Table). Also, ΔAICc with null model was only 6 (S4 Table). The model effects indicated a significantly lower β coefficient for the struggle rate in squirrels in spring, summer, and autumn than in winter (Table 7, Fig 6). Similar to breath rate, the CONDITION effect was included in the model, and also not statistically significant ($\beta = 667$, $p = 0.373$). The squirrels showed a higher struggle rate as their body condition level increased (Table 7).

Vocalization

Among the analyzed variables only EXPERIENCE and CONDITION had a weak effect on vocalization. Within ΔAICc = 2 there were five models, which, apart from CONDITION and EXPERIENCE, included also SITE and AGE (S4 Table). The highest ranked model had AICc lower by only 2.6 from the null model (S4 Table). Interestingly, the squirrel with no experience (i.e. first-trapped) presented lower vocalization than squirrels with the experience of being previously trapped ($\beta = -0.201$, $p = 0.031$) (Table 8, Fig 7). CONDITION also had a nonsignificant effect on the vocalization, but the opposite trend compared to previous models; i.e. squirrels with better condition presented a lower value of vocalization ($\beta = -1.344$, $p = 0.069$) (Table 8).

Discussion

In this study, we focused on the response to stress of two red squirrel populations: a) to environmental stress (revealed by the level of stress hormones in hair and/or feces) and b) a reaction to acute human-induced stress (measured as a reaction to handling at a trapping event).

Table 5. Effect of SEASON and CONDITION on fecal cortisol concentration in squirrels in the highest ranked generalized linear mixed model (0*–reference category), REPRODUCTIVE STATUS, AGE, SITE, and EXPERIENCE were excluded during model selection (marginal $R^2 = 0.081$ and conditional $R^2 = 0.653$ for the highest ranked model).

Source	Beta	SE	t	p	Lower CI	Upper CI
Intercept	3.906	1.0048	3.888	<0.001	1.914	5.898
SEASON (autumn)	-0.586	0.3135	-1.870	0.064	-1.208	0.035
SEASON (summer)	-1.266	0.2810	-4.504	<0.001	-1.823	-0.709
SEASON (spring)	-0.663	0.2191	-3.028	0.003	-1.098	-0.229
SEASON (winter)	0*					
CONDITION	0.583	1.7274	0.337	0.737	-2.842	4.007

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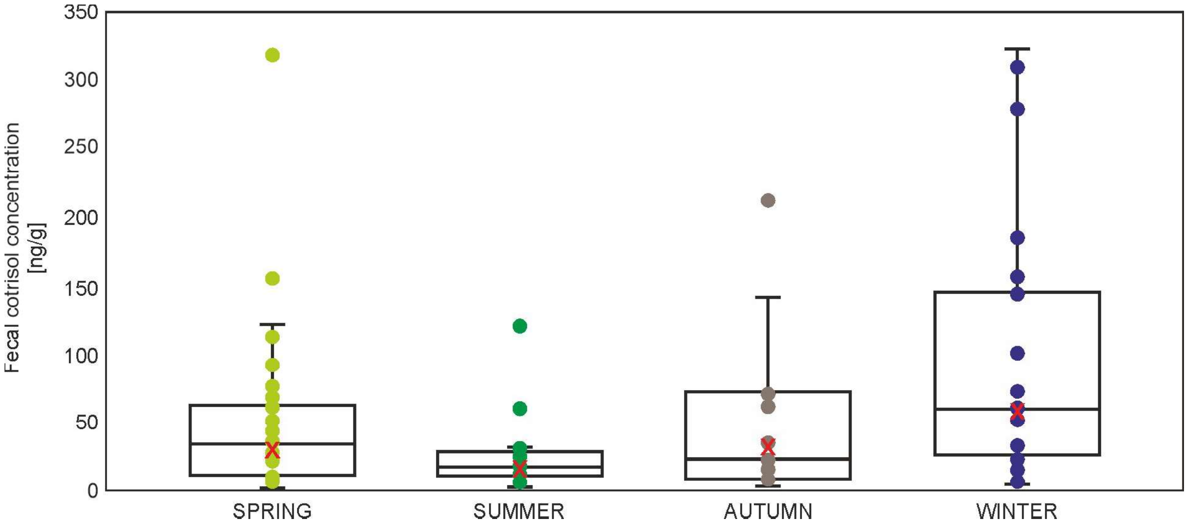

Fig 4. Boxplot with individual data points and mean (red X) fecal cortisol concentration in squirrels with regard to SEASON (marginal means from generalized linear mixed model, S3 Table).

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Contrary to our expectations, no pronounced site effect was found in long-term and medium-term stress. Herein, the only differences in cortisol and cortisone concentrations clearly depended on the season. This was confirmed by all three models for the concentrations of hormones (in hair and feces) (Tables 3–5, Figs 2–4). The squirrels in our study had higher levels of cortisol and cortisone concentrations in winter and autumn, which may be related to higher predation risk during foraging on the ground (higher visibility because of lack of leaves), reduced food quality, or more extreme weather conditions (snow and cold) [38].

Stress hormones in hair and feces

The stress response of animals to various external factors (e.g., predation pressure) is a typical challenge for wild animals and results in a variety of adaptive responses [67]. However, urbanization poses a novel and previously unknown challenge for animals, and adaptation processes are likely occurring right before our eyes. Although it is generally assumed that urban populations should have higher levels of cortisol metabolite due to chronic stress [8], previous studies showed diverse results, i.e. higher levels of cortisol metabolites [68,69], lower levels [39,70,71] or no differences [42,72]. Variations among studies might be related to complex responses of neuroendocrine system to chronic stress or species/population/individual-dependent differences in the perception of stressors [8]. Our results are similar to a recent study conducted in

Table 6. Effect of EXPERIENCE, SITE, and CONDITION on breath rate of squirrels in the highest ranked generalized linear mixed model (0*–reference category), REPRODUCTIVE STATUS, AGE, and SEASON were excluded during model selection (marginal R^2 = 0.076 and conditional R^2 = 0.274 for the highest ranked model).

Source	β	SE	t	p	Lower CI	Upper CI
Intercept	3.230	0.1251	25.806	0.000	2.983	3.476
EXPERIENCE (first-trapped)	0.067	0.0228	2.946	0.003	0.022	0.112
EXPERIENCE (re-trapped)	0*					
SITE (urban park)	-0.112	0.0230	-4.850	<0.001	-0.157	-0.066
SITE (urban forest)	0*					
CONDITION	0.334	0.2122	1.577	0.116	-0.083	0.752

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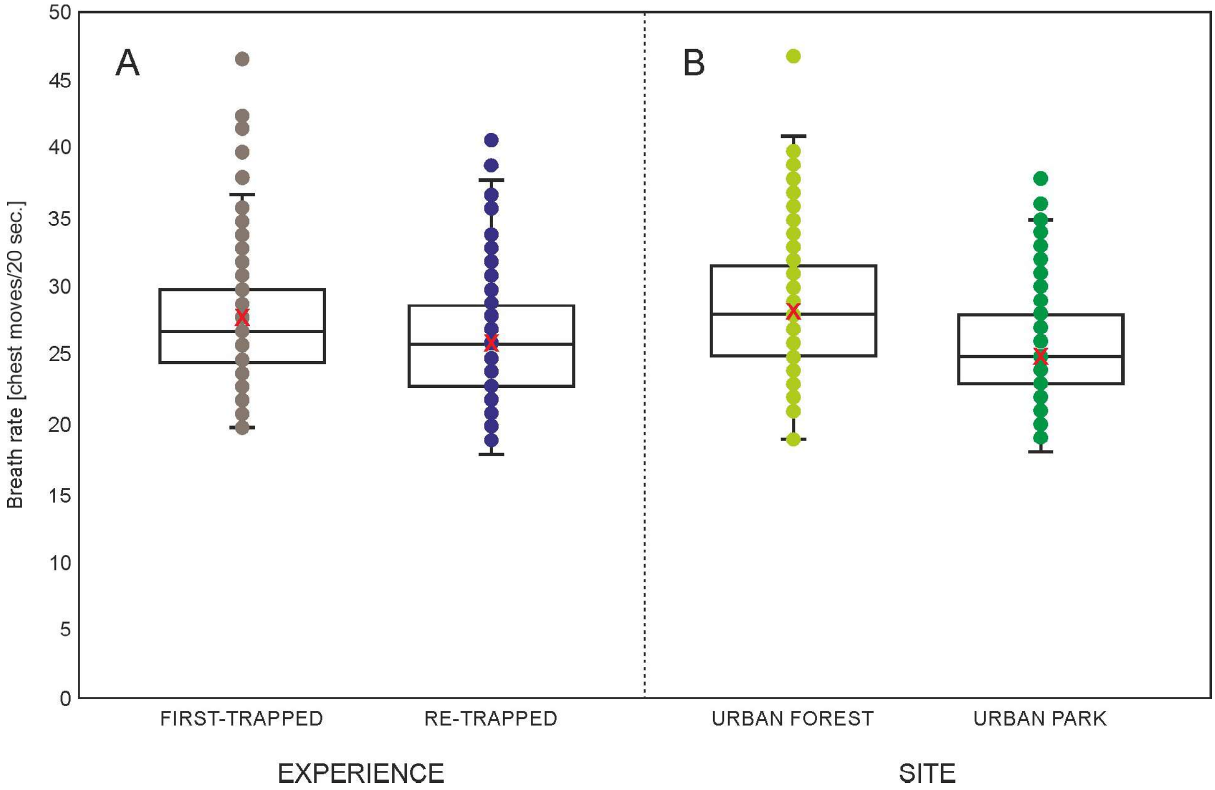

Fig 5. Boxplot with individual data points and mean (red X) breath rate in squirrels with regard to A) EXPERIENCE (first-trapped or retrapped) and B) SITE (urban park or urban forest) (marginal means from generalized linear mixed model, S3 Table).

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Japan, which compared rural and urban populations of the Eurasian red squirrels [42]. Also, Martin and Réale [73] found that human frequentation did not affect cortisol levels in chipmunks, and suggested that human presence was not the main factor responsible for the stress reaction. If animals are exposed to a repeated non-lethal stressor, their stress response may decrease [74]. Indeed, in urban striped field mice *Apodemus agrarius* lowered concentrations of cortisol were observed, which suggested hormonal adjustment to urban conditions [71].

On one hand, urban habitats may be challenging to animals due to various disturbances, but on the other hand, they often offer greater availability of anthropogenic food and/or artificial feeding. According to some studies, cortisol concentration was shown to influence body

Table 7. Effect of SEASON and CONDITION on struggle rate of squirrels in the highest ranked generalized linear mixed model (0*–reference category), REPRODUCTIVE STATUS, AGE, SITE, and EXPERIENCE were excluded during model selection (marginal R^2 = 0.037 and conditional R^2 = 0.494 for the highest ranked model).

Source	β	SE	t	p	Lower CI	Upper CI
Intercept	2.217	0.6443	3.441	<0.001	0.949	3.484
SEASON (autumn)	-0.375	0.1106	-3.388	<0.001	-0.592	-0.157
SEASON (summer)	-0.485	0.1258	-3.858	<0.001	-0.733	-0.238
SEASON (spring)	-0.339	0.1018	-3.329	<0.001	-0.539	-0.139
SEASON (winter)	0*					
CONDITION	0.667	0.7483	0.892	0.373	-0.805	2.139

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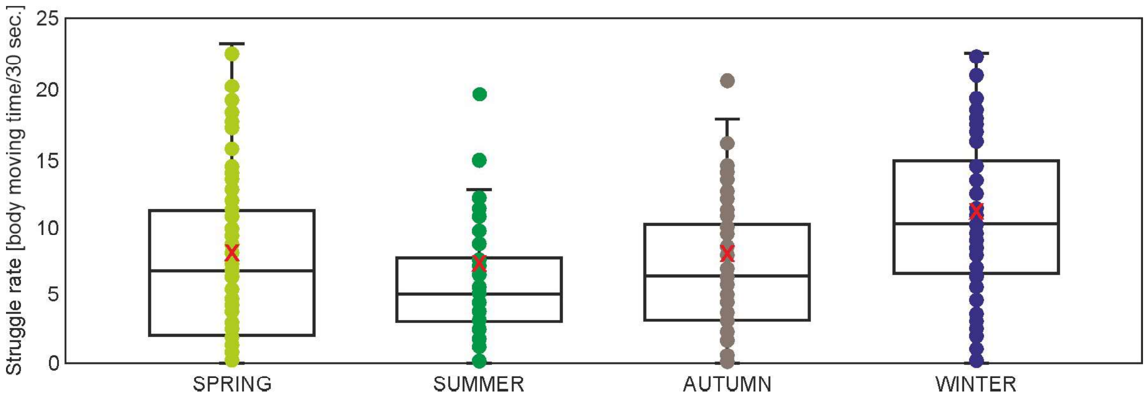

Fig 6. Boxplot with individual data points and mean (red X) struggle rate in squirrels with regard to SEASON (marginal means from generalized linear mixed model, S3 Table).

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condition [37,44] (or body mass [38,40,42]). Some others showed that higher body mass and better body condition may reduce glucocorticoid levels [75,76]. In our case, some effect on stress hormones can be also expected in relation to body condition, because in all three of the highest ranked models this explanatory variable was present (but nonsignificant). Interestingly, in our study, both hair and fecal cortisol concentrations increased with body condition. Squirrels' body mass may be positively correlated with boldness and aggressiveness [77], which may explain this link between body mass and cortisol concentration.

Food abundance relates to physiological stress [19,78]. Supplementary food was already shown to make red squirrels shift their home ranges closer to supplementary food sources [23] and change their activity to adjust to human presence [16]. In our case, both study sites were deciduous and offered plentiful natural food sources but only park squirrels received anthropogenic food [14,20,22]. Yet, a much higher population density in the park might have resulted in higher intra-specific competition [14], which would wear off the positive effect of supplementary feeding in the park.

There was no influence of study site, sex, or reproductive status on stress hormones in all three of the highest ranked models, although these variables were incidentally present in some models within ΔAICc = 2, which indicates the possible effect of these variables on hormone levels. The not significant effect of sex reported in our case was in line with many studies [38,40,41,79,80]. However, the lack of a clear effect of reproductive status may be a result of masking the medium-term and long-term effect by individual variability and a clear influence of the season. It should be noted that during not only spring and summer, but also winter months squirrels are already engaged in mating chases and females may start lactating [81,82]. Indeed, sexually active squirrels were recorded in winter in our study, but also during spring and summer [14]. In previous studies the effects of the reproductive status of squirrels on the

Table 8. Effect of EXPERIENCE and CONDITION on vocalization of squirrels in the highest ranked generalized linear mixed model (0*–reference category), REPRODUCTIVE STATUS, AGE, SITE, and SEASON were excluded during model selection (marginal $R^2 = 0.013$ and conditional $R^2 = 0.185$ for the highest ranked model).

Source	β	SE	t	p	Lower CI	Upper CI
Intercept	1.030	0.4390	2.347	0.019	0.167	1.894
EXPERIENCE (first-trapped)	-0.201	0.0929	-2.163	0.031	-0.384	-0.018
EXPERIENCE (re-trapped)	0*					
CONDITION	-1.344	0.7375	-1.822	0.069	-2.795	0.107

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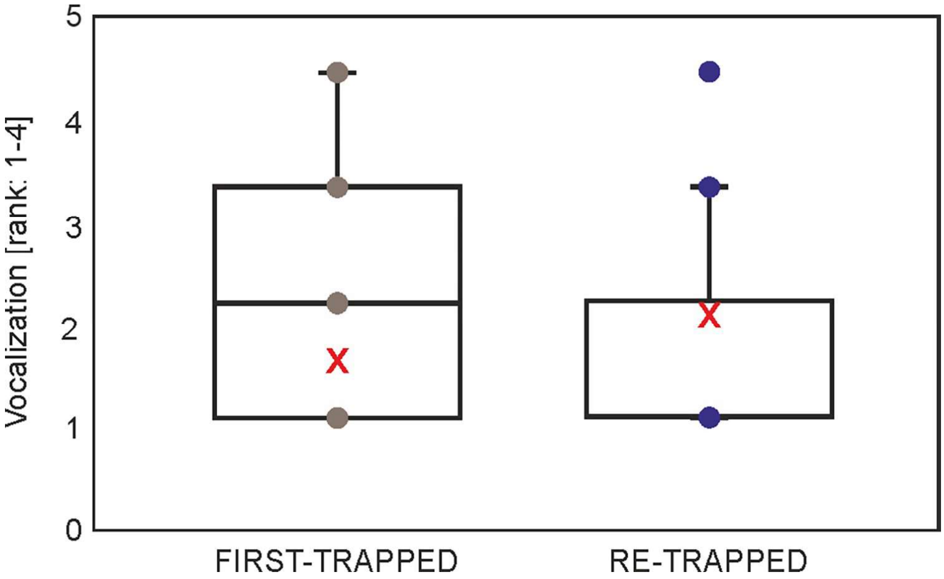

Fig 7. Boxplot with individual data points and mean (red X) vocalization in squirrels with regard to EXPERIENCE (first-trapped or retrapped) (marginal means from generalized linear mixed model, S3 Table).

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level of glucocorticoids levels were inconsistent. No effect as found in our case was in line with Santicchia *et al.* [40] but stands in contrast to other [38].

Breath rate, struggle rate, and vocalization

We used three simple measures (breath rate, struggle rate, and vocalization) to compare squirrel reaction to acute human-induced stress between the study areas. As assumed, forest squirrels had significantly higher breath rates (suggesting they had stronger response to handling) than squirrels in a park. Urban squirrels are shown to adjust to human presence by altering their behavior, i.e. shortening the flight distance [15]. They become more tolerant to contact with humans, by staying on the ground and approaching people, which benefits squirrels with supplementary feed [20,22]. Also, in fox squirrels responses to human cues were reduced in urban as compared to less urban environments [83]. Increased respiration rate is one of the physiological components of stress, which shift metabolism toward energy mobilisation and away from energy conservation [84]. The breath rates were found to be indicators of acute stress in great tits [46]. We showed that first-trapped individuals were more stressed that the re-trapped ones, which points to the habituation (to a certain degree) of the stressor regardless of the study site.

Struggle rate can be interpreted as a part of behavioral stress response of an individual, but it is unknown if the stress response of an individual to handling (i.e. its magnitude) is correlated with the response to natural stress [85]. Nevertheless, the mean struggle rate of red squirrels was already shown to be higher in low density than in high density areas [47], while American red squirrels which struggled the most, also tended to be the most aggressive [45]. In our study, there was no difference in the struggle rate between forest and urban squirrels, this index was mostly affected by season (i.e. the struggle rate was highest in winter) but body condition (with a positive effect) was also included in the model. It is worth noting that the struggle rate is generally in line with the results of stress hormones, in which the seasonal effect is also pronounced. Glucocorticoids are hypothesized to serve as a major mechanism to cope with inclement weather [86]. It is thus possible that the seasonally elevated levels related to the struggle rate during handling, i.e. more pronounced activity of individuals in the face of imminent threat. It is worth noting, that a high inter-individual difference ocurred, indicated by relatively high conditional comparying to marginal R^2 (Table 7). This may indicate different coping strategies in squirrels that mask the effects of the study site.

Alarm calls are vocalizations that alert other animals to impending danger [87]. These calls serve to warn conspecifics and signal the predator that its presence has been detected thus reducing chances of being preyed upon [87]. Here we measured the vocalization of squirrels as a response to handling (i.e. human-induced stress). Reactions of squirrels to some disturbance vary and range from ´freezing´ (remaining still) to alarm calling [18]. In our case, first-trapped squirrels vocalized less than during the subsequent trappings. Assumingly, during the first, and more stressful trapping, squirrels used 'freezeing' and/or little vocalization, and during next trapping they used alarm calls to warn conspecifics. Nevertheless, the explained variance of fixed and random effects was the lowest among all indicators (Table 8). It is still questioned if alarm calls are a form of altruism or nepotism in relation to relatives [87]. Here we assumed that during handling squirrels produced alarm calls, because these can serve typically as a warning call [53]. However, a call-type may also have more functions [51], e.g. being a mixed alarm and discomfort call.

Conclusion

To sum up, in our study, the two squirrel populations affected by different level of human disturbance differed only in terms of their breath rate. This suggests, that squirrels in these two populations did not differ in medium-term and long-term stress in general, but they can differ in acute response to handling. No differences in the medium-term and long-term stress (i.e. cortisol and cortisone concentrations) between study sites suggests that both populations are similarly affected by environmental factors (e.g. season or weather conditions). The lack of clear effects, in this case, may also suggest that the populations are heterogeneous, which was shown in the body condition of individuals and breeding activity, specifically in the urban park population [14]. Indeed, it is thought that no single specific behavioral phenotype is favored by selection, because unpredictable and changing environmental conditions will favor different phenotypes and heterogeneous populations allow for adjustment to variable selective forces [58]. The varied response to human-induced stressors (e.g., handling) was likely due to the limited contact of forest squirrels with humans, whereas urban park squirrels have adapted to this ongoing anthropogenic stressor. This also indicates that breath rate was the best indicator of the acute stress response to handling [46]. This was confirmed by the higher breath rate in first-trapped squirrels. The indicators of acute stress, such as struggle rate and vocalization, appear to be less straightforward, as they are likely influenced by other factors that might not always be easy to interpret. For instance, it seems that the struggle rate was affected by environmental conditions (seasonal effects), and/or elevated glucocorticoids which have also been shown to influence behaviors [88]. Additionally, vocalization was probably intensified by the initial interaction with a human (handling), but its elevated level was only evident during the subsequent encounter. The summary above clearly demonstrates that in order to accurately assess the effects of anthropogenic stressors, it is advisable to employ a broader range of indicators and diverse analytical methods, including behavioral analyses. This approach enables a comprehensive and holistic examination of the matter at hand, as well as the identification of indicators suitable for future studies on the impact of urbanization on wildlife.

Supporting information

S1 Table. Average (±SD) values of indicators calculated from raw data for given groups with regard to SITE.
(DOCX)

S2 Table. Ranking of the models (ten highest ranked models and null model) explaining the long-term and medium-term stress in squirrels in generalized linear mixed models with gamma distribution and log link function (ΔAICc—AICc differences, ωi—Akaike weights, Rank—rank of the models based on AICc values; bolded text in the row indicates chosen model (for variable explanation, see: Methods).
(DOCX)

S3 Table. Indicator values (marginal averages), which are presented in Figs 2–7.
(DOCX)

S4 Table. Ranking of the models (ten highest ranked models and null model) explaining the acute stress in squirrels in generalized linear mixed models with gamma or negative binomial distribution and log link function (ΔAICc—AICc differences, ωi—Akaike weights, Rank—rank of the models based on AICc values; bolded text in the row indicates chosen model (for variable explanation, see: Methods).
(DOCX)

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Table S11. Average (±SD) values of indicators calculated from raw data for given groups with regard to SITE.

	URBAN FOREST		*URBAN PARK*	
	Average	*Standard Deviation*	*Average*	*Standard Deviation*
Hair cortisol concentration [ug/g]				
SEASON				
SPRING	0.039	0.037	0.063	0.045
SUMMER	0.028	0.026	0.017	0.024
AUTUMN	0.027	0.011	0.030	0.028
EXPERIENCE				
FIRST-TRAPPED	0.032	0.034	0.039	0.040
RETRAPPED	0.033	0.026	0.051	0.044
REPRODUCTIVE STATUS				
FEMALE BREEDING	0.050	0.048	0.051	0.031
FEMALE NONBREEDING	0.024	0.019	0.035	0.033
MALE BREEDING	0.038	0.034	0.079	0.062
MALE NONBREEDING	0.029	0.022	0.034	0.030
Hair cortisone concentration [ug/g]				
SEASON				
SPRING	0.039	0.010	0.048	0.015
SUMMER	0.043	0.017	0.039	0.017
AUTUMN	0.058	0.013	0.055	0.022
EXPERIENCE				
FIRST-TRAPPED	0.047	0.019	0.052	0.019
RETRAPPED	0.045	0.013	0.046	0.017
REPRODUCTIVE STATUS				
FEMALE BREEDING	0.040	0.011	0.046	0.013
FEMALE NONBREEDING	0.053	0.018	0.055	0.021
MALE BREEDING	0.038	0.011	0.053	0.017
MALE NONBREEDING	0.048	0.015	0.040	0.013
Fecal cortisol concentration [ng/g]				
SEASON				
SPRING	37.60	41.19	84.09	80.32
SUMMER	20.46	14.79	16.22	12.18
AUTUMN	32.10	27.19	51.39	59.54
WINTER			90.33	93.16
EXPERIENCE				
FIRST-TRAPPED	17.38	18.01	93.10	103.45
RETRAPPED	35.38	29.46	55.07	63.55
REPRODUCTIVE STATUS				
FEMALE BREEDING	39.84	23.39	69.99	74.84
FEMALE NONBREEDING	26.11	40.14	81.41	89.29
MALE BREEDING	31.93	28.92	57.42	69.38
MALE NONBREEDING	25.24	16.89	36.81	51.81

Breath rate [chest moves/20 sec.]				
SEASON				
SPRING	29.621	5.031	26.135	3.742
SUMMER	26.400	3.069	27.636	4.756
AUTUMN	29.674	4.888	25.596	3.527
WINTER	28.300	4.547	24.226	2.880
EXPERIENCE				
FIRST-TRAPPED	30.786	5.094	26.354	3.873
RETRAPPED	27.952	4.290	25.151	3.503
REPRODUCTIVE STATUS				
FEMALE BREEDING	27.375	3.777	26.027	3.069
FEMALE NONBREEDING	28.838	4.330	25.808	3.879
MALE BREEDING	28.867	3.662	24.513	3.634
MALE NONBREEDING	28.673	5.276	25.750	3.756
Struggle rate [body moving time/30 sec.]				
SEASON				
SPRING	6.110	4.814	7.519	5.159
SUMMER	5.216	3.443	6.785	4.151
AUTUMN	8.272	5.698	7.073	6.217
WINTER	11.46	4.487	10.45	6.334
EXPERIENCE				
FIRST-TRAPPED	8.960	4.112	8.573	5.701
RETRAPPED	6.598	5.319	7.595	6.097
REPRODUCTIVE STATUS				
FEMALE BREEDING	7.598	3.437	7.544	5.851
FEMALE NONBREEDING	7.593	5.245	7.086	5.415
MALE BREEDING	8.941	4.425	9.727	7.288
MALE NONBREEDING	6.365	5.390	8.047	5.626
Vocalization [rank: 1-4]				
SEASON				
SPRING	1.667	0.959	1.593	1.002
SUMMER	1.806	1.046	2.043	1.186
AUTUMN	1.548	0.803	1.936	1.171
WINTER	1.200	0.632	1.761	1.139
EXPERIENCE				
FIRST-TRAPPED	1.500	0.793	1.573	0.999
RETRAPPED	1.659	0.946	1.985	1.181
REPRODUCTIVE STATUS				
FEMALE BREEDING	1.500	0.756	2.139	1.125
FEMALE NONBREEDING	1.757	1.038	1.867	1.256
MALE BREEDING	2.077	1.038	1.765	1.046
MALE NONBREEDING	1.436	0.764	1.649	1.010

Table S1. Ranking of the models (ten highest ranked models and null model) explaining the long-term and medium-term stress in squirrels in generalized linear mixed models with gamma distribution and log link function (ΔAICc - AICc differences, ω_i - Akaike weights, Rank - rank of the models based on AICc values; bolded text in the row indicates chosen model (for variable explanation, see: methods).

Models	*ΔAICc*	ω_i	*Rank*
Hair cortisol concentration			
SEASON + CONDITION + AGE	**0.0**	**0.155**	**1**
SITE + SEASON + CONDITION + AGE	0.5	0.120	2
SEASON + CONDITION + AGE + EXPERIENCE	1.1	0.089	3
SITE + SEASON + CONDITION + AGE + EXPERIENCE	1.4	0.077	4
SEASON + CONDITION	2.0	0.057	5
SITE + CONDITION + AGE + REPRODUCTIVE ST.	2.3	0.049	6
SEASON + CONDITION + AGE + REPRODUCTIVE ST.	2.4	0.047	7
SEASON + AGE	3.3	0.030	8
SITE + CONDITION + AGE + EXPERIENCE + REPRODUCTIVE ST.	3.3	0.030	9
SEASON + CONDITION + EXPERIENCE	3.4	0.030	10
…			
null model	20.5	0.000	64
Hair cortisone concentration			
SEASON + CONDITION	**0.0**	**0.214**	**1**
SEASON	1.0	0.130	2
SEASON + CONDITION + AGE	1.9	0.083	3
SITE + SEASON + CONDITION	2.6	0.058	4
SEASON + CONDITION + EXPERIENCE	2.7	0.055	5
SITE + SEASON	3.4	0.039	6
SEASON + EXPERIENCE	3.6	0.035	7
SEASON + AGE	3.7	0.034	8
CONDITION	4.0	0.029	9
SEASON + CONDITION + EXPERIENCE + AGE	4.4	0.030	10
…			
null model	5.1	0.017	14
Faecal cortisol concentration			
SEASON + CONDITION	**0.0**	**0.119**	**1**
SITE + SEASON + CONDITION	0.1	0.114	2
SEASON + CONDITION + AGE	0.3	0.103	3
SITE + SEASON + CONDITION + AGE	0.5	0.093	4
SEASON + CONDITION + EXPERIENCE	1.2	0.066	5
SITE + SEASON + CONDITION + EXPERIENCE	1.3	0.062	6
SEASON + CONDITION + EXPERIENCE + AGE	1.5	0.056	7
SITE + SEASON + CONDITION + EXPERIENCE + AGE	1.6	0.054	8
SEASON + CONDITION + REPRODUCTIVE ST.	2.8	0.029	9
SEASON	3.0	0.027	10
…			
null model	18.6	0.000	57

Table S3. Indicator values (marginal averages), which are presented in figures 2-7.

Figure 2 - Mean (±SE) hair cortisol concentration in squirrels with regard to SEASON (marginal means from generalized linear mixed model) [ug/g]

SEASON	*Marginal mean*	*Standard Error*
SPRING	0.03	0.03
SUMMER	0.01	0.01
AUTUMN	0.02	0.02

Figure 3 - Mean (±SE) hair cortisone concentration in squirrels with regard to SEASON (marginal means from generalized linear mixed model) [ug/g]

SEASON	*Marginal mean*	*Standard Error*
SPRING	0.046	0.012
SUMMER	0.041	0.012
AUTUMN	0.057	0.012

Figure 4 - Mean (±SE) fecal cortisol concentration in squirrels with regard to SEASON (marginal means from generalized linear mixed model) [ng/g]

SEASON	*Marginal mean*	*Standard Error*
SPRING	30.4	26.4
SUMMER	16.6	14.7
AUTUMN	32.8	29.4
WINTER	58.6	51.5

Figure 5. Mean (±SE) breath rate in squirrels with regard to A) EXPERIENCE (first-trapped or retrapped) and B) SITE (urban park or urban forest) (marginal means from generalized linear mixed model). [chest moves/20 sec.]

EXPERIENCE	*Marginal mean*	*Standard Error*
FIRST-TRAPPED	28.3	2.9
RE-TRAPPED	26.5	2.7

SITE	*Marginal mean*	*Standard Error*
URBAN PARK	25.9	2.7
URBAN FOREST	28.9	3.0

Figure 6. Mean (±SE) struggle rate in squirrels with regard to SEASON (marginal means from generalized linear mixed model). [body moving time/30 sec.]

SEASON	*Marginal mean*	*Standard Error*
SPRING	8.0	4.8
SUMMER	6.9	4.2
AUTUMN	7.7	4.7
WINTER	11.2	6.8

Figure 7. Mean (±SE) vocalization in squirrels with regard to EXPERIENCE (first-trapped or retrapped) (marginal means from generalized linear mixed model). [rank: 1-4]

EXPERIENCE	*Marginal mean*	*Standard Error*
FIRST-TRAPPED	1.5	0.6
RE-TRAPPED	1.9	0.7

Table S3. Ranking of the models (ten highest ranked models and null model) explaining the acute stress in squirrels in generalized linear mixed models with gamma or negative binomial distribution and log link function (ΔAICc - AICc differences, ω_i - Akaike weights, Rank - rank of the models based on AICc values; bolded text in the row indicates chosen model (for variable explanation, see: methods).

Models	*ΔAICc*	*ω_i*	*Rank*
Breath rate			
SITE + CONDITION + EXPERIENCE	**0.0**	**0.465**	**1**
SITE + EXPERIENCE	1.3	0.243	2
SITE + CONDITION	3.4	0.085	3
SITE	3.9	0.066	4
SITE + CONDITION + AGE + EXPERIENCE	3.9	0.066	5
SITE + AGE + EXPERIENCE	4.6	0.047	6
SITE + CONDITION + AGE	7.4	0.011	7
SITE + AGE	7.6	0.010	8
SITE + SEASON + CONDITION + EXPERIENCE	10.3	0.003	9
SITE + SEASON + EXPERIENCE	11.3	0.002	10
…			
null model	22.2	0.000	28
Struggle rate			
SEASON + CONDITION	**0.0**	**0.257**	**1**
SEASON + CONDITION + EXPERIENCE	1.6	0.116	2
SEASON	2.7	0.067	3
SEASON + CONDITION + AGE	2.7	0.067	4
SITE + SEASON + CONDITION	2.8	0.063	5
CONDITION	3.6	0.043	6
SEASON + CONDITION + EXPERIENCE + AGE	4.1	0.033	7
SITE + SEASON + CONDITION + EXPERIENCE	4.4	0.028	8
SITE + CONDITION	4.5	0.027	9
SEASON + CONDITION + REPRODUCTIVE ST.	4.8	0.023	10
…			
null model	6.0	0.013	18
Vocalization			
CONDITION + EXPERIENCE	**0.0**	**0.172**	**1**
CONDITION	0.3	0.148	2
CONDITION + EXPERIENCE + AGE	1.2	0.094	3
CONDITION + AGE	1.2	0.094	4
SITE + CONDITION + EXPERIENCE	1.6	0.077	5
SITE + CONDITION	2.2	0.057	6
null model	2.6	0.047	7
SITE + CONDITION + EXPERIENCE + AGE	2.8	0.042	8
SITE + CONDITION + AGE	3.2	0.035	9
EXPERIENCE	3.2	0.035	10

Rozdział 4.

Jackowiak M., Krauze-Gryz D., Beliniak A., Jasińska K. D., Gryz J., Żyfka-Zagrodzińska E., Matracka A. The heavy burden of city life: factors affecting mercury bioaccumulation in urban red squirrels.

Manuskrypt w trakcie recenzji w Environmental Science and Pollution Research.

Title: The heavy burden of city life: factors affecting mercury bioaccumulation in urban red squirrels

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ABSTRACT

Mercury, together with cadmium and lead, is one of the most harmful metal contaminants, which can bioaccumulate and biomagnify. In Poland, the emission of mercury is one of the highest in Europe due to coal combustion-based power production. In this study, we focused on two populations of the red squirrel inhabiting Warsaw to assess total mercury (THg) concentrations in hair samples: the urban forest located in an outer district of Warsaw, and the urban park located in the city center. Squirrels were live-trapped, and their sex, age, and breeding status were assessed. Mercury determination was done in a laboratory by means of atomic absorption spectrometry. In general, the concentration of THg in hair samples from sub-adult individuals was lower than in adults. Yet, due to the low sample size of sub-adults, only adults were used in further analysis. The site of a sample collection, the sex of the squirrel, and their reproductive status affected the level of contamination (higher values were reported for the park and for breeding females). Body mass positively affected THg contamination in the urban park but not in the forest. The proportion of internally incorporated (ingested with food) vs. superficially adsorbed mercury was much higher in the urban park than in the urban forest. The bioaccumulation was clearly higher in the urban park than in the urban forest, which can be explained by both current and/or historical pollution but also by altered behavior of park animals as a response to the presence of humans (i.e., more time spent on the ground). We also showed that individual characteristics (i.e. sex, breeding, body mass) could affect the level of mercury intoxication. All this points to the importance of future studies on the variation in mercury concentration within mammal populations inhabiting different urban green areas.

Keywords: trace element, body mass, sex and breeding activity, age, hair samples, pollution

1. INTRODUCTION

Mercury, together with cadmium and lead, is one of the most harmful metal contaminants, which can bioaccumulate (e.g. Rimmer et al., 2010; Peterson et al., 2015) and biomagnify (e. g. Lavoie et al., 2013; Seco et al., 2021). For decades, environmental monitoring of mercury contamination has been one of the most important trends in environmental conservation. Mercury is emitted during various technological processes and the combustion of fossil fuels (Pirrone et al., 2010). In Poland, the emission of mercury is one of the highest in Europe (Zyśk et al., 2011), due to coal combustion-based power production (Głodek and Pacyna, 2009), with particular importance of brown coal combustion (Zyśk et al., 2011).

Mercury occurs in an environment as metallic, inorganic, and organic forms, the last being the most toxic. Mercury can be accumulated in various environmental reservoirs (e.g., Bull et al., 1977), including birds and mammals (Boening, 2000; Scheuhammer et al., 2007; Scheuhammer et al., 2015). Mercury, in its organic, bioavailable form, can be easily taken with food and effectively absorbed in the gastrointestinal tract (Wolfe et al., 1998). Mercury can also be inhaled as a vapor and, to some extent, absorbed through the skin (Wolfe et al., 1998). It readily transfers across the blood-brain barrier and damages the central nervous system (López-Berenguer et al., 2020), thus affecting behavior and cognition (Carpenter, 2001). It also interferes with enzymes, disturbing numerous biochemical pathways (Wolfe et al., 1998) and disturbing cardiovascular (Fernandes Azevedo et al., 2012) and immune systems (Das et al., 2008; Desforges et al., 2016). Mercury can cause lesions to the kidneys and liver (Sonne et al., 2007) and affects the reproductive system (Massányi et al., 2020). Finally, it also penetrates the placenta (Nehring et al., 2017), selectively concentrating in the fetus's brain, affecting the fetus's development (Castoldi et al., 2008); it is also excreted into milk and absorbed by offspring (Wagemann et al., 1988; Habran et al., 2011; Lyytikäinen et al., 2015).

The highest concentration of mercury was observed in the kidneys, liver, and muscles (Wren, 1986; Khabarova et al., 2018). However, organ sampling is invasive, thus its usability is limited. Measuring trace elements in the hairs of mammals is thought to be a simple, reliable, and minimally invasive way of assessing exposure to environmental pollution (i.e., Duffy et al., 2005; Dobrzański et al., 2014). Excreted trace elements are incorporated into hair (Wolfe et al., 1998; Grajewska et al., 2020), in which their concentration is proportionate (yet, generally higher) to the concentration in organs (Evans et al., 1998; Gerstenberger et al., 2006; Dainowski et al., 2015; Peterson et al., 2016a; Peterson et al., 2016b). Mercury load in hair samples corresponds well to environmental exposure to mercury (Eccles et al., 2020). Also, hair has considerable stability for long periods of time (Dietz et al., 2006; Dietz et al., 2009; Bocharova et al., 2013).

As one of the most important contaminants, mercury levels were tested in many mammalian species. The most important factor shaping interspecific, spatial, and temporal mercury concentration is diet variability (Bocharova et al., 2013; Hallanger et al., 2019; but see also Lippold et al., 2022). Typically, the highest mercury levels were recorded in marine, piscivorous mammals like seals (Sergeant and Armstrong, 1973; Skaare et al., 1994) and toothed whales (Dietz et al., 2013), but also other carnivores, including terrestrial apex predators like polar bear (*Ursus maritimus*) (Dietz et al., 2013; Lippold et al., 2022) or smaller arctic fox (*Vulpes lagopus*) (Bocharova et al., 2013; Treu et al., 2018), raccoon (*Procyon lotor*) or striped skunk (*Mephitis mephitis*) (Peterson et al., 2020). Most of the studies focused on mercury levels in the Arctic and marine biota, where its accumulation can be a serious ecological problem (Dietz et al., 2013; Krey et al., 2015; Dietz et al., 2021). High concentrations were noted in small insectivorous mammals

(Talmage and Walton, 1993; Sánchez-Chardi et al., 2007), while the lowest values were obtained for non-insectivorous, small mammals (Bull et al., 1977; Gerstenberger et al., 2006; Lurz et al., 2017) or ungulates (Frøslie et al., 1984; Gamberg et al., 2016). Mercury can be a potential threat to populations and ecosystems (Poissant et al., 2008; Dietz et al., 2013; Rea et al., 2013; Herring et al., 2018; Dietz et al., 2021; Dietz et al., 2022). Taking into account the increasing role of urban habitats for wildlife (Baker and Harris, 2007) and the potential higher exposure of wildlife to chemical contaminants in cities (Newman, 2006), the biocontamination of urban mammal populations should be monitored. Indeed, previous studies pointed to a high concentration of chemical contaminants in the tissues of urban mammals (Dip et al., 2001; Lurz et al., 2017).

Squirrels (Sciuridae) may be valuable biological sentinels for environmental pollution and good indicators of mercury presence in terrestrial habitats (Gerstenberger et al., 2006; Jenkins et al., 1980; Lurz et al., 2017). Although red squirrel (*Sciurus vulgaris*) has been classified as a least concern (LC) species, the observed global population trend is declining (Shar et al., 2016). Thus, any new threats should be identified (Lurz et al., 2017). Moreover, red squirrel is one of the species that adjusted well to urban habitat (Rézouki et al., 2014; Reher et al., 2016; Fey et al., 2016; Uchida et al., 2019; Kostrzewa and Krauze-Gryz, 2020; D. Krauze-Gryz et al., 2021a; b; Beliniak et al., 2022; Fingland et al., 2022). Its abundant presence in cities (Beliniak et al., 2022) and within areas of various anthropogenic pressure and habitat transformation (Babińska-Werka and Żółw, 2008; Krauze-Gryz et al., 2021a; Beliniak et al., 2022) make the red squirrel an ideal target species for studying mercury contamination in urban ecosystems.

In this study, we focused on two populations of red squirrels inhabiting Warsaw to assess total mercury (THg) concentrations in hair samples. The first was an urban forest located in an outer district of the city; the other was an urban park located in the center of Warsaw. Moreover, we compared the contribution of internally incorporated (intTHg) and externally deposited (extTHg) mercury in squirrel hair samples between both locations. As we found in our previous studies, the two populations differed (among others) in terms of body mass, reproductive activity, and food composition (Krauze-Gryz et al., 2021a; Beliniak et al., 2022), the factors that may potentially affect mercury accumulation (Mierle et al., 2000; Lodenius et al., 2014). We assumed that the Hg value in red squirrel hair would be higher in the urban park than in the urban forest, possibly due to heavy traffic and high particulate matter concentration in the city center as compared to an outer district (Majewski et al., 2011). We also predicted that higher Hg values would be recorded for females (Yates et al., 2005; McKinney et al., 2017) and in a breeding season (Lurz et al., 2017), as they are known to gnaw on bones or consume soil to obtain minerals (e.g., calcium) to satisfy the increased demand during lactation (e.g., Bosch and Lurz, 2012). As mercury bioaccumulates (e.g., Peterson et al., 2015), we may also assume that its levels will be higher in older animals (Mierle et al., 2000), and with a higher body mass (Lodenius et al., 2014).

2. MATERIAL AND METHODS

2.1. Study area

The study was conducted on two study sides: an urban forest (Natolin Reserve) and an urban park (Royal Łazienki Museum), both located in Warsaw (52°14'13.37" N, 21°1'3.11" E), the capital city of Poland. The city has approximately two million people, and it is located in the central part of the country. The main coal-fired heat plants are ‘Żerań’, ‘Siekierki’, and the smaller peaking heat plant ‘Kawęczyn’ (Fig. 1). These are characterized by relatively low emissions of mercury (as compared to others located in other parts of the country that use mainly brown coal; Zyśk et al., 2011). However, geochemical studies of soils in Warsaw parks (the urban park) showed soil enrichment in mercury (as well as other heavy metals; Dusza-Dobek, 2012).

The first study site, the urban forest, was located approximately 10 km from the city center. The reserve covers 105 ha, and it has been protected since 1991. It has been closed to the public and permission is needed for an entrance. The oldest stands are more than 250 years old, dead or fallen trees are left for natural decomposition, and only natural regeneration occurs, and in practice, very little human intervention is allowed. To the west of the reserve are built-up areas, whereas on the other side, it is surrounded by farmland. The sampling site was 4.5, 14.5, and 17 km from the three power plants (Fig. 1).

The second study site, the urban park, is located in the city center and covers 76 ha. This is one of the most popular attractions among local inhabitants and visitors. Busy streets and built-up areas surround it. The park has more than 90 species of trees and shrubs, both natural and foreign species, and tree stands can reach more than 150 years, which provides a natural food base for animals (Babińska-Werka and Żółw, 2008). Animals in this park, including red squirrels, are commonly fed by visitors (Kostrzewa and Krauze-Gryz, 2020; Krauze-Gryz et al., 2021a). Human-delivered nuts comprise the bulk of the diet of the red squirrels in this park (Krauze-Gryz et al., 2021a). The sampling site was located 4.5, 8.7, and 9 km from the main power plants (Fig. 1).

2.2. Sample collection

Squirrels were live-trapped with 40 traps in the urban forest and 30 traps in the urban park. The study lasted from July 2018 to December 2020 (Beliniak et al., 2022). In both areas, we trapped in the same month, in total, during thirteen trapping sessions (i.e., in 2018: Jul, Sept, Nov;

in 2019: Jan, Mar, May, Jul, Sept; in 2020: Mar, May, Jul, Oct, Dec). We used standard wire mesh live traps (51 × 15 × 15 cm) (manufactured by "Jerzyk" Jerzy Chilecki, Białowieża, Poland). We flushed every trapped squirrel into a wire mesh handling cone (Lurz et al., 2000) to minimize stress during handling. Each newly trapped squirrel was individually marked with numbered ear tags 2x8 mm (National Tag&Band, Newport, KY, USA). Squirrels were weighted to the nearest 10 g (Pesola spring balance). We also defined sex and reproductive status. Females were defined as nonbreeding (anoestrous, small vulva, no longitudinal opening) or breeding. The latter included postoestrous and pregnant (swollen vulva with longitudinal opening, enlarged belly during pregnancy) or lactating (large nipples, milk excretion could be stimulated). Males were recorded as nonbreeding (abdominal testes or semi-scrotal and scrotum small) or breeding (testes scrotal and scrotum large) (Santicchia et al., 2018). We also defined age class: sub-adult males had small scrotum and abdominal testes, females had a very small vulva, and the nipples were still invisible. Older animals were considered as adult (Wauters and Dhondt, 1993). The hair samples were collected from the coat using gloved fingers and stored in small, individually-labelled, clear plastic bags. Hair samples (5-15 mg) were taken once per trapping session, so samples collected from the same individual were at least two months apart.

Samples were collected throughout the year and in all seasons. They came from both sexes. Samples from sub-adults and adults were collected in the park. Only adult individuals were sampled in the urban forest. This was due to a lower trapping rate of sub-adults in the forest (Beliniak et al., 2022).

The year was divided into four seasons: spring (1st March–31st May), summer (1st June–31st August), autumn (1st September–30th November), and winter (1st December–28th February).

2.3. Laboratory analysis

THg determination

All of the collected hair samples were delivered to the laboratory, where mercury was determined using atomic absorption spectrometry. We used the device for direct mercury determination – AMA-254 (Altec, Czech Republic), dedicated for THg determination. There was no need to conduct sample preparation prior to analysis because of an automatic mineralization during the process. To determine THg, a sample with a known mass was applied to the nickel nacelle on a dispenser. The dispenser placed the sample in a combustion chamber, where the sample dried and was exposed to pyrolytic mineralization in an oxygen stream at 550°C. Mercury vapor, released during decomposition, moved through the catalytic column on the gold amalgamator. After being rapidly evaporated from the amalgamator, preconcentrated mercury was measured in two measuring cells by atomic absorption technique under 254 nm UV light. The whole procedure took about five minutes. The result was THg content in a sample (in ng) and THg concentration (in ppm, for further analysis recalculated to µg/kg of dry weight), calculated on the basis of an absorbance calibration curve. The limit of detection for AMA-254 was 0.01 ng.

Moreover, we evaluated how much mercury in a sample was an effect of atmospheric deposition (extTHg) and how much was incorporated into hair structure as a result of food intake (intTHg). Thus, for another 38 samples (urban forest – 16, urban park – 22), THg determination was replicated with an additional rinsing procedure. Before analysis, the replicated samples were rinsed a few times in 0.1 molar hydrochloric acid to remove unbound mercury from the hair surface. By comparing THg concentration between unrinsed and replicated, rinsed subsamples, a concentration of mercury adsorbed on hair (extTHg) for each sample was assessed. An average percentage share of intTHg and extTHg in samples for each location separately and for both locations jointly was estimated.

Quality assurance and control

To ensure high repeatability, for 30 samples THg determination was replicated. With a sufficient sample volume it was separated into two subsamples. On average, the accordance between THg determination in both subsamples was 84.62%; SE=0.03 (with no difference between an urban park and urban forest – 84.55%; SE=0.04 vs. 84.71%; SE=0.05, respectively). Variation of THg concentration in replicated samples fluctuated between 62.87 and 99.71%, with most of the results accordance over 90%.

To maintain a high quality of research and to check the correctness of mercury determination, European Reference Material ERM-DB001, with a human hair matrix dedicated to trace elements analysis, was used. Reference material was used once per each analytical series, but at least once per 10 samples. Reference material weights were about 10 mg, where mercury concentration was homogeneous, in accordance with the certificate of analysis, and what corresponds to typical sample mass (about 5-15 mg). Recovery of the reference material ranged from 91.42% to 99.34% (mean 95.56%; SE=0.01). To ensure the proper mercury determination result, mercury in a blank sample was determined after each hair sample. An acceptable threshold for mercury value in the blank sample was below one ppb.

2.4. Statistical analysis

We used linear mixed-effects models to find factors affecting THg values in red squirrel hair samples. As exploratory variables, we used the site of sample collection (urban forest, urban park), season (spring, summer, autumn, winter), sex (female, male), and interaction between sex and reproductive status (breeding, non-breeding). Next, we run two models, using the body mass of squirrels as an exploratory variable, separately for the urban forest and the urban park. As squirrels were individually marked and could be trapped (thus sampled) more than once, we used squirrel ID (individual_ID) as a random effect. As THg concentration data was not normally distributed (confirmed by the Shapiro-Wilk test), we used log-transformed (ln) data for the analysis. Akaike Information Criterion (AIC) was used to evaluate the fit of all of the models. Significant differences between intTHg and extTHg in hair samples were confirmed by using Student's paired t-test (significance level 0.05).

All analyses were performed using R (v.4.1.2, R Core Team 2021) and the 'lme4' package (Bates et al., 2015). The linear mixed-effect model was fitted using the 'lmer' function.

3. RESULTS

The mean THg concentration in squirrel hair was 100.65 µg/kg (SE=8.73). The concentration of THg in hair samples from sub-adult individuals differed from adults (ONE-WAY ANOVA, F =19.3, df = 1 and 187, $P < 0.001$) (Fig. 2). The mean value for samples from sub-adult individuals equaled 35.27 µg/kg (SE=11.89) (N=12) (Table S1). In comparison, the mean value for adults was 105.08 µg/kg (SE=9.19) (N=177) (Table S2-S3). As the number of samples collected from sub-adult individuals was low, we used only data for adults in further analysis.

Amongst five built models (including the null model), the one that included site and interaction between sex and reproductive status was selected as the best model (Table 1).

The THg value was affected by the site of a sample collection, the sex of a squirrel, and reproductive status (Table 2). Higher values of THg were recorded in the urban park (mean 135.13

µg/kg; SE=14.19) than in the urban forest (mean 60.21 µg/kg; SE=5.53) (Fig. 3). Also, greater variation in THg concentration was observed in squirrels from the urban park (4.34 – 776.5 µg/kg), than from the urban forest (9.07 – 330.00 µg/kg). Higher THg values were recorded in the samples collected from females during the breeding season (mean 165.63 µg/kg, SE=33.25), while for males, higher THg concentration was recorded during the nonbreeding period (mean 98.03 µg/kg; SE=13.17) (Fig. 4) (Table S2-S3).

Next, we analyzed the link between the body mass of squirrels and the concentration of mercury (THg) in hair samples, separately for the urban forest and the urban park (Table 3). In the urban forest, body mass did not affect the THg value in red squirrels' hair samples, while in the urban park, the influence of body mass was significant (Table 3). In the case of park squirrels, higher THg values were recorded in hair samples from squirrels with higher body mass (Fig. 5).

There was also a difference between THg concentration in acid-rinsed and non-rinsed samples ($t=4.557$, $df=37$, $p<0.001$). The intTHg concentration in hair samples was, on average, 46.67 µg/kg, which was about 74% of the mean THg value before rinsing, which means that about a quarter of determined mercury in hair was extTHg. The extTHg and intTHg concentrations in hair samples differed significantly ($t=2.881$, $df=37$, $p<0.01$). Only 6% of THg in the hair sample was extTHg in squirrels from urban park, while 94% was intTHg ($t=4.430$, $df=21$, $p<0.001$). In the case of urban forest squirrels, the proportion between superficially adsorbed and internally incorporated THg was almost equal, with 54% of extTHg and 46% of intTHg ($t=-0.788$, $df=15$, $p=0.439$) (Table S4).

4. DISCUSSION

In our study, we showed differences in mercury concentration in hair samples of red squirrels inhabiting the urban forest and the urban park in Warsaw. First, we showed that mercury contamination was age and body-mass dependent. We also proved that samples from the urban park were more mercury-contaminated than the ones from the urban forest. At the same time, in the case of park squirrels, less mercury was adsorbed on the hair surface (rather than ingested) than in the case of forest squirrels. Finally, we pointed to the relationship between the breeding status of a squirrel and the mercury level in its hair sample.

Diet and feeding habits are usually considered the main factors shaping differences in intra- or interspecific mercury concentration. Although some individuals of red squirrels in our study had high mercury levels (about 800 µg/kg), the mean value was about 120 µg/kg, which was similar to the results obtained in a recent pilot study carried out in different study areas (including Warsaw), and the species in general (Lurz et al., 2017). For grey squirrels (*Sciurus carolinensis*) in urban habitats, mercury values in hair samples were higher, on average about 1000 µg/kg (Jenkins et al., 1980). The mercury levels obtained in hair samples in our study were similar to values obtained in other studies and locations for (mostly) herbivorous small mammals, e.g., the bank vole (*Myodes glareolus*) or the wood mouse (*Apodemus sylvaticus*) (Bull et al., 1977) or a few North American species (Gerstenberger et al., 2006). They also resembled levels obtained for some large, strictly herbivorous mammals like the reindeer (Frøslie et al., 1984; Gamberg et al., 2016) or were higher than those noted for the moose or the red deer (*Cervus elaphus*) (Frøslie et al., 1984). Nevertheless, the aforementioned studies were based on liver samples, and the values for hair samples can be higher due to mercury excretion. In turn, in comparison to mostly or strictly carnivorous species like the Arctic fox, the American mink, the river otter, and the polar bear (Yates et al., 2005; Bocharova et al., 2013; Lippold et al., 2022) or insectivorous like shrews

(Talmage and Walton, 1993; Sánchez-Chardi et al., 2007) mercury levels obtained in our study were quite low. The mostly herbivorous diet of red squirrels can probably explain moderate mercury levels in both populations studied in Warsaw. However, we found that park squirrels had over two times higher concentrations of mercury than forest squirrels. In comparison, in Jacksonville, USA, the mercury levels of grey squirrels were similar over the urban area (Jenkins et al., 1980). In our case, the food composition of the two populations was different. In essence, park squirrels were supplementarily fed with nuts (walnuts and hazelnuts, provided by park visitors). In contrast, only natural seeds occurred in a diet of forest squirrels (Krauze-Gryz et al., 2021a). Because mercury levels in nuts and seeds are typically low (Rodushkin et al., 2008), the diet variation is probably not responsible for observed differences. On the other hand, differences in mercury concentration may be explained by the variation in food selection by certain individuals (Krauze-Gryz et al., 2021b) rather than the whole population. Also, local differences in mercury emission or environmental pollution may explain variations in mercury contamination (Lurz et al., 2017). Elevated mercury concentration in mammals could also be related to the distance from the emission sources (Talmage and Walton, 1993). In our study, the urban forest was located at a greater distance from two (out of three) emission sources than the urban park. Additionally, earlier studies showed a high variation of metal concentration (including mercury) in the soils of Warsaw (Tomassi-Morawiec, 2016). In detail, there was a significant soil enrichment in mercury in the Łazienki Park in comparison to other locations of Warsaw and the geochemical background of mercury in Poland (Dusza-Dobek, 2012). Thus, high levels of soil mercury in the park could explain higher levels of mercury in park squirrels, due to the bioaccumulation from plants to animals.

It is not clear how squirrels acquire mercury in an urban environment (Jenkins et al., 1980); however, our comparison of acid-rinsed and non-rinsed hair samples showed a significant difference between internal (i.e., digested with food) and external mercury concentration in hair, but depending on the location. A higher share of internal mercury in the hair of urban park squirrels points to greater food than atmospheric exposure. This again points to the influence of elevated levels of mercury in soils (and, as a consequence, in plants and seeds, the food sources for squirrels) of the urban park on the variation in mercury concentration between the two populations. An important factor in shaping differences between the internal and external distribution of mercury in hair can be the behavior of squirrels in the two locations. In general, forest squirrels were mainly arboreal as compared to park squirrels that spent much time on the ground (Krauze-Gryz et al., 2021b). Squirrels that moved mostly in the crowns of the trees and brushed against tree leaves can be more exposed to atmospheric mercury deposition, which is finally deposited on the hair surface, as leaves keep a significant part of atmospheric contaminants (see Lohr and Pearson-Mims, 1996; Morani et al., 2011; Simon et al., 2014).

Mercury concentrations in our study were also dependent on body mass, age, sex, and reproductive status. In our previous paper, we showed that squirrels inhabiting the urban forest were heavier and had better body condition than those in the park (Beliniak et al., 2022). In general, mercury concentration can increase with body mass and individual age (Lodenius et al., 2014). Thus, we may assume that mercury concentration should increase with the age and body mass of a squirrel. Nevertheless, this association does not have to be so straightforward (Lodenius et al., 2014; Gamberg et al., 2016; McKinney et al., 2017). Mercury can be redistributed in organisms, leading to changes in its concentration in various tissues, e.g., being released to the blood system when fat reserves are being used. Low body mass may also be due to a high concentration of pollutants in the body (Bremle et al., 1997; McKinney et al., 2017). In our case, the body mass of squirrels was rather stable over a year (Beliniak et al., 2022), and a clear positive correlation between body mass and hair mercury concentration was recorded for the urban park squirrels, while in the urban forest squirrels body mass did not affect mercury levels. There is no clear reason why the relationship between body weight and mercury content should be different in the two cases. However, this suggests that in the case of park squirrels, mercury is mostly ingested with food. The high content of external mercury (i.e., deposited on hair) in forest squirrels can disguise any interaction between body mass and mercury concentration. Interestingly, the season of sample collection did not affect mercury concentration in hair. In the winter season, due to intensive combustion processes, mercury emission increases (Zielonka et al., 2005), so higher mercury concentration in hair molted in spring can be expected. Nevertheless, the molting pattern in squirrels is quite variable. It depends on age, sex, reproductive activity, and condition (Bosch and Lurz, 2012), which may explain this lack of relation between the season and mercury level.

Despite a small number of hair samples from sub-adults, we clearly showed lower mercury concentration in younger individuals than in adult ones. As mercury accumulates in tissues, a longer time of individual exposition results in a growing concentration in time. Such differences in mercury concentration between adults and juveniles were already reported in, e.g., grey squirrels (Jenkins et al., 1980), Arctic foxes *Vulpes lagopus* (Bocharova et al., 2013) or European otters *Lutra lutra* (Yates et al., 2005; Lodenius et al., 2014).

In many studies, sex did not play a significant role in shaping mercury levels (Yates et al., 2005; Gerstenberger et al., 2006; Bocharova et al., 2013; Lodenius et al., 2014), Nevertheless, in some studies (but see Lodenius et al., 2014) females had higher mercury concentrations than males (Gamberg et al., 2005; Yates et al., 2005; Lyytikäinen et al., 2015; Lurz et al., 2017; McKinney et al., 2017), even independently from their reproductive status (McKinney et al., 2017). This pattern corresponds to our result, where females were much more exposed to mercury. In the case of Warsaw squirrels, the highest mercury levels were recorded for pregnant or lactating females. It could be the result of higher nutritional needs during these periods, resulting in much intense foraging and gnawing on bones or consuming soil to obtain minerals (Bosch and Lurz, 2012). In the American minks (*Neogale vison*), higher mean mercury values in females probably reflected greater food consumption by females (Gamberg et al., 2005). A high concentration of mercury in pregnant and lactating females can be worrisome. Although lactation is thought to be the main exposure route, also a prominent part of transferred mercury is transported to offspring during the gestation period (Habran et al., 2011; Lyytikäinen et al., 2015; Grajewska et al., 2019).

5. CONCLUSIONS

Urban-dwelling mammals face numerous threats like habitat fragmentation, limited natural resources, or environmental pollutants. In turn, these novel habitats may offer numerous anthropogenic shelters or food sources. In this study, we compared the two populations inhabiting the same city but areas under various anthropogenic pressures. The difference between the levels of contamination between the two squirrel populations was significant. Nevertheless, the sources of mercury were probably both current and/or historical pollution, and the level at which it potentially affected the two populations was also different. It is not clear why the bioaccumulation was clearly higher in the urban park than in the urban forest. The factors could be attributed to the altered behavior of animals in response to the presence of humans (i.e., time spent on the ground or within the tree crowns). We also showed that individual characteristics could affect the level of mercury. All this points to the importance of future studies on the variation in mercury concentrations within mammal populations inhabiting different urban green areas. Our results suggest that mercury remains a problem, not only for marine fauna but also for terrestrial organisms, and should still be monitored. Moreover, further studies should consider the importance of mercury exposition to populations and the functioning of the terrestrial food web.

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ETHICS DECLARATIONS

ETHICAL APPROVAL

Trapping and handling squirrels complied with current laws on animal research in Poland and were carried out with the permit of the Local Ethical Committee (WAW2/072/2018). Access to the Natolin Forest Reserve (the urban forest) and red squirrel capture was allowed with permission from the General and Regional Directorates for Environmental Protection.

CONFLICTS OF INTERESTS

The authors declare no competing interests.

CONSENT TO PARTICIPATE

All authors contributed to the study's conception and design.

CONSENT TO PUBLISH

All the authors gave their consent for this article to be published.

AUTHOR CONTRIBUTIONS

Mateusz Jackowiak, Dagny Krauze-Gryz, Agata Beliniak, and Karolina Danuta Jasińska wrote the first draft of the manuscript. All authors contributed to the study's conception, design, and investigation. Formal analysis was performed by Karolina Danuta Jasińska, data curation by

Mateusz Jackowiak and Agata Beliniak, and methodology and founding acquisition by Mateusz Jackowiak, Agata Beliniak, and Dagny Krauze-Gryz. Jakub Gryz, Ewa Żyfka-Zagodzińska and Anita Matracka were involved in the investigation, review and editing of the manuscript. All authors commented on previous versions of the manuscript, and all authors read and approved the final manuscript.

FIGURES AND TABLES

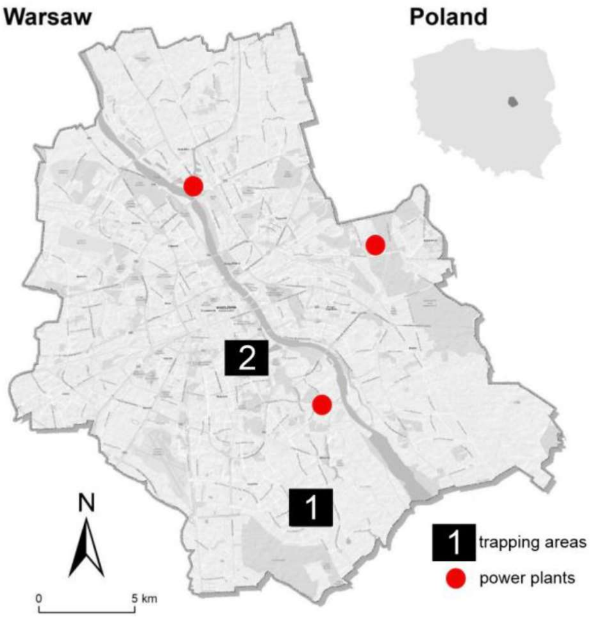

Figure 1. Study areas: 1. an urban forest (Natolin Forest Reserve) and 2. an urban park (Royal Łazienki Museum) in Warsaw, where red squirrels were live-trapped and their hair samples collected. Red dots show the locations of the main power plants.

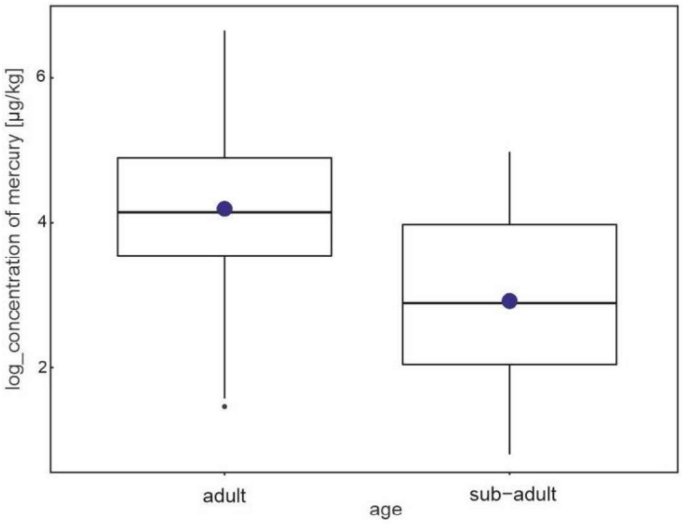

Figure 2. Differences in THg (µg/kg) concentration in hair samples (blue dot stands for mean) between adult and sub-adult red squirrels in Warsaw (samples from an urban forest and an urban park pooled together).

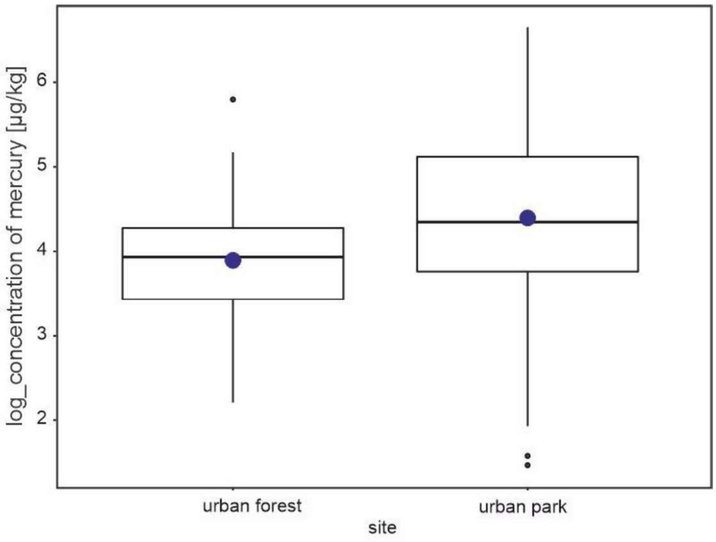

Fig. 3. Differences in THg (µg/kg) concentration in hair samples (blue dot stands for mean) of red squirrels in an urban forest and an urban park in Warsaw. As no sub-adults were sampled in the forest, only adult individuals were taken into analysis.

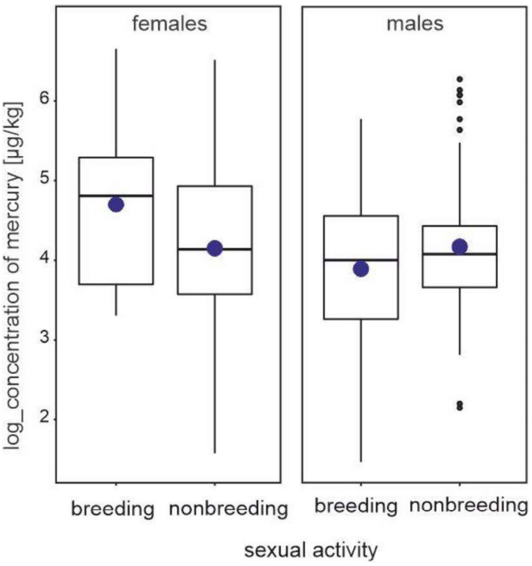

Fig. 4. Differences in THg (µg/kg) concentration in hair samples (blue dot stands for mean) of adult female and male red squirrels during breeding and non-breeding period, collected in Warsaw (samples from an urban forest and an urban park pooled together).

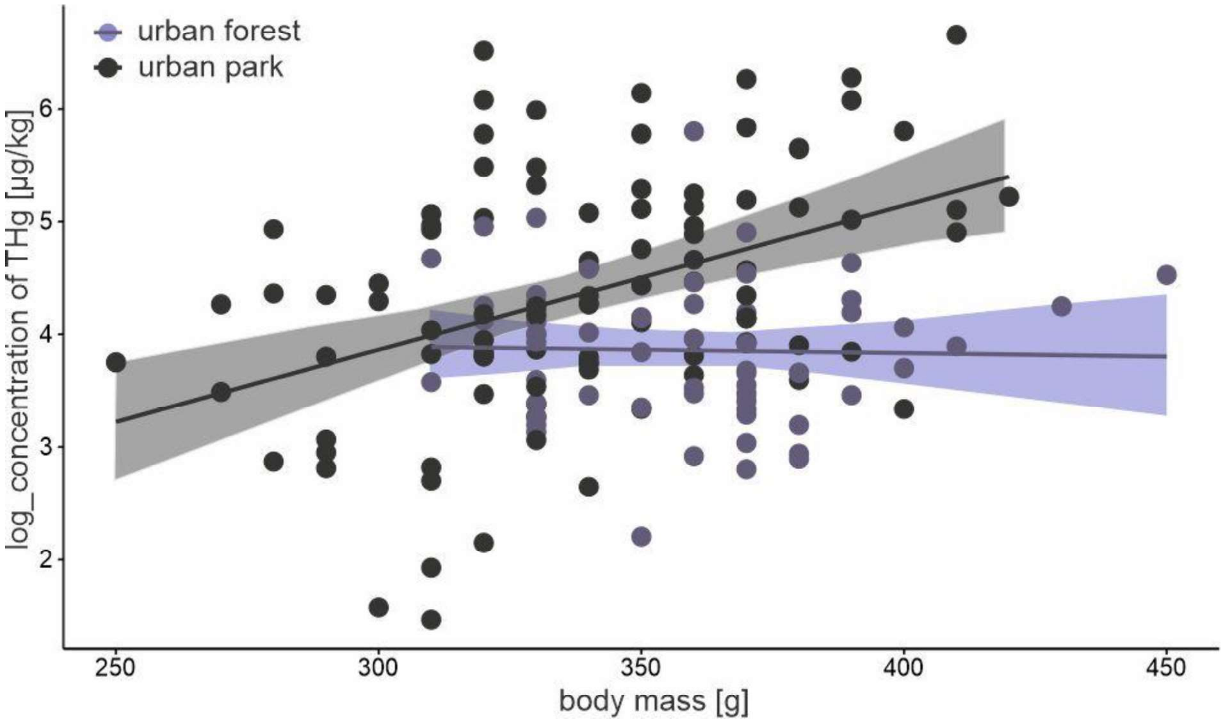

Fig. 5. Relationship between body mass and concentration of THg [µg/kg] in hair samples of red squirrels collected in the urban forest and the urban park in Warsaw (samples from females and males pooled together), as predicted by models summarized in Table 3.

Table 1. Akaike Information Criterion (AIC) for linear mixed-effects models analyzing factors affecting THg (µg/kg) values in red squirrel hair samples from the two sites: an urban forest and an urban park in Warsaw

Model	AIC
site + sex*reproductive status + (1|individual_ID)	464.95
site + sex*reproductive status + season + (1|individual_ID)	470.64
site +(1|individual_ID)	471.01
site + sex + (1|individual_ID)	473.69
Null model	473.99

Table 2. Effects of a site, sex, and breeding condition on THg concentration values in hair samples from the two sites: an urban forest and an urban park in Warsaw. The intercept stands for the urban forest, female, and breeding period.

variables	estimate (SE)	t value	*p*-value
Intercept	4.3878 (0.24)	18.527	**<0.001**
site: urban park	0.4464 (0.18)	2.451	**0.015**
sex: male	-0.6653 (0.27)	-2.434	**0.016**
activity: nonbreeding	-0.4497 (0.21)	-2.115	**0.036**
male*nonbreeding	0.6909 (0.29)	2.396	**0.018**

Table 3. Relationship between body mass of adult red squirrels (females and males pooled together) and concentration of THg in hair samples collected in two sites in Warsaw: an urban park and an urban forest.

study site	variables	estimate (SE)	t value	*p*-value
urban forest	Intercept	3.4131 (1.060)	3.221	**0.002**
	body mass	0.0014 (0.001)	0.471	0.639
urban park	Intercept	-0.1270 (1.078)	-0.118	0.906
	body mass	0.0133 (0.003)	4.237	**<0.001**

The heavy burden of city life: factors affecting mercury bioaccumulation in urban red squirrels

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Table S1. THg values in hair samples of sub-adult red squirrels collected in the urban park in Warsaw, n.d. – no data

season	sex	N samples	THg [µg/kg]		
			min	max	mean
spring	female	2	2.24	53.04	27.64
summer	female	1	35.27	35.27	35.27
summer	male	2	4.51	23.85	14.18
autumn	female	2	10.77	13.68	12.23
autumn	male	3	6.04	146.10	53.50
winter	female	2	54.01	65.34	59.68

Table S2. THg values in hair samples of adult red squirrels collected in the urban park in Warsaw, n.d. – no data

season	sex	reproductive status	N samples	THg [µg/kg]		
				min	max	mean
spring	female	breeding	12	27.25	776.50	222.35
spring	female	non-breeding	4	14.09	189.60	88.18
spring	male	breeding	8	4.34	322.45	103.74
spring	male	non-breeding	20	21.36	464.10	95.74
summer	female	breeding	1	197.40	197.40	197.40
summer	female	non-breeding	1	51.88	51.88	51.88
summer	male	non-breeding	9	8.59	321.90	109.62
autumn	female	breeding	3	28.12	234.70	99.74
autumn	female	non-breeding	16	4.84	524.50	113.38
autumn	male	breeding	1	132.80	132.80	132.80
autumn	male	non-breeding	5	16.76	398.45	109.59
autumn	male	n.d.	1	17.65	17.65	17.65
winter	female	breeding	3	90.81	198.60	153.77
winter	female	non-breeding	7	21.45	674.00	208.23
winter	male	breeding	6	16.64	134.80	76.57
winter	male	non-breeding	9	34.31	531.10	236.93

Table S3. THg values in hair samples of adult red squirrels collected in the urban forest in Warsaw, n.d. – no data

season	sex	reproductive status	N samples	THg [µg/kg]		
				min	max	mean
spring	female	breeding	5	28.25	176.40	94.83
spring	female	non-breeding	3	39.51	97.40	66.82
spring	male	breeding	10	20.81	72.54	39.73
spring	male	non-breeding	13	31.81	106.90	62.48
spring	male	n.d.	2	33.95	102.80	68.38
summer	female	breeding	1	40.41	40.41	40.41
summer	female	non-breeding	4	16.49	71.32	46.58
summer	male	non-breeding	11	24.40	63.01	45.41
autumn	female	non-breeding	9	18.51	330.00	94.39
autumn	male	breeding	1	80.49	80.49	80.49
autumn	male	non-breeding	5	9.07	84.02	47.97
winter	female	non-breeding	5	18.09	141.85	47.40
winter	male	non-breeding	2	54.90	66.15	60.53

Table S4. THg, intTHg and extTHg values in hair samples of red squirrels collected in urban park and urban forest in Warsaw

location	N samples	THg [μg/kg]			intTHg [μg/kg]			extTHg [μg/kg]		
		min	max	mean	min	max	mean	min	max	mean
urban park	22	16.76	239.50	84.61	4.08	192.80	64.55	0.13	137.80	20.06
urban forest	16	27.93	102.80	55.11	6.90	48.42	22.09	4.10	78.01	33.03

9. Podsumowanie

Na podstawie przeprowadzonych badań udowodniono, że zachowanie i parametry populacji wiewiórek z miejskiego rezerwatu leśnego (terenu o niskim stopniu przekształcenia antropogenicznego) są podobne do populacji z obszarów pozamiejskich, natomiast wiewiórki z parku miejskiego (o wysokim stopniu przekształcenia antropogenicznego) wykazują szereg zmian, spowodowanych dostosowaniem do warunków środowiska i obecności ludzi.

Aktywność dobowa była typowa dla gatunku na terenach pozamiejskich – wiewiórki prezentowały bimodalny wzorzec aktywności, czyli widoczne były dwa szczyty aktywności (po wschodzie słońca oraz przed zachodem). Również typowo dla gatunku zwierzęta te były aktywne przed wschodem słońca, jak również po jego zachodzie. Wiewiórki z parku miejskiego (o wysokim stopniu przekształcenia antropogenicznego) miały tylko jeden szczyt aktywności (który zaczynał się kilka godzin po wschodzie słońca i trwał do wczesnego popołudnia). Nie zaobserwowano u nich również aktywności przed wschodem i po zachodzie słońca. Najprawdopodobniej wiewiórki z parku dostosowały swoją aktywność do obecności ludzi odwiedzających park, co zwiększa szanse na zdobycie dodatkowego pokarmu. W przeciwieństwie do rezerwatu leśnego, w parku najwięcej zwierząt zaobserwowano zimą, co sugeruje, że wiewiórki te są w stanie utrzymać w tym czasie wysoką aktywność (prawdopodobnie dzięki dokarmianiu w ciągu całego roku). Wbrew oczekiwaniom, duża dostępność pokarmu uzupełniającego (w postaci dokarmiania przez ludzi) nie skutkowała większą masą ciała. Przeciwnie, to wiewiórki z rezerwatu leśnego były cięższe oraz miały lepszą kondycję ciała w porównaniu do zwierząt z parku. Najprawdopodobniej dostępność wysokokalorycznych nasion (orzechów laskowych, orzeszków grabowych i żołędzi) na terenie rezerwatu pozwoliła na osiągnięcie wysokiej masy ciała. Pokarm antropogeniczny w parku, mimo że dostępny przez cały rok, może być gorszej jakości i traktowany przez zwierzęta jako uzupełniające źródło pokarmu. Być może jednak, całoroczny dostęp do obfitych zasobów pokarmowych w parku powoduje, że gromadzenie zapasów tłuszczu (powodujące zwiększenie masy ciała) przed zimą nie jest konieczne. Wiewiórki zamieszkujące park miejski mogą mieć również niższą masę ciała w związku z konkurencją międzyosobniczą (wynikającą z wysokiego zagęszczenia populacji). Pomimo, że wiewiórki w parku miejskim miały gorszą kondycję oraz niższą masę ciała, udział aktywnych płciowo samic był większy, co może być spowodowane wyższą dostępnością pokarmu uzupełniającego. Obie populacje różnił jedynie jeden (z trzech analizowanych) wskaźników reakcji na nagły stres: u wiewiórek z rezerwatu leśnego zaobserwowano wyższą częstotliwość oddechu. Taka odmienna reakcja najprawdopodobniej była spowodowana ograniczonym kontaktem z ludźmi, w przeciwieństwie do wiewiórek z parku, które przyzwyczaiły się do bliskiej obecności człowieka. Kolejną różnicą między populacjami była zawartość metalu ciężkiego w sierści (rtęci) – u osobników z rezerwatu miejskiego była prawie dwukrotnie niższa niż u zwierząt z parku miejskiego. Może to być wynikiem różnego poziomu zanieczyszczenia środowiska oraz odmiennego składu pokarmu, ponieważ metale ciężkie mogą być dostarczane do organizmu razem z pokarmem.

Pomimo wielu wykazanych wcześniej różnic, zaobserwowano również podobieństwa między obiema miejskimi populacjami. Niezależnie od terenu badań, najwięcej aktywnych płciowo samic zaobserwowano wiosną, choć aktywne płciowo osobniki rejestrowane były również zimą na obu obszarach. Zwierzęta z populacji miejskich wcześniej przystępują do rozrodu dzięki większej dostępności pokarmu i wyższym temperaturom (Selonen i in. 2016). W przeciwieństwie do założeń, nie zaobserwowano różnic w reakcjach na długoterminowy i średnioterminowy stres. Również dwa wskaźniki (z trzech) reakcji na nagły stres były podobne (wskaźnik zaniepokojenia “struggle rate” i wokalizacja). Wskaźnik zaniepokojenia (“struggle rate”) zależał jedynie od pory roku i był najwyższy zimą, a wokalizacja była najmniej intensywna w przypadku zwierząt złapanych po raz pierwszy. U zwierząt na obu terenach poziomy metabolitów hormonu stresu zależały od pory roku – wyższe wartości odnotowano jesienią i zimą. Brak różnic między populacjami wskazuje, że zwierzęta z obu terenów są w podobnym stopniu poddane czynnikom środowiska. W przypadku obu populacji poziom zanieczyszczenia sierści metalem ciężkim (rtęcią) zależał od wieku – wyższe wartości wykazano u osobników dorosłych niż młodocianych. Taki wynik był spodziewany, ponieważ rtęć akumuluje się w tkankach, a dłuższy czas ekspozycji powoduje większą koncentrację tego pierwiastka. Stężenia rtęci w sierści u zwierząt z obu terenów były najwyższe dla samic, które były w ciąży lub karmiły. Może to wynikać z wyższego zapotrzebowania na składniki odżywcze w tym okresie u samic, przez co spożywają więcej pokarmu i mają bardziej specyficzną dietę.

Wyniki omawianych badań pokazują, że wiewiórka pospolita to gatunek plastyczny, który dostosowuje się do środowiska, w którym żyje. Są to jedne z pierwszych badań porównujących dwie miejskie populacje tych zwierząt żyjących na terenach o różnym stopniu antropopresji, w przeciągu wszystkich pór roku. Wyniki są również potwierdzeniem, że wiewiórka pospolita może być traktowana jako gatunek modelowy, służący do oceny zanieczyszczenia środowiska metalami ciężkimi. Dodatkowo wyniki wyraźnie wskazują, że zastosowanie szerokiego zakresu wskaźników i różnych metod analitycznych, w tym analiz behawioralnych, jest niezbędna do oceny wpływu antropogenicznych czynników stresogennych na zwierzęta. Jednocześnie pojawia się potrzeba dalszych badań w celu potwierdzenia tych wyników oraz lepszego poznania, jak zwierzęta radzą sobie w warunkach miejskich oraz identyfikacji wskaźników, które pozwolą skutecznie ocenić wpływ urbanizacji na dziką przyrodę.

10. Kierunki przyszłych badań

Uzyskane wyniki wykazały szereg różnic w funkcjonowaniu dwóch populacji wiewiórek poddanym odmiennej antropopresji. Pozwala to na lepsze zrozumienie mechanizmów dostosowania się zwierząt do zmienionych warunków środowiska miejskiego oraz wskazanie przyszłych kierunków badań.

1) Wyniki omawianych badań wskazują na wiele różnic między miejskimi populacjami wiewiórek żyjącymi terenach o odmiennym stopniu przekształcenia antropogenicznego (park miejski i rezerwat leśny). Badania te skupiają się jedynie na dwóch terenach. Cenne by było poszerzenie badań tak, by objęły większą liczbę terenów zielonych o różnym stopniu przekształcenia antropogenicznego a także położonych w gradiencie urbanizacji, obejmującym również tereny pozamiejskie.
2) Badania przeprowadzone były na terenie tego samego miasta oraz w tych samych miesiącach, dlatego można założyć, że oddziaływanie czynników abiotycznych na obie populacje powinno być podobne. Jednak znaczna zmienność warunków środowiska w obrębie miasta wynikająca z jego heterogenności, takich jak zanieczyszczenie światłem czy wpływ miejskiej wyspy ciepła, może oddziaływać na funkcjonowanie zwierząt w mieście. Uwzględnienie tych czynników i zbiór danych w większej liczbie lokalizacji pozwoliłoby na lepsze zrozumienie mechanizmów odpowiadających za zmiany zachowania czy parametrów miejskich populacji wiewiórek.
3) W omawianych pracach oceniono wpływ człowieka jako jeden z kluczowych czynników powodujących zmiany w populacji żyjącej na terenie parku. W badaniach jednak nie była bezpośrednio oceniania dostępność pokarmu pochodzenia antropogenicznego ani naturalnego czy skład pokarmu poszczególnych osobników. Wykazano wcześniej, że wiewiórki z parku prezentują różne strategie związane z pozyskiwaniem pokarmu, tj. niektóre osobniki spędzają więcej czasu na ziemi i pozytywnie reagują na ludzi (zbliżają się i proszą o jedzenie), inne natomiast zdają się unikać ludzi i polegać głównie na pokarmie naturalnym (Krauze-Gryz i in. 2021*a*). Przyszłe badania poszczególnych osobników, ukierunkowane na znalezienie związku pomiędzy stopniem wykorzystania pokarmu antropogenicznego vs. naturalnego a kondycją, długością życia czy parametrami rozrodu poszczególnych osobników, pozwolą zrozumieć, która ze strategii jest wyrazem lepszego dostosowania.

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12. Oświadczenia współautorów

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