FAKE PAPER (SCQ)

Hyperactive sloths leads to isolation of a new compound

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The observation of hyperactive three-toed sloths, *Bradypus variegates,* on the island of Barro Colorado is due to the addition of *Panamae caenaela* to their diets. *P. caenaela* is only found on the Barro Colorado Island which explains why only hyperactive sloths were found on the Barro Colorado Island. Using gas chromatography/ mass spectrometry analysis, we were able to isolate the compound responsible for the hyperactive behaviour of the sloth. The compound was determined to be N-methyl-1-(3,4-methylenedioxyphenyl)-2aminopropane. We have named it MMAP for short.

The Barro Colorado Island (BCI) of Panama was formed in 1914. The island was separated from the mainland when the Chagras River was dammed to form the infamous Panama Canal[4]. After the formation of the island, the Smithsonian Tropical Research Institute built a research station there in 1923 to study the 1500 hectare tropical rainforest[3,7]. The creation of BCI provided the opportunity to study species from the beginning of geographical isolation. The BCI rainforest is the most studied rainforest in the world due to its unique history and the amount of biodiversity found on the island[6,10,12].

A recent survey of the three-toed sloth, inhabited on the Barro Colorado Island of Panama found that the three-toed sloth of that island displayed some unusual behaviour from their mainland cousins[16]. The brown-throated three-toed sloth, Bradypus variegates, is a remarkably slow moving, nocturnal and diurnal mammal that spends most of its life in the middle layers and the tops of trees where it hangs upside down from branches or sits in the forks between tree canopies[8,9,11,14]. Sloths are known to sleep or rest up to twenty hours a day[2], however, the sloths on BCI observed exhibited signs of increased activity. It was noted that they were more mobile than their mainland cousins, normally traveling at an average speed of 5 m/min. The mainland three-toed sloth is usually observed traveling at an average speed of 3m/min. Although the mainland three-toed sloths are capable of reaching a speed of 5m/min, this is rarely seen as such high speeds are reserved for escape response from predators[13,15,17]. Sloths will leave their forest canopy to defecate and to relieve themselves about once a week[1,5]. On occasion, Bradypus variegates also leaves the trees and crawls along the forest floor or swims in the flooded forest to find other plants for food[18,19]. In the survey, increased frequency of descending from tree canopies were found in the sloths of BCI, up to two or three times a week. The BCI sloths also seem to be more sensitive to intense light stimulation. In response to increased light, their acitivity rates increased.

The three-toed sloths are herbivores that only eat tree leaves, shoots and other foliage found in the canopy where they live[8]. The leaves of the Cecropia tree and liana woody plants found in the tropical rainforest comprise most of the three-toed sloths' diets[16,20]. However, a recent identification of an epiphytic plant, *Panamae caenaela,* exclusive to BCI is commonly seen in the BCI sloths' diet due to its relatively easy digestibility by the animal[18]. We propose that the reason why BCI sloths are exhibiting these peculiar behavioural differences from their mainland predecessor is due to the slight difference in their diet of *P. caeneala*.

A series of loss-of-function and gain-of-function experiments were conducted to determine whether *P. caenaela* caused the sloth to become hyperactive. Using chromatography we have isolated different compounds from the epiphytic plant. The different compounds were tested on the sloth to determine which is the ingredient that causes increased activity in the sloth. The structure of the compound was subsequently determined using mass spectrometry.

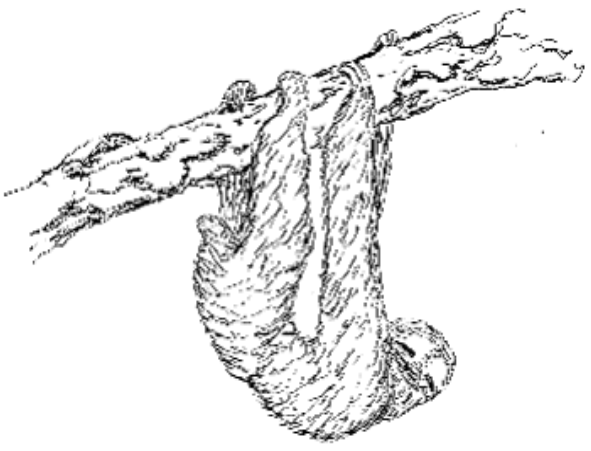

Figure 1 Picture of a brown-throated three-toed sloth, *Bradypus variegates*.

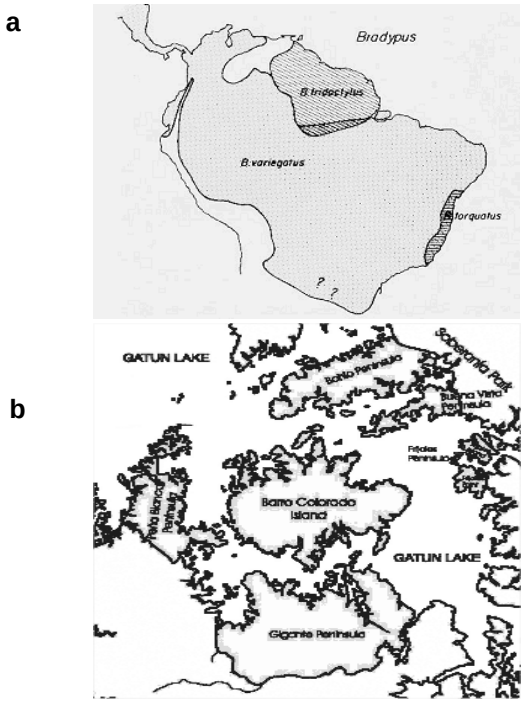

Figure 2 Distribution of three-toed slothes, *Bradypus variegates*, in Central and South America. **a,** *Bradypus variegatus* is endemic to the tropical and subtropical regions of the American continent. These animals are best suited for highly humid and woody areas, near rivers, and in open jungles. Their continuous distribution can range from Honduras in Central America into South America (roughly between 15oN and 25oS Latitude). In South America, *Bradypus variegatus* can be found in coastal Ecuador, through Columbia and Venezuela (except for Llanos, and the Orinoco river delta), continuing to through the forested areas of Ecuador, Peru, Bolivia, through-out Brazil, and extending to the northern portion of Argentina. *Bradypus variegatus* can be found living in the trees of neotropical deciduous forests as well as tropical rainforests. **b,** Location of Barro Colorado Island (BCI) of Panama where distribution of hyperactive three-toed sloths was found.

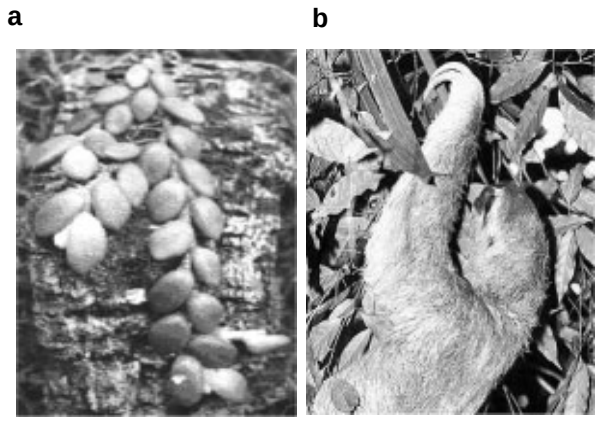

Figure 3 **a,** Picture of the epiphytic plant, *Panamae caenaela*, which grows exclusive on BCI and commonly contributes to the diets of the BCI slothst. **b,** The three-toed sloths are herbivores that only eat tree leaves, shoots and other foliage found in the canopy where they live. The leaves of the Cecropia tree and liana woody plants found in the tropical rainforest comprise most of the three-toed sloths' diets.

Determination of ingredient in food source responsible for increased activity

LOF

P. caenaela was removed from the diet of 5 hyperactive sloths from the Barro Colorado Island for 1 week. The diet consisted of controlled plant material such as ymbahuba leaves[26]. The Respiratory Quotient (RQ) was used to gauge the activity levels of the sloth[38]. It was found that the RQ value of the sloths was significantly higher after the removal of P. caenaela from their diets, from 0.79 to 0.96 ± 0.02. (RQ value of 1.0 indicates energy expenditure from carbohydrates and 0.7 indicates energy expenditure from fat. A value in between demonstrates energy expenditure is dependent on both fat and carbohydrates[32,35,38].) The increased RQ value shows a significant change from the sloth's initial dependence on fat and carbohydrates for energy expenditure to mainly carbohydrates. This further indicates that the sloth's activity levels have decreased, since energy expenditure is derived primarily from carbohydrates in the food source and not internal stores of body fat[31]. It was also observed that the sloth was only seated with the hind limbs wrapped around any vertical object available and with the head down upon the chest, seemingly less alert. Adding to that we checked the heart rates and respiration differences before and after *P. caenaela* was removed from their diets. After the removal of *P. caenaela*, we measured a heart rate of 70.9 ± 6.7 beats per minute in unrestrained conditions, which was definitely lower than before the diet (89.9 ± 6.2 bpm). The respiration rate also decreased to an average of 13 breaths per minute (range 10 to 18) in comparison to an initial average respiratory rate of 45 breaths/min (range 40 to 54). Both decreased respiration rate and heartbeat rate leads to the conclusion that the sloths became less active after removal of *P. caenaela* from their diet.

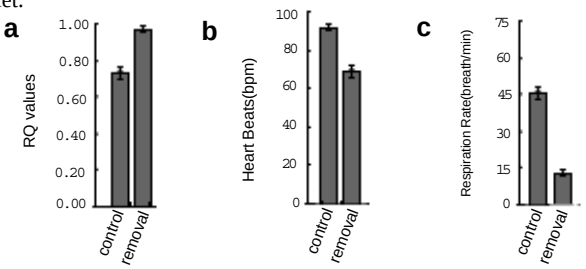

Figure 4 The comparative measures of RQ values, heart beats and respiration rates of *Bradypus variegates* in LOF procedure. **a,** the respiratory quotient (RQ) values of the sloths was significantly higher after the removal of *P. caenaela* from their diets, from 0.79 to 0.96 ± 0.02. **b,** after the removal of *P. caenaela*, we measured a heart rate of 70.9 ± 6.7 beats per minute in unrestrained conditions, which was definitely lower than before the diet (89.9 ± 6.2 bpm). **c,** the respiration rate decreased to an average of 13 breaths per minute (range 10 to 18) in comparison to an initial average respiratory rate of 45 breaths/min (range 40 to 54).

GOF

When we added *P. canaela* to the diet of 5 sloths from the mainland for one week, we measured a significant increase in activity. The RQ value of the sloths decreased from 0.93 to 0.72 ± 0.03. This decrease shows a significant shift of their energy dependency from carbohydrates to fat. The dependency of energy from fat shows that the activity of sloths has increased and that the energy from food is solely not enough[32,35,38]. The respiration rate and heart rate were also measured before and after the change in diet. The respiration rate increased from an average of 10 breaths/min to 46 breaths/min (range 38-52). And the heartbeat rate increased from 66.2 ± 5.9bpm to 83.8 ± 6.8bpm. Furthermore, we observed that the sloths were moving around more and seemed to be more agitated. These observations and measurements indicate that *P.caenaela* is sufficient and necessary for the increase in activity.

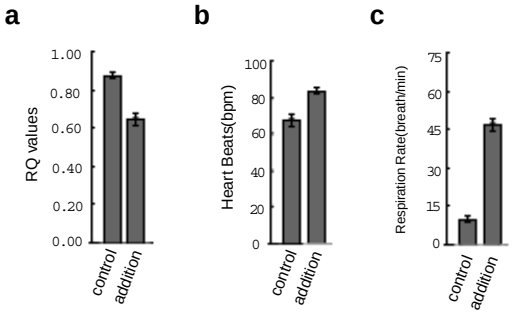

Figure 4 The comparative measures of RQ values, heart beats and respiration rates of *Bradypus variegates* in GOF procedure. **a,** the respiratory quotient (RQ) values of the sloths decreased from 0.93 to 0.72 ± 0.03 after the addition of *P. caenaela* from their diets. Such decrease shows a significant shift of their energy dependency from carbohydrates to fat. **b,** after the addition of *P. caenaela*, the heartbeat rate increased from 66.2 ± 5.9bpm to 83.8 ± 6.8bpm. **c,** the respiration rate increased from an average of 10 breaths/min to 46 breaths/min (range 38-52).

Fraction #	Heart beat before feeding with fractioned compound	Heartbeat after feeding with fractioned compound
1	64.5 ± 5.4 bpm average	67.2 ± 4.5
2	62.3 ± 4.4	61.2 ± 4.3
3	63.8 ± 4.3	67.4 ± 5.2
4	66.4 ± 4.3	80.7 ± 5.6
5	65.7 ± 5.1	62.0 ± 5.2
6	61.7 ± 4.9	67.1 ± 4.2
7	65.4 ± 4.1	62.4 ± 4.9
8	63.4 ± 5.1	65.6 ± 5.0

Table 1 Heat beat rates after each fraction was added to the sloth's diet

Determination of the compound in *P. caenaela* responsible for the increased activity in sloths

A chloroform-methanol extraction was preformed using *P. caenaela* leaves[37]. This yielded an organic and aqueous phase. Each phase was dried and tested on 5 mainland sloths by adding it to their diets. The organic phase increased the sloths' activity. Their heartbeats were increased from an average of 66.4 ± 4.3 bpm to 80.7 ± 5.6 bpm. Therefore the organic phase must contain the active compound.

The organic phase was further separated by gas chromatography. The elution took a total of 12.45 minutes and yielded 8 fractions. Testing of the fractions individually on the sloths showed that fraction 4 yielded the greatest increase in activity (See Table 1and Figure 5). Fraction 4 was then analyzed with a mass spectrometer and the subsequent structure was determined (See Figure 6). The putative compound is determined to be N-methyl-1-(3,4-methylenedioxyphenyl)-2aminopropane (MMAP).

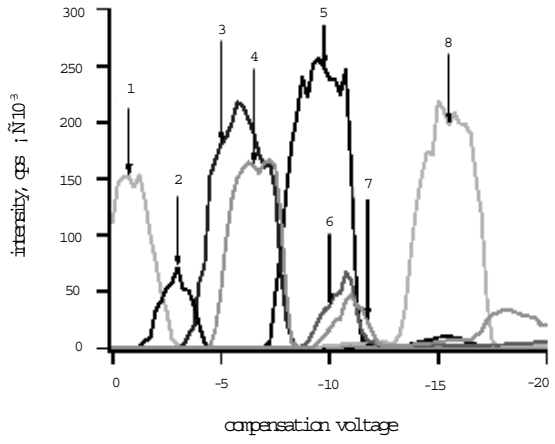

Figure 5 IS-CV spectra of a 10 ppb solution of the extracts in 9/1 MeOH/water +0.2mM ammonium acetate. Carrier gas was 40/60 nitrogen/helium. Eight fractions were tested and the fourth curve (fraction) represents our newly discovered compound,N-methyl-1-(3,4-methylenedioxyphenyl)-2aminopropane (MMAP).

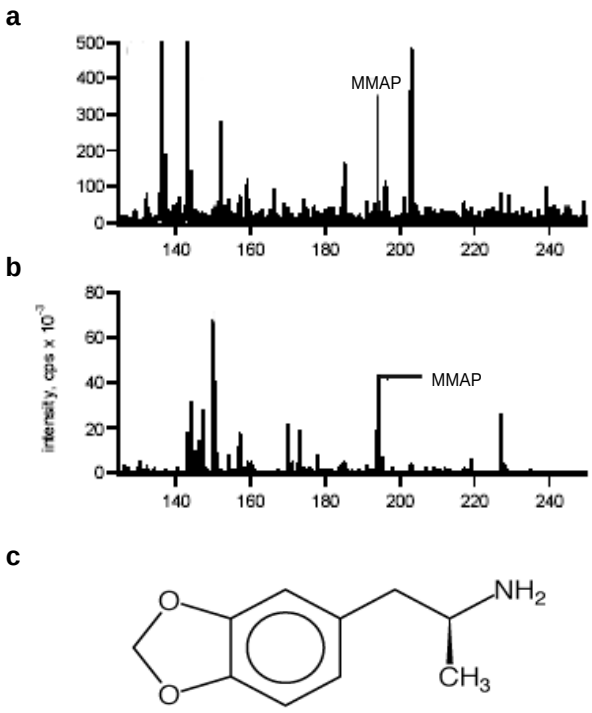

Figure 6 Mass spectra of a 10 ppb solution of the extracts in 99/1 methanol/urine. 40/60 nitrogen/helium carrier gas. **a & b,** ESI-MS and ESI-FAIMS-MS spectra **c,** chemical structure of the newly isolated compound, MMAP, which is similar to MDMA.

Discussion

The experimental results showed that the active compound found within P*anamae caenaela* was in fact the cause of the increased activity within the *Bradypus Variegates* three-toed sloth found on the Barro Colorado Island. The isolated compound was shown (from the mass spectra results) to be similar to the compound MDMA a highly stimulatory drug[20,21,24].

The comparative studies between sloths from the mainland and the so-called hyper sloths from the island showed the plant's effects to be fairly instantaneous[22,28,33]. The effects also seemed fairly short lived as the hyper sloths from the island soon reverted back to "normal" levels of activity when the plant was removed from their diet. Further experiments to reveal how the compound actually affects the metabolic pathways of the sloth need to be carried out. The performance of western blot should allow us to determine what proteins are present in such plant extracts and it also enables us to find out how these proteins interact with the metabolic mechanisms of the sloths[23,25].

Behavioral studies should also be conducted to determine other aspects of how the plant interacts with different processes within the sloth[27]. Natural predators of the sloth (Jaguar) would likely be far more of a threat to the hyper sloth as it tends to leave the relative safety of the trees more often than its unaffected relatives found on the mainland[29,30]. However there was no detection of an increase in predation compared to the numbers on the mainland, if anything there were as a higher than average survival rate on the island. This could be explained by a heightened sensitivity of the sloths to the surroundings and possible increased chance of escape due to the higher motility of the hyper sloths[36,40].

The behavior of the sloths towards each other was noticeably more aggressive and closer observation needs to be undertaken[16]. But these changes have led to an increase in fights among the males in particular. Another aspect of the plant's effects on the sloth that has been recorded is due to light stimulation. When the light has shone down through the canopy and flashed on to the forest floor, it caused an increase in activity of the sloths[34,39]. The sloths seem to become disorientated and would move in peculiar jerky motions. A similar effect was seen if the sloths were exposed to loud noises, particularly rhythmic beats[26,32]. When this behavior was observed it was termed the super sloth stage.

The plant itself reveals many significant possibilities for uses by pharmaceutical companies. It is endemic to the Barro Cubrado Island, so, careful preservation of the plants is extremely important. The purified form of the compound could prove to be an excellent natural stimulant though further work is obviously needed. Both the plant and the hyper sloths offer a unique possibility to study how geographical isolation can affect species in such an extreme way. From now on the worlds perception of the slowest creature on earth may have to be altered to make room for the super sloths of Barro Colorado Island of Panama.

Methods

Determination of ingredient in food source responsible for increased activity

The sloths were kept at a constant room temperature at 26°C with a 12h light/ 12h dark cycle. They were fed two times a day at 11am and 4 pm with a 160g diet of ymbahuba leaves. When subjecting the sloths to tests with other compounds or leaves, they were only fed 80g of ymbahuba leaves and 80g of the test compound or leaves. In order to measure the RQ value (energy usage), we put the sloths into a closed warm chamber with oxygen and carbon dioxide analyzers attached. The room-temperature air then was guided through the analyzer into the chamber with the sloths inside. During that time the air was dehydrated and carbon dioxide was filtered out. We measured each sloth for the duration of 115 min. During this time we were able to measure the consumption of oxygen and the release of carbon dioxide. With these values we calculated the respiratory quotients.

To measure the heart rate and respiratory rate a portable ECG and respiratory monitor from Vetronic Services (ERM-1800) was attached to the sloth for the duration of 10 minutes while they were able to move freely.

Determination of the compound in *P. caenaela* responsible for the increased activity in sloths

P. caenaela samples were collected from 50 different plants in various locations within the region. The samples were cleaned using a dichloromethane wash and grounded into a fine powder (motar and pestle/ liquid nitrogen). 15mg of the powdered sample was used for the Chloroform-Methanol extraction as described in Chiu et al. The sample was first dissolved in 100ml chloroform, 200ml methanol and 80ml water. The water was added until two phases were formed. The phases were separated, dried and tested on the sloth. The phase that had a positive effect on the sloth was then subjected to gas chromatography/mass spectrometer analysis.

Isolation of compounds

A Varian 3400 CX GC with Saturn ion trap mass spectrometer was used for the analysis; an RTX-5 (5%diphenyl-95% dimethylpolysiloxane, 30 m 0.25mm i.d.) was the chromatography column. Initially the column was programmed to 50°C and held for 1 min then to 225°C(increase of 20°C/min) and held for 1 min, then to 260°C(increase of 50°C/min) and held for 1 min. The carrier gas was ultra grade helium at a flow rate of 1 mL/min. Fractions were collected at 1 min intervals (i.e. 0 -1 min= fraction 1; 1 -2 min = fraction2 etc). Each fraction was dried and tested for activity in the sloth. The dried compounds were added to the sloths diet for testing.

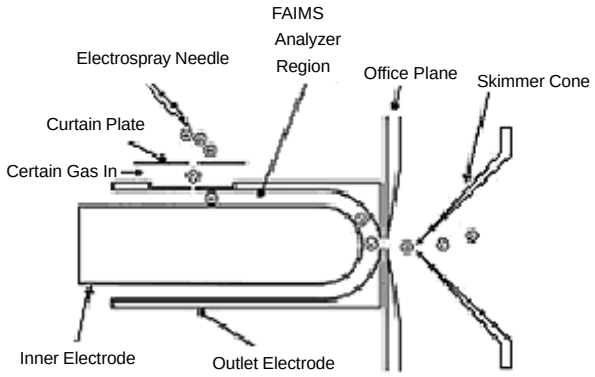

Figure 7 Schematicof the ESI-FAIMS-MS instrument used in this study. The FAIMS was interfaced to a PE-Sciex API-300 mass spectrometer.

Determination of structure

Figure 7 shows the FAIMS device used in the analysis. This geometry consists of an inner cylinder (radius = 8 mm) with a hemispherical tip facing the mass spectrometer, and an outer cylinder (radius = 10 mm) machined to keep the spacing between the cylinders constant at 2 mm. An asymmetric waveform, with dispersion voltage (DV) of -4000 V (P2 mode) at a frequency of 750 kHz, was applied to the inner cylinder of the FAIMS. The compensation voltage (CV) was scanned to transmit the series of our compound, which were detected by an API-300 triple quadrupole mass spectrometer. A 40/60 nitrogen/helium gas mixture was found to significantly improve sensitivity for these compounds, relative to pure nitrogen. Flow injection analysis (FIA) used a 50 µL sample loop, with manual sample loading. Flow from a pump was split after the injection loop to provide a flow of approximately 1 µL/min to the electrospray needle. Concentration of analytes ranged from 0.5 ppb to 100 ppb in 99/1 methanol/ urine.

Our compound was monitored by stepwise selection of the m/z and optimum CV of each drug, at a dwell time of 200 ms with a pause time of 500 ms between ions. This cycle through our samples required 5.6 seconds, and about 10 such repeat measurements were acquired for each FIA peak (less than 1 minute peak width).

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